

Article

Macrophages Upregulate Estrogen Receptor Expression in the Model of Obesity-Associated Breast Carcinoma

Daniela Nahmias Blank ¹, Esther Hermano ¹, Amir Sonnenblick ², Ofra Maimon ¹, Ariel M. Rubinstein ¹, Emmy Draï ¹, Bella Maly ³, Israel Vlodavsky ⁴, Aron Popovtzer ^{1,5}, Tamar Peretz ^{1,5}, Amichay Meirovitz ^{1,5,†} 
and Michael Elkin ^{1,5,*,†}

¹ Sharett Institute of Oncology, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel

² Oncology Division, Tel Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 64239, Israel

³ Department of Pathology, Hadassah-Hebrew University Medical Center, Jerusalem 91120, Israel

⁴ Cancer and Vascular Biology Research Center, The Rappaport Faculty of Medicine, Technion, Haifa 31096, Israel

⁵ Hebrew University Medical School, Jerusalem 91120, Israel

* Correspondence: melkin@hadassah.org.il; Tel.: +972-2-6776598

† These authors contributed equally to this work.



Citation: Nahmias Blank, D.; Hermano, E.; Sonnenblick, A.; Maimon, O.; Rubinstein, A.M.; Draï, E.; Maly, B.; Vlodavsky, I.; Popovtzer, A.; Peretz, T.; et al. Macrophages Upregulate Estrogen Receptor Expression in the Model of Obesity-Associated Breast Carcinoma. *Cells* **2022**, *11*, 2844. <https://doi.org/10.3390/cells11182844>

Academic Editor: J. Dinny Graham

Received: 2 August 2022

Accepted: 8 September 2022

Published: 12 September 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Breast cancer (BC) and obesity are two heterogeneous conditions with a tremendous impact on health. BC is the most commonly diagnosed neoplasm and the leading cause of cancer-related mortality among women, and the prevalence of obesity in women worldwide reaches pandemic proportions. Obesity is a significant risk factor for both incidence and worse prognosis in estrogen receptor positive (ER+) BC. Yet, the mechanisms underlying the association between excess adiposity and increased risk/therapy resistance/poorer outcome of ER+, but not ER–negative (ER–), BC are not fully understood. Tumor-promoting action of obesity, predominantly in ER+ BC patients, is often attributed to the augmented production of estrogen in ‘obese’ adipose tissue. However, in addition to the estrogen production, expression levels of ER represent a key determinant in hormone-driven breast tumorigenesis and therapy response. Here, utilizing in vitro and in vivo models of BC, we show that macrophages, whose adverse activation by obesogenic substances is fueled by heparanase (extracellular matrix-degrading enzyme), are capable of upregulating ER expression in tumor cells, in the setting of obesity-associated BC. These findings underscore a previously unknown mechanism through which interplay between cellular/extracellular elements of obesity-associated BC microenvironment influences estrogen sensitivity—a critical component in hormone-related cancer progression and resistance to therapy.

Keywords: breast cancer; heparanase; obesity; estrogen; estrogen receptor α ; macrophages

1. Introduction

Breast carcinoma (BC) is the most commonly diagnosed cancer type and the leading cause of cancer-related mortality among women [1]. BC is a highly heterogeneous disease [2,3]. The widely employed classification, referred to as PAM50, classifies BC into five molecular intrinsic subtypes: luminal A, luminal B, HER2-enriched, basal-like, and normal [3,4]. Over 75% of BCs express estrogen receptor α (ER) in >1% of the tumor cells by IHC, and overlap with luminal A and B subtypes [2]. ER α acts as a key driver of BC development, progression and dissemination [5–11]. Hormonal therapies for ER α -positive (ER+) BC target ER α either directly, by selective ER modulators and down-regulators, or indirectly, by abolishing estrogen production by inhibitors of aromatase, the rate-limiting enzyme in estrogen biosynthesis. Hence, ER expression level (governed by multiple and poorly-understood mechanisms [6–8]) is an important determinant in BC tumorigenesis, as well as in the tumor response to hormonal therapy (reviewed in [7,12]).

Obesity is a known risk factor in ER+/luminal BC subtypes [13–18]. Furthermore, once diagnosed, obese ER + BC patients (mostly postmenopausal) have worse clinical outcomes than their lean counterparts [13,17,19–23], and evidence is accumulating that the association between obesity and risk/mortality of ER+ BC is stronger [13,24] than earlier estimated [15,25]. A recent large population-based case-cohort study [13] demonstrated that obese women with Luminal A and B (i.e., ER+) BC subtypes had a 1.8– and 2.2– fold increased BC mortality risk, respectively, compared to normal weight women (defined as BMI <25 Kg/m²) [13]. Additionally, obesity was strongly associated with aggressive characteristics of luminal tumors (1.7– and 1.8– fold increased risk of stage III/IV disease and grade 3/4 tumors, respectively) [13]. Of note, obesity was not associated with breast cancer-specific mortality among women who had non-luminal (ER–) tumors [13], in agreement with previous reports [14,26,27].

At least 15 different types of cancer have been linked to obesity [15,28]. Thus, the predominant association between obesity and ER+/luminal, rather than ER–/non-luminal, BC subtypes [13,14,26,27] is puzzling. It is often explained by enhanced estrogen signaling, due to increased production of estrogen in chronically-inflamed “obese” adipose tissue compartments, in the context of adverse activation of macrophages (M ϕ) [16–18,20,29–32]. Indeed, M ϕ mobilization is a characteristic feature of obesity-associated BC [33,34]. In fact, M ϕ are regarded as the key immunocyte type within the breast microenvironment, where they adopt maladaptive phenotypes and mediate the BC-promoting action of obesity [16,17,33–35]. Among the components of the obese milieu, free saturated fatty acids (SFAs), the levels of which are chronically increased in obesity [36], are well-known for their ability to trigger adverse M ϕ activation via a toll-like receptor (TLR)-dependent mechanism [35,37–39]. The occurrence/severity of M ϕ -driven breast adipose tissue inflammation is higher in obese, as compared to normal weight, women [16]. Similar inflammation has been noted in subcutaneous and intra-abdominal visceral fat depots of obese patients/experimental animals [30,40]. It was suggested that inflammatory mediators, secreted by adversely-activated M ϕ , may upregulate expression of aromatase by stromal cells of “obese” adipose tissue, leading to an increase in estrogen levels, and, thus, selectively promoting ER+ BC in obese postmenopausal patients [13,18,41,42].

However, in addition to estrogen, changes in ER expression levels can profoundly affect breast tumorigenesis and BC response to hormonal therapy. Given the recent evidence linking M ϕ to induction of steroid receptor expression in hormone-responsive tumor types other than BC [43,44], we hypothesized that in obesity-associated BC, M ϕ residing in the tumor microenvironment can regulate cellular levels of ER expressed by carcinoma cells, increasing their sensitivity to the hormone and, thus, contributing to enhanced estrogen signaling. Investigating obesity-associated BC in immunocompetent mice, we found that expression of ER by the tumor cells was markedly increased under obese conditions. Moreover, this increase correlated with augmented tumor growth, as well as with the infiltration/activation of M ϕ in tumor and fat tissue of obese mice. In vitro, M ϕ , stimulated by TLR-triggering obese milieu components (i.e., SFA) augmented ER α expression in BC cells of human/murine origin. We also found that augmentation of ER α expression in BC cells by M ϕ was dependent on heparinase, the sole mammalian endoglycosidase enzyme that degrades heparan sulfate proteoglycan chains at the cell surface and is essential for TLR4-mediated M ϕ activation [45–47]. Of note, heparinase was recently shown to sustain M ϕ reactivity in several obesity-related [30,48,49] and unrelated [45,50,51], M ϕ -driven inflammatory conditions. Worth mentioning is the fact that, similarly to obesity per se, expression of heparinase correlates with worse outcome in ER+, but not ER–, breast tumors [52]. Collectively, our findings attest to upregulation of ER expression (especially in the face of enhanced production of estrogen in fat depots) as an important, and previously unidentified, mechanism of obesity-accelerated BC progression, likely explaining the dichotomy of the obesity effects on the risk/poorer outcome between luminal (i.e., ER+) and non-luminal (i.e., ER–) BC subtypes.

2. Materials and Methods

2.1. Clinical Data Analysis

Breast tumor tissue specimens and clinical data from 58 ER+ breast carcinoma female patients were available from the Sharett Oncology Institute, Hadassah Medical Center, Jerusalem. A summary of clinical and pathological characteristics of the study population is shown in Table 1. The use of these data and formalin-fixed, paraffin-embedded breast carcinoma tissues in research was approved by the Human Subjects Research Ethics Committee of the Hadassah Medical Center. Tissue microarray construction was performed as previously described [30,53]. Immunodetection of ER α was performed as in [30,53], using ER α monoclonal antibody NCL-L-ER 6F11 (Novocastra, Newcastle, UK). ER α positivity and staining intensity (scored as weak (=1), moderate (=2), or strong (=3)) was determined in accordance with [54,55] by an expert pathologist (B.M.). Immunodetection of heparanase was performed using polyclonal rabbit anti-heparanase antibody (733) directed against a synthetic peptide (¹⁵⁸KKFKNSTYRSSVD¹⁷¹) corresponding to the N-terminus of the 50-kDa subunit of the heparanase enzyme [30,53]. The antibody was diluted 1:100 in 10% goat serum in PBS. Control slides were incubated with 10% goat serum alone. Color was developed as described in [30,53], slides were visualized with a Zeiss axioscope microscope and manually read by an expert pathologist (B.M.). To define tumor as heparanase-positive, a cut-off point of 25% immuno-stained tumor cells was chosen, based on an initial overview of the cases, in order to improve signal-to-noise ratios. Cut-off was chosen before any attempt at correlating heparanase expression with the obese status of the patients.

Table 1. Clinical and pathological characteristics of the study population ($n = 123$ subjects).

Criteria	Range	No. Cases (%)
Tumor size (cm)	0.5–10.5	
<2		31 (25.2)
2–5		50 (40.7)
>5		3 (2.4)
Unknown		39 (31.7)
Tumor type		
Ductal		109 (88.6)
Lobular		5 (4.1)
Others		9 (7.3)
Lymph node status		
LN pos		38 (30.9)
LN neg		37 (30.1)
Unknown		48 (39)
Grade		
1		19 (15.5)
2		46 (37.4)
3		56 (45.5)
Unknown		2 (1.6)
ER status		
Negative		40 (32.5)
Positive		78 (63.4)
Unknown		5 (4.1)

2.2. Cell Culture

MCF7 and T47D cells were cultured in DMEM; E0771, 4T1 and THP-1 cells were cultured in RPMI medium, supplemented with 1 mM glutamine, 50 µg/mL streptomycin, 50 U/mL penicillin and 10% fetal calf serum (FCS, Biological Industries) at 37 °C and 8% CO₂. For estrogen depletion, cells were cultured for 14 days in phenol red-free DMEM supplemented with 5% charcoal-stripped FCS (Biological Industries, Beit-Haemek, Israel), antibiotics, and supplemented with 1 mM glutamine, prior to estrogen treatment.

2.3. Macrophage Isolation and Treatment

Primary mouse macrophages were obtained applying the isolation procedure described in [30,49,51]. For experiments with fatty acids: palmitic (16:0) and stearic (18:0) acid (Sigma-Aldrich, Rehovot, Israel) were diluted in 95% ethanol and conjugated with fatty acid free-BSA at a 2:1 molar ratio before treatment, yielding a stock concentration of 3 mM. For experiments, the fatty acid-BSA solution was further diluted in the culture medium yielding a final concentration of 200 µM. LPS was diluted to a final concentration of 0.1 ng/mL. TAK-242, TLR4 inhibitor (InvivoGen, Toulouse, France) was diluted to a final concentration of 30 µM.

2.4. MTS Assay

Cells were seeded in 96-well culture plates in phenol red-free DMEM, supplemented with 5% charcoal-stripped FCS. MTS assay (Promega, Madison, WI, USA) was performed according to the manufacturer's instructions. Each experiment was performed at least 3 times. Each data point shows the mean of pentaplicate cultures.

2.5. Orthotopic Immunocompetent Mouse Model of Obesity-Associated Breast Cancer

Ten week old, female *wt* C57BL/6J mice (Envigo; Jerusalem, Israel) and heparanase-knock out (*Hpse*-KO) mice [56] on C57BL/6J background were fed *ad libitum* high-fat diet (HFD) (Teklad TD.06414, 60% of total calories from fat), or control diet (CD) (Teklad 2018S) for 15 consecutive weeks. In experimental week 12, when HFD-fed animals became obese, E0771 cells were injected orthotopically into the 4th left mammary fat pads of both HFD-fed (obese) and control diet-fed (lean) *wt* and *Hpse*-KO mice (5×10^5 cells per injection). The tumor volume was monitored until experimental week 15. Animals were then sacrificed, tumor tissue samples collected and snap-frozen for RNA/protein extraction.

For tumor irradiation studies tumor-bearing obese and lean mice were anesthetized 7 days before sacrifice and their tumors irradiated conformally to 5Gy, using surface brachytherapy, essentially as described in [57]. Briefly, radiation was delivered to the tumor-bearing mammary gland by using a brachytherapy after-loader (¹⁹² Nucletron microSelectron HDR, Veenendaal, The Netherlands); the brachytherapy sleeve was positioned over the tumor (above a 0.5 cm width silicon bolus). The dose distribution calculations were based on CT simulation; the dose of 4.5Gy was calculated to 1.0 cm isodose line, in order to achieve 5Gy to 90% of the tumor volume, while relatively protecting the surrounding normal tissues. The prescribed dose was confirmed by film dosimetry. Variation of dose inside the tumors was estimated to be within ±15% of the prescribed dose. Tumor growth was monitored immediately prior to irradiation and at the indicated times after irradiation. All experiments were performed in accordance with the Hebrew University Institutional Animal Care and Use Committee.

2.6. Immunoblotting

Cell and tumor tissue lysates were homogenized in lysis buffer containing 0.6% SDS, 1 mM Tris-HCl, pH 7.5, supplemented with a mixture of protease inhibitors (Roche) and phosphatase inhibitors (Thermo Scientific, Waltham, MA, USA). Equal protein aliquots were subjected to SDS-PAGE (10% acrylamide) under reducing conditions and proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Darmstadt, Germany). Membranes were blocked with 3% BSA for 1 h at room temperature and probed with

the appropriate antibody, followed by horseradish peroxidase-conjugated secondary antibody (KPL, Milford, MA, USA) and a chemi-luminescent substrate (Biological Industries, Beit-Haemek, Israel). Band intensity was quantified by densitometry analysis using ImageJ software.

2.7. Antibodies

Immunoblot analysis and Immunofluorescence staining were carried out with the following antibodies: anti-F4/80 (AbD Serotec), anti-ER α D6R2W (13258 Cell Signaling), and ab3575 (Abcam, Cambridge, UK), anti-Actin C-2 (sc-8432 Santa Cruz, Santa Cruz, CA, USA), and anti-GAPDH (RPCA Encor, Gainesville, FL, USA).

2.8. Immunofluorescence

For immunofluorescence analysis, DyLight 488 goat anti-rat and CyTM3 donkey anti-rabbit (The Jackson Laboratory) antibodies were used as secondary antibodies. Nuclear staining was performed with 1,5-bis[[2-(di-methylamino)ethyl]amino-4,8-dihydroxyanthracene-9,10-dione (DRAQ5) (Cell Signaling Technology). Images were captured using a Zeiss LSM 5 confocal microscope and analyzed with Zen software (Carl Zeiss) and ImageJ software.

2.9. Analysis of Gene Expression by Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from cultured cells or snap-frozen tumor tissue samples using TRIzol (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions, and quantified by spectrophotometry. Single-stranded cDNA was amplified from 1 μ g of total RNA using a qScript cDNA Synthesis Kit (Quanta, Beverly, MA, USA). Real-time quantitative PCR (qRT-PCR) analysis was performed with an automated rotor gene system RG-3000A (Corbett Research, Sydney, Australia). The PCR reaction mix (20 μ L) was composed of 10 μ L QPCR sybr master mix (Quanta, Beverly, MA, USA), 5 μ L of diluted cDNA (each sample in triplicate) and a final concentration of 0.3 μ M of each primer. Hypoxanthine guanine phosphoribosyl transferase (HPRT) primers were used as an internal standard. The following primers were utilized:

mouse HPRT sense: 5'-GTC GTG ATT AGC GAT GAT GAA -3', antisense: 5'-CTC CCA TCT CCT TCA TGA CAT C-3';

mouse Estrogen Receptor Alpha sense: 5'- GGG CTG ACT TCA CTT ACA TTT C -3', antisense: 5'- GGA GCA TCT ACA GGA ACA CAG -3';

human HPRT sense: 5'- GCT ATA AAT TCT TTG CTG ACC TGC T -3', antisense: 5'- ATT ACT TTT ATG TCC CCT GTT GAC TG -3';

human Estrogen Receptor Alpha sense: 5'- TGA TGA AAG GTG GGA TAC GA -3', antisense: 5'-AAG GTT GGC AGC TCT CAT GT -3';

2.10. Statistical Analysis

The results are presented as the mean \pm SD unless otherwise stated. p values \leq 0.05 were considered statistically significant. Statistical analysis of in vitro and in vivo data was performed by unpaired Student's t -test. Pearson Chi-Square test was applied to analyze the relationship between heparanase and estrogen receptor alpha expression of breast carcinoma patients, using SPSS software (SPSS Inc., Chicago, IL, USA). All statistical tests were two-sided.

3. Results

3.1. Increased ER α Expression Levels in Obesity-Associated E0771 Murine Breast Carcinoma

Tumor-promoting action of obesity in ER+ breast tumors is often explained by enhanced estrogen signaling, owing to induction of aromatase expression in "obese" adipose tissue and augmented extragonadal synthesis of estrogen [30,41,42,58]. However, increase in estrogen signaling may also occur due to the upregulation of ER protein levels in BC cells. To test the impact of obesity on ER expression in BC, we applied an immunocompetent orthotopic mouse model of obesity-associated breast cancer, based on murine BC cell line

E0771, growing in female syngeneic C57BL/6J mice with high fat diet (HFD)-induced obesity [30,34,59]. E0771 cells express relatively low levels of ER α and are considered as a luminal B subtype of BC [60–62]. HFD-fed C57BL6 mice represent a reliable model of diet-induced obesity and related pathological conditions, i.e., inflammation [63], FA accumulation [64], and obesity-accelerated tumor growth [34,59,65]. Importantly, female (as opposed to male) C57BL/6J mice are protected against HFD/obesity-induced hyperinsulinemia/hyperglycemia [66], therefore allowing scrutinization of the specific contribution of obesity-related inflammatory events to BC progression.

In agreement with earlier reports [30,34,59,65], following 12 weeks on HFD, the experimental mice became obese, as evidenced by a 38% increase in their body weight, compared to control diet-fed lean mice ($p < 0.001$). E0771 cells were then implanted orthotopically in obese and lean mice, as described in Methods, and tumor growth was monitored for 14 days. Consistent with previous findings [30,34,59,65], growth of E0771 tumors was augmented in obese, as compared to lean, mice (on day 14 tumors from obese mice were 2.4-fold larger than tumors from lean mice, $p < 0.004$). As expected, the extent of M ϕ infiltration, the hallmark of obesity-accelerated breast tumor growth [33,34], was increased 1.9-fold ($p < 0.03$) in tumors grown in obese versus lean mice (Supplementary Figure S1A,B). Additionally, the obese state attenuated the sensitivity of E0771 tumors to radiotherapy—backbone treatment modality in ER + BC (Supplementary Figure S1C). Given the role of ER α in growth and radio-resistance of BC [5–8], we examined ER α expression levels in tumors derived from obese versus lean mice. Immunoblot and qPCR analysis of the tumor tissue revealed that expression of the full length 66-kDa ER α protein and mRNA was significantly increased in E0771 tumors growing in obese, as compared to lean, mice (Figure 1A–C). Of note, there was no difference in the levels of 46-kDa ER isoform (known to antagonize the full-length ER-mediated responses in BC [67,68]) between tumors growing in obese versus lean mice.

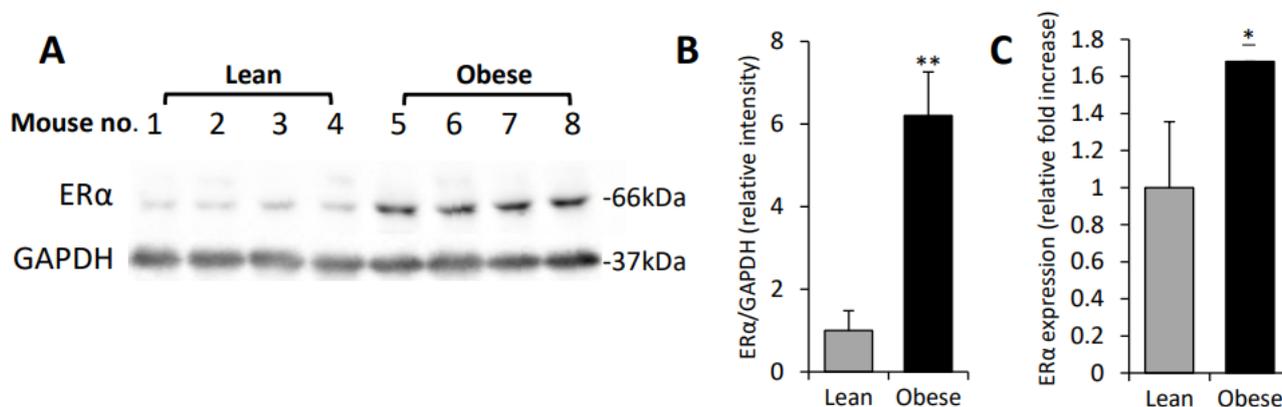


Figure 1. Upregulation of ER α in obesity-associated murine breast carcinoma model. Female C57BL6 mice fed for 12 weeks with either HFD (obese) or control diet (lean), were injected with E0771 cells into the 4th mammary fat pad of all mice, as described in Methods. Tumors were harvested 14 days post cell injection and lysates of tumor tissue derived from lean and obese mice were analyzed for ER α expression by immunoblotting and qRT-PCR. (A) Protein levels of full length 66 kDa ER α in the orthotopic E0771 tumors derived from lean and obese. (B) The band intensity was quantified using ImageJ software; intensity ratio for ER α /GAPDH is shown, $n \leq 4$ mice per condition. (C) qRT-PCR was used to determine the levels of ER α mRNA in tumor tissue samples collected from lean and obese mice. Error bars represent \pm SD. * $p < 0.04$; ** $p < 0.001$.

3.2. Macrophages Stimulated by Obese Milieu Components Mediate Upregulation of ER α in BC Cells

Emerging evidence indicates that tumor-infiltrating immunocytes (including M ϕ) are capable of inducing ER α expression in hormone-responsive tumor types [43,69] via cytokine-mediated mechanism. Based on these observations, along with the notion that M ϕ represent the principal pro-cancerous immunocyte population implicated in obesity-

accelerated BC tumorigenesis [35,42], and the well-documented increase in M ϕ infiltration in E0771 tumors under obese conditions (References [30,33,34] and Supplementary Figure S1B), we sought to investigate whether M ϕ are capable of modulating ER expression in the setting of obesity-associated BC. To mimic conditions occurring in breast tumor-bearing obese patients/experimental animals (i.e., adverse activation of M ϕ [35,41] due to the presence of increased concentrations of sFA in circulation [36]), murine and human (i.e., E0771, MCF7) ER + BC cell lines were incubated with medium conditioned by primary M ϕ (isolated as described in Methods), either unstimulated or stimulated by SFA (i.e., palmitate, dominant SFA present in fat depots/circulation of female patients [70] and responsible for adverse M ϕ activation under obese conditions [35,37,39,71]). As shown in Figure 2, SFA-stimulated M ϕ -conditioned medium markedly upregulated ER α protein (Figure 2A,B,D,E), as well as mRNA expression (Figure 2C,F) of ESR1 gene, encoding for ER α , in E0771 and MCF7 cells. The effect of SFA-stimulated M ϕ on ER expression was evident even in the 4T1 murine BC cell line (Supplementary Figure S2A), which is often considered ER–negative, but in fact expresses extremely low levels of ER α [72]. Similar effects were observed in MCF7 cells incubated with a medium conditioned by SFA-stimulated human M ϕ differentiated from promonocytic cell line THP-1 [73] (Supplementary Figure S2B).

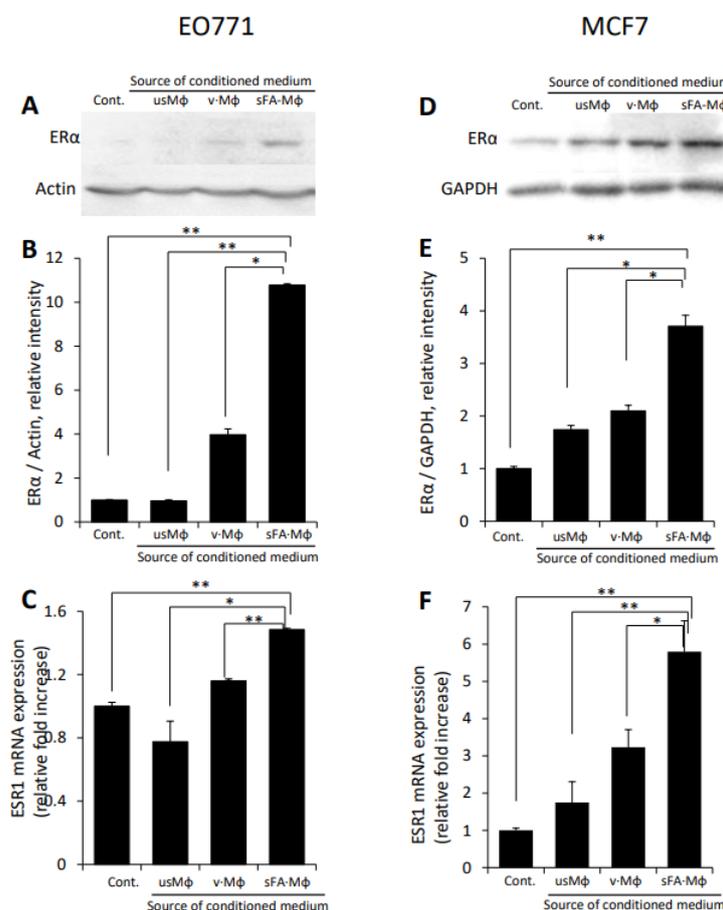


Figure 2. Upregulation of ER α expression in murine and human BC cells by SFA-stimulated macrophages. E0771 (A–C) and MCF-7 (D–F) BC cells either remained untreated (Cont.) or were incubated (24 h, 37 °C) with medium conditioned by either unstimulated macrophages (usM ϕ), or macrophages stimulated (16 h) by vehicle (BSA) alone (v-M ϕ) or by SFA (i.e., palmitic acid) conjugated with fatty acid free-BSA at a 2:1 molar ratio (SFA-M ϕ). (A,D) ER α protein levels were determined by immunoblotting. (B,E) The band intensity was quantified using ImageJ software; intensity ratio for ER α /actin or ER α /GAPDH is shown. (C,F) The expression of mouse (C) and human (F) ESR1 gene, encoding for ER α , was determined by qRT-PCR. The data shown are representative of ≥ 3 independent experiments; error bars represent \pm SD. * $p < 0.02$; ** $p < 0.0015$.

Since M ϕ activation by SFA under the obese state involves a TLR4-dependent mechanism [35,37,39,71], we next tested the effect of TLR4 inhibition on the ability of SFA-stimulated M ϕ to induce ER expression. As shown in Figure 3A,B, specific TLR4 inhibitor TAK-242 abolished the ability of SFA-stimulated M ϕ to induce ER α expression in E0771 (A) and MCF7 (B) cells, in agreement with TLR4-activating properties of SFA [37–39].

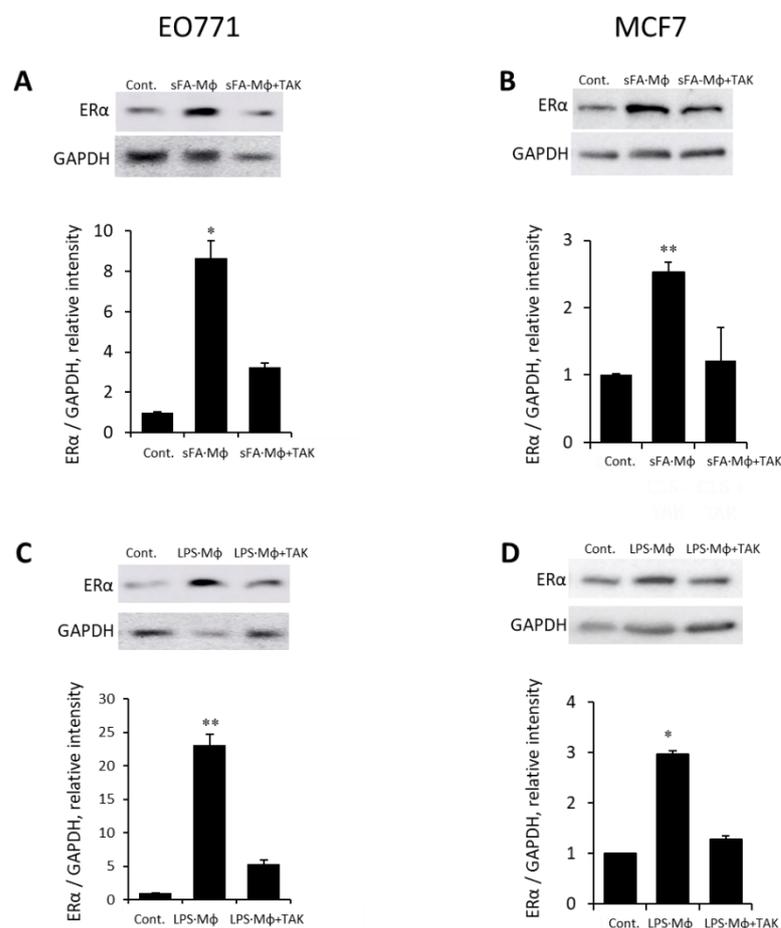


Figure 3. Ability of macrophages to upregulate ER expression in BC cells in response to stimulation by obese milieu components depends on TLR4 signaling. (A,B). Mouse E0771 (A) and human MCF7 (B) BC cells either remained untreated (Cont.) or were incubated (24 h, 37 °C) with medium conditioned by macrophages stimulated with 200 μ M SFA (i.e., palmitic acid), alone (SFA-M ϕ) or in the presence of 30 μ M of TLR4-specific inhibitor TAK-242 (SFA-M ϕ + TAK). ER α protein levels were determined by immunoblotting (top) and quantified using ImageJ software (bottom); the intensity ratio for ER α /GAPDH is shown. (C,D). Macrophages stimulated by conditions mimicking metabolic endotoxemia upregulated ER α expression in BC cells. E0771 (C) and MCF7 (D) cells were either remained untreated (Cont.) or were incubated (24 h, 37 °C) with medium conditioned by macrophages stimulated by 0.1 ng/mL LPS alone (LPS-M ϕ) or in the presence of 30 μ M of TLR4-specific inhibitor TAK-242 (LPS-M ϕ + TAK). ER α protein levels were determined by immunoblotting (top) and quantified using ImageJ software (bottom). The intensity ratio for ER α /GAPDH is shown. The data are representative of 3 independent experiments; error bars represent \pm SD. * $p < 0.007$; ** $p < 0.0009$.

Of note, the canonic ligand of TLR4 lipopolysaccharide [LPS] per se is a component of the systemic obese milieu, since it is chronically present (at extremely low levels, typically ranging between 0.01 to 0.2 ng/mL) in the circulation of obese individuals/experimental animals [74–76]. This phenomenon, known as ‘metabolic endotoxemia’ [74–76], occurs due to impaired intestinal permeability under obese conditions [74–78]. Interestingly, we

found that medium conditioned by M ϕ stimulated by LPS at a concentration reflecting metabolic endotoxemia (i.e., 0.1 ng/mL) also upregulated ER in E0771 and MCF7 cells, while pretreatment of M ϕ by TAK-242 prior to LPS stimulation abolished this upregulation (Figure 3C,D), further validating involvement of TLR4-mediated signaling. Since TLR4 could also be expressed by BC cells per se, to exclude the possible effect of carry-over of TLR4 ligand via the M ϕ -conditioned medium, we compared levels of ER α protein in untreated BC cells (E0771, MCF-7) versus those incubated with medium conditioned by LPS (0.1 ng/mL)-stimulated M ϕ , or those incubated with standard medium containing the same concentration of LPS. As shown in Supplementary Figure S3, ER α protein levels were significantly increased in BC cells treated with medium conditioned by LPS-stimulated M ϕ , as compared to either untreated cells or cells treated directly with LPS, confirming macrophage specific function.

3.3. Macrophages Stimulated by SFA Increase the Sensitivity of BC Cells to Estrogen

To verify the functional significance of ER α upregulation, depicted in Figure 2, we next assessed the effect of medium conditioned by SFA-stimulated M ϕ on the proliferative response of MCF7 cells to estrogen. For this purpose, cells were first depleted of estrogen for 2 weeks (as detailed in Methods) and then treated with 17 β estradiol in the presence or absence of medium conditioned by either unstimulated or SFA-stimulated M ϕ (Figure 4). As expected, 17 β estradiol increased proliferation of MCF7 under all conditions. However, the presence of medium conditioned by SFA-stimulated M ϕ (but not vehicle-stimulated M ϕ) significantly augmented proliferative response to estradiol (Figure 4), consistent with the upregulation of ER α levels (Figure 2). Importantly, stimulated M ϕ also enhanced MCF7 proliferation under conditions of estrogen depletion (i.e., without addition of exogenous estrogen—Figure 4, white bars). In vitro estrogen depletion procedure ensures that only extremely low residual estrogen levels are present in the medium. In addition, this procedure mimics depletion of estrogen that occurs in BC patients treated with aromatase inhibitors. In this context, it is worthy to note that enhanced MCF7 proliferation, triggered by stimulated (as compared to unstimulated) M ϕ , was observed even in the presence of residual levels of estrogen (Figure 4). These data suggest that in the setting of obesity, upregulation of ER levels in BC cells by SFA stimulated M ϕ may enable their proliferative response even to extremely low levels of estrogen, thus providing a molecular/cellular explanation for the observation that obese patients are more likely to experience ER+BC recurrence after treatment with aromatase inhibitors [23] and generally contributing to the worse response to hormonal therapy in BC [13,19,79]. Collectively, these results confirmed the ability of obese milieu component-activated M ϕ to increase the sensitivity of BC cells to estrogen through upregulation of ER α .

3.4. M ϕ -Mediated Augmentation of ER Expression in BC Is Dependent on Heparanase, the Endoglycosidase Enzyme Essential for M ϕ Reactivity

Heparanase enzyme is preferentially expressed in obesity-associated breast tumors in clinical/experimental settings [30] and associated with worse prognosis in ER+, but not ER- BC [52]. Heparanase is the only known mammalian endoglycosidase capable of cleaving heparan sulfate (HS) at the cell surface and ECM. Of note, intact extracellular HS was shown to inhibit TLR4 signaling and M ϕ activation, while enzymatic cleavage of HS relieves this inhibition [80]. Furthermore, soluble fragments released through enzymatic cleavage of HS stimulate TLR4 signaling [80–84]. Thus, heparanase enzyme is causally involved in M ϕ activation by TLR4 ligands [45–48,50], including FA [30,49]. In light of the above data, we next investigated the role of heparanase in M ϕ -mediated induction of ER under obese conditions.

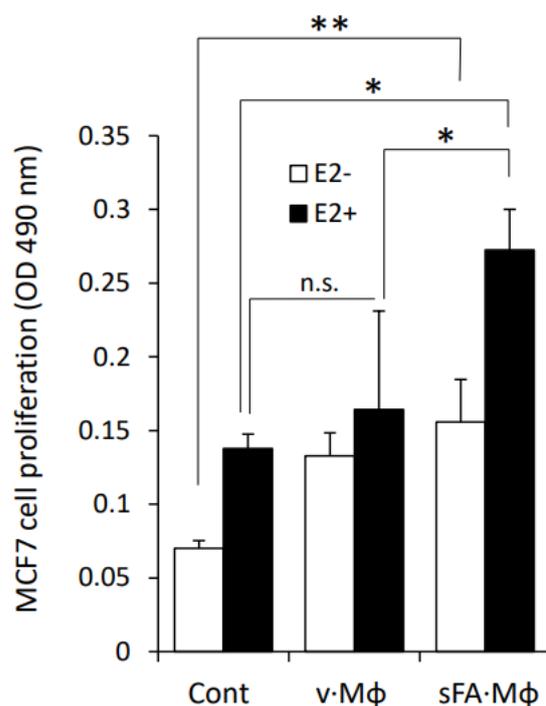


Figure 4. Effect of SFA-stimulated macrophages on estrogen sensitivity of MCF7 cells. Prior to estrogen treatment, MCF7 cells were maintained for 2 weeks in phenol red-free medium supplemented with charcoal-stripped FCS (5%), and then incubated (96 h, 37 °C) with the medium conditioned by either unstimulated macrophages (Cont), or macrophages stimulated (16 h) by vehicle (BSA) alone (v-Mφ) or by 200 μM palmitate (SFA) conjugated with fatty acid free-BSA at a 2:1 molar ratio (SFA-Mφ), in the absence (white bars) or presence (black bars) of 10^{-9} M estradiol (E2). Cell proliferation was analyzed by MTS assay, * $p < 0.02$, ** $p < 0.003$; n.s: no statistical difference. Error bars represent \pm SD.

First, utilizing the obesity-associated E0771 BC model in heparanase-null *Hpse*-KO mice, we found that, unlike in wild-type (*wt*) animals, the obese state failed to upregulate ER α expression in BC tumors under conditions of heparanase deficiency (Figure 5A–C). Of note, heparanase deficiency has been previously shown by Hermano et al., to abolish BC-promoting action of obesity in vivo [30]. Data from these experiments were used in the present study for comparative purposes. It should be noted that heparanase deficiency did not affect growth of E0771 tumors in lean mice (as no statistically significant difference was detected between volume of tumors derived from *wt* lean (105 mm³) versus *Hpse*-KO lean (154 mm³) mice on day 14, $p > 0.06$). In agreement with the in vivo findings (Figure 5), in vitro Mφ derived from heparanase-null mice (KO-Mφ), differently from Mφ derived from *wt* mice (*wt*-Mφ), failed to upregulate ER α in murine (E0771, Figure 6A) and human (T47D, Figure 6B) BC cell lines following SFA stimulation. On the other hand, in vitro treatment with recombinant heparanase enzyme prior to stimulation by SFA (mimicking overexpression of the enzyme in obesity-associated breast tumors [30]) resulted in a 4-fold increased ability of SFA-stimulated Mφ to upregulate ER α expression in E0771 and MCF7 cells (Supplementary Figure S4). Corroborating the above findings in the clinical setting, immunohistochemical analysis of ER + BC tissue specimens derived from 58 patients revealed that heparanase positivity significantly correlated with higher levels of ER α expression (quantified as in [54] in the tumor tissue): a nearly 2-fold higher proportion of high ER α expression was detected in heparanase-positive versus heparanase-negative tumors (73% vs. 40%, chi-square test $p = 0.0135$, Figure 7).

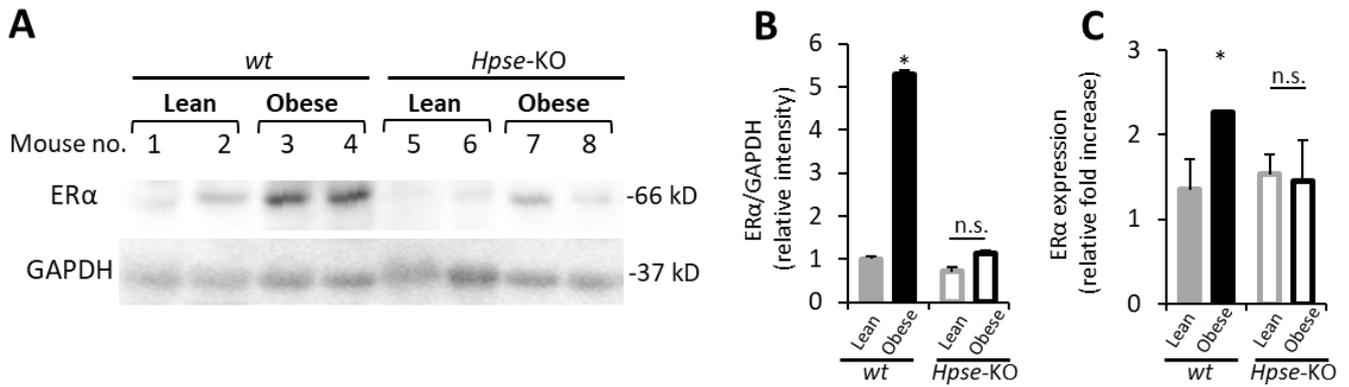


Figure 5. Heparanase deficiency abolishes effect of obesity on ERα expression in the murine model of BC. Wild-type (*wt*) and heparanase-null (*Hpse*–KO) female C57BL6 mice were made obese by administration of HFD for 12 consecutive weeks, as described in Methods. Control (lean) mice were feed with a regular diet. HFD-fed animals of both *wt* and *Hpse*–KO genotypes became obese, as evidenced by their significantly increased body weight compared to control diet-fed lean mice (*wt*: 38% increase $p < 0.001$; *Hpse*–KO: 49% increase, $p < 0.03$). Then, all mice were injected with E0771 cells into the 4th mammary fat pad, as described in Methods. Tumors were harvested 14 days post cell injection and lysates of tumor tissue were analyzed for expression of ERα protein (A,B) and mRNA (C). (A,B) Levels of full length 66 kDa ERα protein in the orthotopic E0771 tumors derived from lean and obese mice, were analyzed by immunoblotting (A). The band intensity was quantified using ImageJ software; intensity ratio for ERα/GAPDH is shown, $n \leq 5$ mice per condition (B). Error bars represent \pm SD; * $p < 0.0002$; n.s: no statistical difference. (C) qRT-PCR was used to determine the levels of ERα mRNA in tumor tissue samples collected from lean and obese mice.

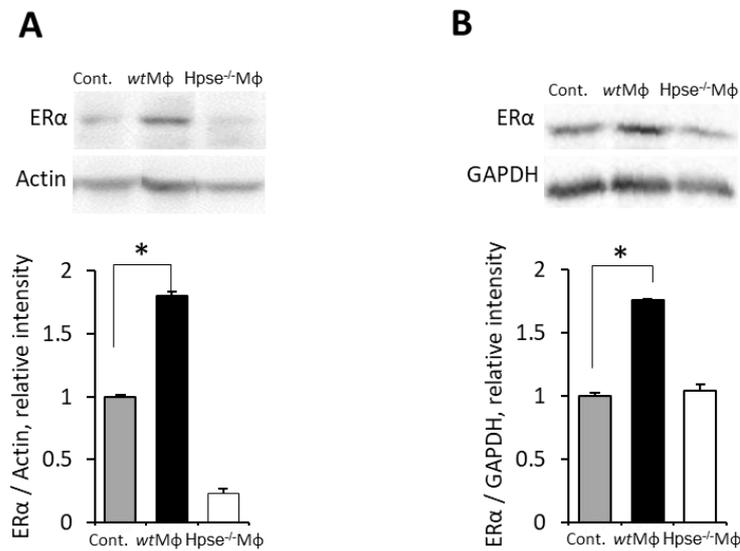


Figure 6. Heparanase-deficient macrophages stimulated by SFA failed to upregulate ERα levels in BC cells. E0771 (A) and T47D (B) cells either remained untreated (Cont.) or were incubated (24 h, 37 °C) with medium conditioned by macrophages derived from either wild-type (wtMφ) or heparanase-knockout (Hpse^{-/-} Mφ) mice, and stimulated by saturated fatty acid, either palmitic (A) or stearic (B), as described in Methods. ERα protein levels were determined by immunoblotting (top) and quantified using ImageJ software (bottom). Error bars represent \pm SD. * $p < 0.002$.

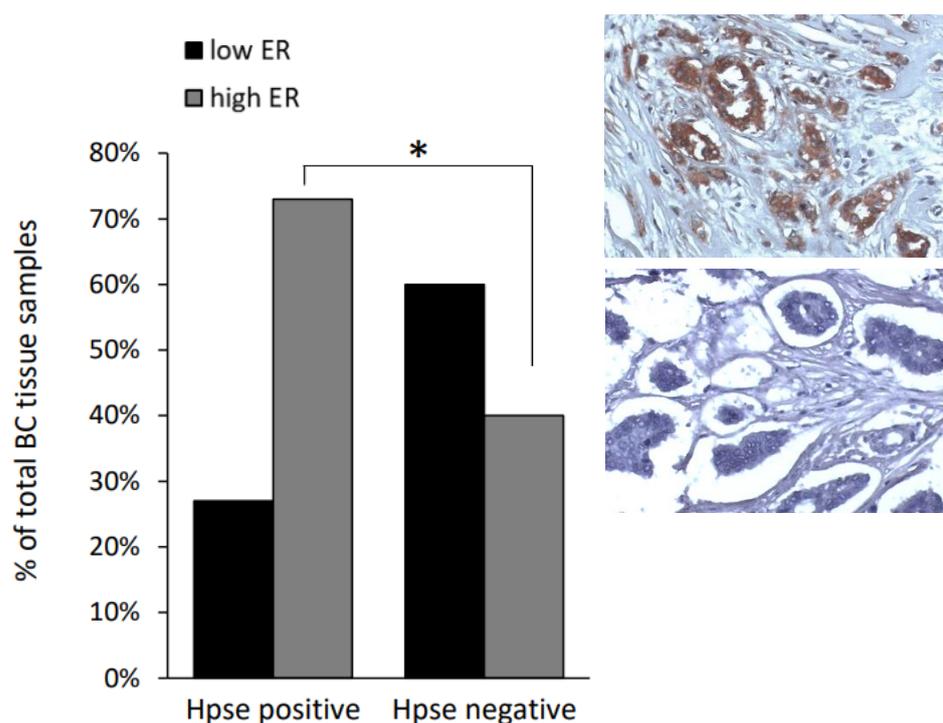


Figure 7. Sections of human ER + BC tissue samples ($n = 58$) were processed for immunohistochemistry with anti-ER α and anti-heparanase (Hpse) antibodies, as described in Methods. **Inset:** representative images of heparanase-positive (top) and heparanase-negative (bottom) breast tumor tissue specimens (invasive ductal carcinoma), original magnification $\times 200$. To define tumor as Hpse-positive, a cutoff point of 25% immuno-stained tumor cells was used, as in Ref. [53]. The intensity of ER α staining was scored as weak (1), moderate (2), or strong (3), as described in Methods; tumors with staining score 1 were categorized as “low ER” (black bars); tumors with staining score 2 and 3 as “high ER” (grey bars). Chi-squared analysis was then used to assess the relationship between heparanase positivity and high versus low ER levels. Significant correlation between expression of heparanase and high ER levels was noted: an almost 2-fold higher proportion of high ER expression was detected in heparanase-positive versus heparanase-negative tumors (73% vs. 40%, chi-square test * $p = 0.0135$).

Of note, although heparanase is expressed by both M ϕ [46–49] and BC cells [52] numerous observations have repeatedly indicated that in the setting of breast tumors, carcinoma cells per se appear to represent the major cellular source of the enzyme (reviewed in [52]). Given the above notion, the secreted nature of heparanase, and the role of extracellular heparan sulfate in modulation of TLR signaling ([80–82]), it is plausible that in the BC under obese conditions the excessive enzyme overexpressed by the tumor cells is a main contributor to adverse activation of macrophages. Collectively, these data highlight a novel mechanism through which interplay between elements of the obesity-associated BC microenvironment, both cellular (i.e., M ϕ , upregulating ER α expression; carcinoma cells, supplying heparanase) and extracellular (obese milieu components, HS), influences estrogen sensitivity—a critical component in hormone-related cancer progression and resistance to endocrine therapy.

4. Discussion

Breast tumors remain a major cause of morbidity/mortality worldwide, despite remarkable progress in the diagnosis/endocrine therapy of ER + BC [28]. Approximately 30% of patients with hormone-responsive BC develop resistance. Moreover, the alarming global increase in the prevalence of obesity [85,86], a well-characterized risk factor for both incidence and worse prognosis of ER+/luminal BC subtypes, threatens to limit the

progress in the management of this disease, highlighting the need for further in-depth characterization of obesity related ER–driven tumorigenesis.

The diverse spectrum of obesity effects in mammary tumorigenesis, as well as association between obesity and ER+ tumors predominantly in postmenopausal patients [13,14,26,27] (in which circulating levels of the hormone drop after cessation of ovarian estrogen production), are far from being fully characterized [41,42]. The ER+BC promoting action of obesity, mainly in postmenopausal women, has been attributed to increase in circulating [87] and adipose tissue levels of estrogen, as a result of upregulation of aromatase enzyme in fat depots of obese patients [13,16,18,30,41,42]. Yet, postmenopausal circulating levels of the hormone in these patients appear to be insufficient to activate signaling through ER α expressed in the uterine tissue [88] (as endometrial thickening does not occur in the majority of the cases [42]). This notion implies that, in addition to increased estrogen levels, the extent of ER α expression may serve as an important determinant of estrogen-driven BC progression under obese conditions.

Here we showed that tumor-associated macrophages (TAMs) can augment estrogen signaling in BC cells through upregulation of ER α expression. We found that the obese state upregulated ER α expression in a murine model of obesity-associated BC (Figure 1), and demonstrated contribution of M ϕ , adversely-activated by obesogenic substances (e.g., SFA) through TLR4-dependent mechanism, to ER α upregulation in mouse/human BC cells (Figures 2 and 3; Supplementary Figure S2). We also showed that M ϕ -mediated increase of ER α levels in BC cells influenced estrogen sensitivity (Figure 4)—a critical component in hormone-related cancer progression and response to endocrine therapy [7]. Indeed, ER α is the key driver of breast tumorigenesis in ~75% of cases, and, at the same time, higher ER α load is often considered as predicting better prognosis in the general population of systemically-treated ER + BC patients, which is typically explained by the ability to effectively target ER α by existing hormonal therapy. Noteworthy is the fact that a recent study based on a cohort of >30,000 BC patients found no clear evidence for an association between higher ER load and better outcome [89]. In this respect, our present findings suggest that in the sub-population of obese postmenopausal ER+BC patients (especially in the face of increased estrogen production in fat depots [13,16,20,29,32]), upregulation of ER α expression by the tumor may promote BC progression and render it less sensitive to standard therapeutic approaches (i.e., estrogen deprivation (Figure 4), and ionizing radiation (Supplementary Figure S1C)).

M ϕ infiltrating the tumor tissue or adjacent adipose tissue have been shown to contribute to several important features of malignant growth, and correlate with poor prognostic signs (i.e., high tumor grade, high mitotic activity, and metastasis [17,90–92]). Yet, the emerging role of M ϕ (and other tumor-residing immunocytes) in the regulation of steroid receptor expression in hormone-responsive tumors (i.e., through secretion of cytokines capable of inducing ERS1 gene expression) only recently came to be appreciated [43,44,69]. When considered in the context of hormone-dependent tumors, our findings suggest a bidirectional action of M ϕ in obesity-associated breast tumorigenesis, through which TAMs augment estrogen signaling in BC via upregulation of ER α (present study), while adipose tissue M ϕ contribute to this augmentation through an increase in estrogen levels (via induction of aromatase expression in stromal cells of ‘obese’ adipose tissue [16,30,41,42]). Interesting to note, M ϕ , per se, reportedly express ER and estrogen can affect some of the macrophage functions; yet, the role of ER signaling in the control of the M ϕ reactivity remains poorly understood, and both findings supporting the activating and suppressing effects of the hormone on M ϕ have been published [93].

Taken together, the above findings suggest beneficial effect(s) of approaches aimed at manipulating M ϕ in BC, especially when taken together with previous reports at-testing to M ϕ being an attractive target in mammary tumorigenesis, in general, and obesity-accelerated BC progression, in particular [16,30,33,34,94]. Yet, initial clinical studies (utilizing various agents decreasing M ϕ content) demonstrated mixed results [95,96]. The likely explanation for the somewhat limited benefit of the direct M ϕ targeting approach is

provided by the dynamic nature and highly heterogeneous phenotypes of M ϕ [92,97]. This notion is particularly relevant in the context of obesity: a recent report revealed that ‘obese’ visceral adipose tissue contains seven distinct M ϕ populations with potentially different functions/cytokine profiles [98]. Given this complexity, our findings pointing to the ability of heparanase to dictate the ER-inducing phenotype of M ϕ in BC (Figures 5 and 6), and, therefore, control one of the fundamental features of hormone-dependent breast tumorigenesis, namely, the level of ER expression, suggest that modulation of the enzyme activity (rather than targeting M ϕ per se [95]) may offer an appealing alternative approach to uncouple obesity and breast cancer progression in a rapidly growing population of obese patients. Of note, a recent study (based on microarray datasets comprising >7000 BC patients, as well as prospective data from the BIG 2–98 repository [52]) demonstrated that overexpression of heparanase in BC tumors was associated with worse outcome/increased risk of recurrence in luminal (ER+) subtypes, but did not affect outcome/risk in non-luminal (ER–) subtypes [52]. This pattern of association is essentially similar to one reported for obesity, per se, which correlates with worse prognosis in BC patients bearing luminal tumors, while no such association was found in non-luminal tumors [13,14,26,27], and further supports the contribution of the enzyme to BC-promoting effects of obesity through M ϕ -mediated upregulation of ER.

Collectively, the data presented here reveal previously unknown crosstalk occurring between tumor, adipose and immune compartments in hormone-responsive obesity-associated BC. Moreover, as techniques to effectively screen for the M ϕ infiltration/heparanase expression in tumor tissue become available, and heparanase-targeting approaches are pre-clinically and clinically developed [99], our findings provide a basis for further studies aimed at manipulating estrogen signaling and suppressing breast cancer-promoting consequences of excess adiposity in an increasingly obese population.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells11182844/s1>, Figure S1: Effect of HFD-induced obesity on tumor growth, radiotherapy resistance and macrophage infiltration in murine breast carcinoma model; Figure S2: 4T1 murine BC cells; Figure S3: E0771 (left) and MCF7 (right) cells were either remained untreated (Cont.) or incubated (24 h, 37 °C) with LPS or medium conditioned by macrophages stimulated by LPS at concentration 0.1 ng/mL (M ϕ -LPS). ER α protein levels were determined by immunoblotting (top) and quantified using ImageJ software (bottom). Intensity ratio for ER α /GAPDH is shown. The data are representative of at least 3 independent experiments; error bars represent \pm SD. * $p < 0.007$ Figure S4: Heparanase augments ability of sFA-stimulated macrophages to upregulate ER α expression in BC cells.

Author Contributions: D.N.B., E.H., A.M.R., E.D. and A.M. conducted experiments; O.M. and B.M. acquired data; T.P. generated/characterized clinical databases; IV generated/provided reagents and animals; D.N.B., E.H., A.S., E.D., B.M., A.P., A.M. and M.E. analyzed data; A.M. and M.E. designed research studies, A.S., I.V., A.P., T.P. and A.M. reviewed and edited the manuscript, A.M. and M.E. were responsible for conceptualization and study design; M.E. supervised the study, wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by grants from the Israel Science Foundation (# 1715/17 and 2292/21), the Israel Cancer Research Fund, the Israel Cancer Association, the Zuker Arthur and Ludmilla Fund through the Keren Kayemet LeIsrael and the Gassner fund.

Institutional Review Board Statement: The use of formalin-fixed, paraffin-embedded human breast carcinoma tissues and clinical data was approved by the Human Subjects Research Ethics Committee of the Hadassah Medical Center. Animal experiments were approved by Institutional Animal Care Committee of the Hebrew University.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this article.

Conflicts of Interest: The authors declare no potential conflicts of interest.

References

1. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2019. *CA Cancer J. Clin.* **2019**, *69*, 7–34. [[CrossRef](#)]
2. Prat, A.; Pineda, E.; Adamo, B.; Galvan, P.; Fernandez, A.; Gaba, L.; Diez, M.; Viladot, M.; Arance, A.; Munoz, M. Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast* **2015**, *24* (Suppl. S2), S26–S35. [[CrossRef](#)]
3. Cancer Genome Atlas, N. Comprehensive molecular portraits of human breast tumours. *Nature* **2012**, *490*, 61–70. [[CrossRef](#)] [[PubMed](#)]
4. Caan, B.J.; Sweeney, C.; Habel, L.A.; Kwan, M.L.; Kroenke, C.H.; Weltzien, E.K.; Quesenberry, C.P., Jr.; Castillo, A.; Factor, R.E.; Kushi, L.H.; et al. Intrinsic subtypes from the PAM50 gene expression assay in a population-based breast cancer survivor cohort: Prognostication of short- and long-term outcomes. *Cancer Epidemiol. Biomark. Prev.* **2014**, *23*, 725–734. [[CrossRef](#)] [[PubMed](#)]
5. Piperigkou, Z.; Karamanos, N.K. Estrogen receptor-mediated targeting of the extracellular matrix network in cancer. *Semin. Cancer Biol.* **2020**, *62*, 116–124. [[CrossRef](#)] [[PubMed](#)]
6. Hua, H.; Zhang, H.; Kong, Q.; Jiang, Y. Mechanisms for estrogen receptor expression in human cancer. *Exp. Hematol. Oncol.* **2018**, *7*, 24. [[CrossRef](#)]
7. Thomas, C.; Gustafsson, J.A. The different roles of ER subtypes in cancer biology and therapy. *Nat. Rev. Cancer* **2011**, *11*, 597–608. [[CrossRef](#)]
8. Rong, C.; Meinert, E.; Hess, J. Estrogen Receptor Signaling in Radiotherapy: From Molecular Mechanisms to Clinical Studies. *Int. J. Mol. Sci.* **2018**, *19*, 713. [[CrossRef](#)]
9. Siersbaek, R.; Kumar, S.; Carroll, J.S. Signaling pathways and steroid receptors modulating estrogen receptor alpha function in breast cancer. *Genes Dev.* **2018**, *32*, 1141–1154. [[CrossRef](#)]
10. Elkin, M.; Cohen, I.; Zcharia, E.; Orgel, A.; Guatta-Rangini, Z.; Peretz, T.; Vlodavsky, I.; Kleinman, H.K. Regulation of heparanase gene expression by estrogen in breast cancer. *Cancer Res.* **2003**, *63*, 8821–8826.
11. Elkin, M.; Orgel, A.; Kleinman, H.K. An angiogenic switch in breast cancer involves estrogen and soluble vascular endothelial growth factor receptor 1. *J. Natl. Cancer Inst.* **2004**, *96*, 875–878. [[CrossRef](#)]
12. Lung, D.K.; Reese, R.M.; Alarid, E.T. Intrinsic and Extrinsic Factors Governing the Transcriptional Regulation of ESR1. *Horm Cancer* **2020**, *11*, 129–147. [[CrossRef](#)] [[PubMed](#)]
13. Blair, C.K.; Wiggins, C.L.; Nibbe, A.M.; Storlie, C.B.; Prossnitz, E.R.; Royce, M.; Lomo, L.C.; Hill, D.A. Obesity and survival among a cohort of breast cancer patients is partially mediated by tumor characteristics. *NPJ Breast Cancer* **2019**, *5*, 33. [[CrossRef](#)] [[PubMed](#)]
14. Lauby-Secretan, B.; Scoccianti, C.; Loomis, D.; Grosse, Y.; Bianchini, F.; Straif, K.; International Agency for Research on Cancer Handbook Working. Body Fatness and Cancer—Viewpoint of the IARC Working Group. *N. Engl. J. Med.* **2016**, *375*, 794–798. [[CrossRef](#)] [[PubMed](#)]
15. Pearson-Stuttard, J.; Zhou, B.; Kontis, V.; Bentham, J.; Gunter, M.J.; Ezzati, M. Worldwide burden of cancer attributable to diabetes and high body-mass index: A comparative risk assessment. *Lancet Diabetes Endocrinol.* **2018**, *6*, e6–e15. [[CrossRef](#)]
16. Iyengar, N.M.; Gucalp, A.; Dannenberg, A.J.; Hudis, C.A. Obesity and Cancer Mechanisms: Tumor Microenvironment and Inflammation. *J. Clin. Oncol.* **2016**, *34*, 4270–4276. [[CrossRef](#)]
17. Baumgarten, S.C.; Frasor, J. Minireview: Inflammation: An instigator of more aggressive estrogen receptor (ER) positive breast cancers. *Mol. Endocrinol.* **2012**, *26*, 360–371. [[CrossRef](#)]
18. Cleary, M.P.; Grossmann, M.E. Minireview: Obesity and breast cancer: The estrogen connection. *Endocrinology* **2009**, *150*, 2537–2542. [[CrossRef](#)]
19. Lee, K.; Kruper, L.; Dieli-Conwright, C.M.; Mortimer, J.E. The Impact of Obesity on Breast Cancer Diagnosis and Treatment. *Curr. Oncol. Rep.* **2019**, *21*, 41. [[CrossRef](#)]
20. Gerard, C.; Brown, K.A. Obesity and breast cancer—Role of estrogens and the molecular underpinnings of aromatase regulation in breast adipose tissue. *Mol. Cell Endocrinol.* **2018**, *466*, 15–30. [[CrossRef](#)]
21. Santa-Maria, C.A.; Yan, J.; Xie, X.J.; Euhus, D.M. Aggressive estrogen-receptor-positive breast cancer arising in patients with elevated body mass index. *Int. J. Clin. Oncol.* **2015**, *20*, 317–323. [[CrossRef](#)]
22. Chan, D.S.; Norat, T. Obesity and breast cancer: Not only a risk factor of the disease. *Curr. Treat Options Oncol.* **2015**, *16*, 22. [[CrossRef](#)]
23. Sestak, I.; Distler, W.; Forbes, J.F.; Dowsett, M.; Howell, A.; Cuzick, J. Effect of body mass index on recurrences in tamoxifen and anastrozole treated women: An exploratory analysis from the ATAC trial. *J. Clin. Oncol.* **2010**, *28*, 3411–3415. [[CrossRef](#)]
24. Park, Y.; Colditz, G.A. Diabetes and adiposity: A heavy load for cancer. *Lancet Diabetes Endocrinol.* **2018**, *6*, 82–83. [[CrossRef](#)]
25. Cespedes Feliciano, E.M.; Kwan, M.L.; Kushi, L.H.; Chen, W.Y.; Weltzien, E.K.; Castillo, A.L.; Sweeney, C.; Bernard, P.S.; Caan, B.J. Body mass index, PAM50 subtype, recurrence, and survival among patients with nonmetastatic breast cancer. *Cancer* **2017**, *123*, 2535–2542. [[CrossRef](#)] [[PubMed](#)]
26. Neuhouser, M.L.; Aragaki, A.K.; Prentice, R.L.; Manson, J.E.; Chlebowski, R.; Carty, C.L.; Ochs-Balcom, H.M.; Thomson, C.A.; Caan, B.J.; Tinker, L.F.; et al. Overweight, Obesity, and Postmenopausal Invasive Breast Cancer Risk: A Secondary Analysis of the Women’s Health Initiative Randomized Clinical Trials. *JAMA Oncol.* **2015**, *1*, 611–621. [[CrossRef](#)]
27. Nattenmuller, C.J.; Kriegsmann, M.; Sookthai, D.; Fortner, R.T.; Steffen, A.; Walter, B.; Johnson, T.; Kneisel, J.; Katzke, V.; Bergmann, M.; et al. Obesity as risk factor for subtypes of breast cancer: Results from a prospective cohort study. *BMC Cancer* **2018**, *18*, 616. [[CrossRef](#)] [[PubMed](#)]

28. AACR. AACR Cancer Progress Report 2021. Available online: <https://cancerprogressreport.aacr.org/progress/> (accessed on 1 June 2022).
29. Bhardwaj, P.; Au, C.C.; Benito-Martin, A.; Ladumor, H.; Oshchepkova, S.; Moges, R.; Brown, K.A. Estrogens and breast cancer: Mechanisms involved in obesity-related development, growth and progression. *J. Steroid. Biochem. Mol. Biol.* **2019**, *189*, 161–170. [[CrossRef](#)]
30. Hermano, E.; Goldberg, R.; Rubinstein, A.M.; Sonnenblick, A.; Maly, B.; Nahmias, D.; Li, J.P.; Bakker, M.A.H.; van der Vlag, J.; Vlodaysky, I.; et al. Heparanase Accelerates Obesity-Associated Breast Cancer Progression. *Cancer Res.* **2019**, *79*, 5342–5354. [[CrossRef](#)] [[PubMed](#)]
31. Brown, K.A.; Iyengar, N.M.; Zhou, X.K.; Gucalp, A.; Subbaramaiah, K.; Wang, H.; Giri, D.D.; Morrow, M.; Falcone, D.J.; Wendel, N.K.; et al. Menopause Is a Determinant of Breast Aromatase Expression and Its Associations With BMI, Inflammation, and Systemic Markers. *J. Clin. Endocrinol. Metab.* **2017**, *102*, 1692–1701. [[CrossRef](#)] [[PubMed](#)]
32. Zahid, H.; Simpson, E.R.; Brown, K.A. Inflammation, dysregulated metabolism and aromatase in obesity and breast cancer. *Curr. Opin. Pharm.* **2016**, *31*, 90–96. [[CrossRef](#)] [[PubMed](#)]
33. Arendt, L.M.; McCreedy, J.; Keller, P.J.; Baker, D.D.; Naber, S.P.; Seewaldt, V.; Kuperwasser, C. Obesity promotes breast cancer by CCL2-mediated macrophage recruitment and angiogenesis. *Cancer Res.* **2013**, *73*, 6080–6093. [[CrossRef](#)] [[PubMed](#)]
34. Incio, J.; Tam, J.; Rahbari, N.N.; Suboj, P.; McManus, D.T.; Chin, S.M.; Vardam, T.D.; Batista, A.; Babykutty, S.; Jung, K.; et al. PIGF/VEGFR-1 Signaling Promotes Macrophage Polarization and Accelerated Tumor Progression in Obesity. *Clin. Cancer Res.* **2016**, *22*, 2993–3004. [[CrossRef](#)]
35. Trinchieri, G. Cancer and inflammation: An old intuition with rapidly evolving new concepts. *Annu. Rev. Immunol.* **2012**, *30*, 677–706. [[CrossRef](#)] [[PubMed](#)]
36. Jensen, M.D.; Haymond, M.W.; Rizza, R.A.; Cryer, P.E.; Miles, J.M. Influence of body fat distribution on free fatty acid metabolism in obesity. *J. Clin. Investig.* **1989**, *83*, 1168–1173. [[CrossRef](#)] [[PubMed](#)]
37. Shi, H.; Kokoeva, M.V.; Inouye, K.; Tzamei, I.; Yin, H.; Flier, J.S. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. Clin. Investig.* **2006**, *116*, 3015–3025. [[CrossRef](#)]
38. Lager, S.; Gaccioli, F.; Ramirez, V.I.; Jones, H.N.; Jansson, T.; Powell, T.L. Oleic acid stimulates system A amino acid transport in primary human trophoblast cells mediated by toll-like receptor 4. *J. Lipid Res.* **2013**, *54*, 725–733. [[CrossRef](#)]
39. Tsukumo, D.M.; Carvalho-Filho, M.A.; Carvalheira, J.B.; Prada, P.O.; Hirabara, S.M.; Schenka, A.A.; Araujo, E.P.; Vassallo, J.; Curi, R.; Velloso, L.A.; et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* **2007**, *56*, 1986–1998. [[CrossRef](#)]
40. Bigornia, S.J.; Farb, M.G.; Mott, M.M.; Hess, D.T.; Carmine, B.; Fiscale, A.; Joseph, L.; Apovian, C.M.; Gokce, N. Relation of depot-specific adipose inflammation to insulin resistance in human obesity. *Nutr. Diabetes* **2012**, *2*, e30. [[CrossRef](#)]
41. Bhardwaj, P.; Brown, K.A. Obese Adipose Tissue as a Driver of Breast Cancer Growth and Development: Update and Emerging Evidence. *Front Oncol.* **2021**, *11*, 638918. [[CrossRef](#)]
42. Brown, K.A. Metabolic pathways in obesity-related breast cancer. *Nat. Rev. Endocrinol.* **2021**, *17*, 350–363. [[CrossRef](#)] [[PubMed](#)]
43. Ning, C.; Xie, B.; Zhang, L.; Li, C.; Shan, W.; Yang, B.; Luo, X.; Gu, C.; He, Q.; Jin, H.; et al. Infiltrating Macrophages Induce ERalpha Expression through an IL17A-mediated Epigenetic Mechanism to Sensitize Endometrial Cancer Cells to Estrogen. *Cancer Res.* **2016**, *76*, 1354–1366. [[CrossRef](#)] [[PubMed](#)]
44. Wang, X.; Lin, W.J.; Izumi, K.; Jiang, Q.; Lai, K.P.; Xu, D.; Fang, L.Y.; Lu, T.; Li, L.; Xia, S.; et al. Increased infiltrated macrophages in benign prostatic hyperplasia (BPH): Role of stromal androgen receptor in macrophage-induced prostate stromal cell proliferation. *J. Biol. Chem.* **2012**, *287*, 18376–18385. [[CrossRef](#)]
45. Masola, V.; Zaza, G.; Bellin, G.; Dall’Omo, L.; Granata, S.; Vischini, G.; Secchi, M.F.; Lupo, A.; Gambaro, G.; Onisto, M. Heparanase regulates the M1 polarization of renal macrophages and their crosstalk with renal epithelial tubular cells after ischemia/reperfusion injury. *FASEB J.* **2018**, *32*, 742–756. [[CrossRef](#)]
46. Mayfosh, A.J.; Baschuk, N.; Hulett, M.D. Leukocyte Heparanase: A Double-Edged Sword in Tumor Progression. *Front Oncol.* **2019**, *9*, 331. [[CrossRef](#)] [[PubMed](#)]
47. Gutter-Kapon, L.; Alishekevitz, D.; Shaked, Y.; Li, J.P.; Aronheim, A.; Ilan, N.; Vlodaysky, I. Heparanase is required for activation and function of macrophages. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E7808–E7817. [[CrossRef](#)] [[PubMed](#)]
48. Goldberg, R.; Rubinstein, A.M.; Gil, N.; Hermano, E.; Li, J.P.; van der Vlag, J.; Atzmon, R.; Meirovitz, A.; Elkin, M. Role of heparanase-driven inflammatory cascade in pathogenesis of diabetic nephropathy. *Diabetes* **2014**, *63*, 4302–4313. [[CrossRef](#)]
49. Hermano, E.; Carlotti, F.; Abecassis, A.; Meirovitz, A.; Rubinstein, A.M.; Li, J.P.; Vlodaysky, I.; Rabelink, T.J.; Elkin, M. Dichotomic role of heparanase in a murine model of metabolic syndrome. *Cell Mol. Life Sci.* **2021**, *78*, 2771–2780. [[CrossRef](#)] [[PubMed](#)]
50. Hermano, E.; Meirovitz, A.; Meir, K.; Nussbaum, G.; Appelbaum, L.; Peretz, T.; Elkin, M. Macrophage polarization in pancreatic carcinoma: Role of heparanase enzyme. *J. Natl. Cancer Inst.* **2014**, *106*, dju332. [[CrossRef](#)]
51. Lerner, I.; Hermano, E.; Zcharia, E.; Rodkin, D.; Bulvik, R.; Doviner, V.; Rubinstein, A.M.; Ishai-Michaeli, R.; Atzmon, R.; Sherman, Y.; et al. Heparanase powers a chronic inflammatory circuit that promotes colitis-associated tumorigenesis in mice. *J. Clin. Investig.* **2011**, *121*, 1709–1721. [[CrossRef](#)]
52. Zahavi, T.; Salmon-Divon, M.; Salgado, R.; Elkin, M.; Hermano, E.; Rubinstein, A.M.; Francis, P.A.; Di Leo, A.; Viale, G.; de Azambuja, E.; et al. Heparanase: A potential marker of worse prognosis in estrogen receptor-positive breast cancer. *NPJ Breast Cancer* **2021**, *7*, 67. [[CrossRef](#)] [[PubMed](#)]

53. Cohen, I.; Maly, B.; Simon, I.; Meirovitz, A.; Pikarsky, E.; Zcharia, E.; Peretz, T.; Vlodaysky, I.; Elkin, M. Tamoxifen induces heparanase expression in estrogen receptor-positive breast cancer. *Clin. Cancer Res.* **2007**, *13*, 4069–4077. [[CrossRef](#)]
54. Hammond, M.E.; Hayes, D.F.; Dowsett, M.; Allred, D.C.; Hagerty, K.L.; Badve, S.; Fitzgibbons, P.L.; Francis, G.; Goldstein, N.S.; Hayes, M.; et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *Arch. Pathol. Lab. Med.* **2010**, *134*, 907–922. [[CrossRef](#)] [[PubMed](#)]
55. Cheang, M.C.; Treaba, D.O.; Speers, C.H.; Olivotto, I.A.; Bajdik, C.D.; Chia, S.K.; Goldstein, L.C.; Gelmon, K.A.; Huntsman, D.; Gilks, C.B.; et al. Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival. *J. Clin. Oncol.* **2006**, *24*, 5637–5644. [[CrossRef](#)]
56. Zcharia, E.; Jia, J.; Zhang, X.; Baraz, L.; Lindahl, U.; Peretz, T.; Vlodaysky, I.; Li, J.P. Newly generated heparanase knock-out mice unravel co-regulation of heparanase and matrix metalloproteinases. *PLoS ONE* **2009**, *4*, e5181. [[CrossRef](#)]
57. Meirovitz, A.; Hermano, E.; Lerner, I.; Zcharia, E.; Pisano, C.; Peretz, T.; Elkin, M. Role of heparanase in radiation-enhanced invasiveness of pancreatic carcinoma. *Cancer Res.* **2011**, *71*, 2772–2780. [[CrossRef](#)] [[PubMed](#)]
58. Morgan, M.M.; Arendt, L.M.; Alarid, E.T.; Beebe, D.J.; Johnson, B.P. Mammary adipose stromal cells derived from obese women reduce sensitivity to the aromatase inhibitor anastrozole in an organotypic breast model. *FASEB J.* **2019**, *33*, 8623–8633. [[CrossRef](#)] [[PubMed](#)]
59. Maguire, O.A.; Ackerman, S.E.; Szwed, S.K.; Maganti, A.V.; Marchildon, F.; Huang, X.; Kramer, D.J.; Rosas-Villegas, A.; Gelfer, R.G.; Turner, L.E.; et al. Creatine-mediated crosstalk between adipocytes and cancer cells regulates obesity-driven breast cancer. *Cell Metab.* **2021**, *33*, 499–512.e496. [[CrossRef](#)]
60. Le Naour, A.; Rossary, A.; Vasson, M.P. EO771, is it a well-characterized cell line for mouse mammary cancer model? Limit and uncertainty. *Cancer Med.* **2020**, *9*, 8074–8085. [[CrossRef](#)]
61. Hiraga, T.; Ninomiya, T. Establishment and characterization of a C57BL/6 mouse model of bone metastasis of breast cancer. *J. Bone Min. Metab.* **2019**, *37*, 235–242. [[CrossRef](#)]
62. Gu, J.W.; Young, E.; Busby, B.; Covington, J.; Johnson, J.W. Oral administration of pyrrolidine dithiocarbamate (PDTC) inhibits VEGF expression, tumor angiogenesis, and growth of breast cancer in female mice. *Cancer Biol. Ther.* **2009**, *8*, 514–521. [[CrossRef](#)]
63. Montgomery, M.K.; Hallahan, N.L.; Brown, S.H.; Liu, M.; Mitchell, T.W.; Cooney, G.J.; Turner, N. Mouse strain-dependent variation in obesity and glucose homeostasis in response to high-fat feeding. *Diabetologia* **2013**, *56*, 1129–1139. [[CrossRef](#)] [[PubMed](#)]
64. Kanfi, Y.; Peshti, V.; Gil, R.; Naiman, S.; Nahum, L.; Levin, E.; Kronfeld-Schor, N.; Cohen, H.Y. SIRT6 protects against pathological damage caused by diet-induced obesity. *Aging Cell* **2010**, *9*, 162–173. [[CrossRef](#)] [[PubMed](#)]
65. Mentoor, I.; Nell, T.; Emjedi, Z.; van Jaarsveld, P.J.; de Jager, L.; Engelbrecht, A.M. Decreased Efficacy of Doxorubicin Corresponds with Modifications in Lipid Metabolism Markers and Fatty Acid Profiles in Breast Tumors From Obese vs. Lean Mice. *Front. Oncol.* **2020**, *10*, 306. [[CrossRef](#)] [[PubMed](#)]
66. Pettersson, U.S.; Walden, T.B.; Carlsson, P.O.; Jansson, L.; Phillipson, M. Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. *PLoS ONE* **2012**, *7*, e46057. [[CrossRef](#)] [[PubMed](#)]
67. Chantalat, E.; Boudou, F.; Laurell, H.; Paliarne, G.; Houtman, R.; Melchers, D.; Rochaix, P.; Filleron, T.; Stella, A.; Burlet-Schiltz, O.; et al. The AF-1-deficient estrogen receptor ERalpha46 isoform is frequently expressed in human breast tumors. *Breast Cancer Res.* **2016**, *18*, 123. [[CrossRef](#)]
68. Li, L.; Haynes, M.P.; Bender, J.R. Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4807–4812. [[CrossRef](#)]
69. Majorini, M.T.; Cancila, V.; Rigoni, A.; Botti, L.; Dugo, M.; Triulzi, T.; De Cecco, L.; Fontanella, E.; Jachetti, E.; Tagliabue, E.; et al. Infiltrating Mast Cell-Mediated Stimulation of Estrogen Receptor Activity in Breast Cancer Cells Promotes the Luminal Phenotype. *Cancer Res.* **2020**, *80*, 2311–2324. [[CrossRef](#)]
70. Koutsari, C.; Ali, A.H.; Mundi, M.S.; Jensen, M.D. Storage of circulating free fatty acid in adipose tissue of postabsorptive humans: Quantitative measures and implications for body fat distribution. *Diabetes* **2011**, *60*, 2032–2040. [[CrossRef](#)] [[PubMed](#)]
71. Lumeng, C.N.; Saltiel, A.R. Inflammatory links between obesity and metabolic disease. *J. Clin. Investig.* **2011**, *121*, 2111–2117. [[CrossRef](#)]
72. Fu, J.; Zhang, L.; He, T.; Xiao, X.; Liu, X.; Wang, L.; Yang, L.; Yang, M.; Zhang, T.; Chen, R.; et al. TWIST represses estrogen receptor-alpha expression by recruiting the NuRD protein complex in breast cancer cells. *Int. J. Biol. Sci.* **2012**, *8*, 522–532. [[CrossRef](#)] [[PubMed](#)]
73. Baxter, E.W.; Graham, A.E.; Re, N.A.; Carr, I.M.; Robinson, J.I.; Mackie, S.L.; Morgan, A.W. Standardized protocols for differentiation of THP-1 cells to macrophages with distinct M(IFNgamma+LPS), M(IL-4) and M(IL-10) phenotypes. *J. Immunol. Methods* **2020**, *478*, 112721. [[CrossRef](#)] [[PubMed](#)]
74. Mohammad, S.; Thiemermann, C. Role of Metabolic Endotoxemia in Systemic Inflammation and Potential Interventions. *Front Immunol.* **2020**, *11*, 594150. [[CrossRef](#)]
75. Cao, J.; Peng, J.; An, H.; He, Q.; Boronina, T.; Guo, S.; White, M.F.; Cole, P.A.; He, L. Endotoxemia-mediated activation of acetyltransferase P300 impairs insulin signaling in obesity. *Nat. Commun.* **2017**, *8*, 131. [[CrossRef](#)] [[PubMed](#)]

76. Cani, P.D.; Bibiloni, R.; Knauf, C.; Waget, A.; Neyrinck, A.M.; Delzenne, N.M.; Burcelin, R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* **2008**, *57*, 1470–1481. [[CrossRef](#)] [[PubMed](#)]
77. Amar, J.; Burcelin, R.; Ruidavets, J.B.; Cani, P.D.; Fauvel, J.; Alessi, M.C.; Chamontin, B.; Ferrieres, J. Energy intake is associated with endotoxemia in apparently healthy men. *Am. J. Clin. Nutr.* **2008**, *87*, 1219–1223. [[CrossRef](#)] [[PubMed](#)]
78. Troseid, M.; Nestvold, T.K.; Rudi, K.; Thoresen, H.; Nielsen, E.W.; Lappégard, K.T. Plasma lipopolysaccharide is closely associated with glycemic control and abdominal obesity: Evidence from bariatric surgery. *Diabetes Care* **2013**, *36*, 3627–3632. [[CrossRef](#)]
79. Bergom, C.; Kelly, T.; Bedi, M.; Saeed, H.; Prior, P.; Rein, L.E.; Szabo, A.; Wilson, J.F.; Currey, A.D.; White, J. Association of Locoregional Control With High Body Mass Index in Women Undergoing Breast Conservation Therapy for Early-Stage Breast Cancer. *Int. J. Radiat. Oncol. Biol. Phys.* **2016**, *96*, 65–71. [[CrossRef](#)]
80. Brunn, G.J.; Bungum, M.K.; Johnson, G.B.; Platt, J.L. Conditional signaling by Toll-like receptor 4. *FASEB J.* **2005**, *19*, 872–874. [[CrossRef](#)]
81. Goodall, K.J.; Poon, I.K.; Phipps, S.; Hulett, M.D. Soluble Heparan Sulfate Fragments Generated by Heparanase Trigger the Release of Pro-Inflammatory Cytokines through TLR-4. *PLoS ONE* **2014**, *9*, e109596. [[CrossRef](#)]
82. Johnson, G.B.; Brunn, G.J.; Kodaira, Y.; Platt, J.L. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. *J. Immunol.* **2002**, *168*, 5233–5239. [[CrossRef](#)]
83. Yu, L.; Wang, L.; Chen, S. Endogenous toll-like receptor ligands and their biological significance. *J. Cell Mol. Med.* **2010**, *14*, 2592–2603. [[CrossRef](#)] [[PubMed](#)]
84. Akbarshahi, H.; Axelsson, J.B.; Said, K.; Malmstrom, A.; Fischer, H.; Andersson, R. TLR4 dependent heparan sulphate-induced pancreatic inflammatory response is IRF3-mediated. *J. Transl. Med.* **2011**, *9*, 219. [[CrossRef](#)]
85. WHO. Obesity and Overweight, World Health Organization Fact Sheet. Available online: <http://www.who.int/mediacentre/factsheets/fs311/en/2018> (accessed on 25 May 2022).
86. Ng, M.; Fleming, T.; Robinson, M.; Thomson, B.; Graetz, N.; Margono, C.; Mullany, E.C.; Biryukov, S.; Abbafati, C.; Abera, S.F.; et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: A systematic analysis for the Global Burden of Disease Study 2013. *Lancet* **2014**, *384*, 766–781. [[CrossRef](#)]
87. McTiernan, A.; Wu, L.; Chen, C.; Chlebowski, R.; Mossavar-Rahmani, Y.; Modugno, F.; Perri, M.G.; Stanczyk, F.Z.; Van Horn, L.; Wang, C.Y.; et al. Relation of BMI and physical activity to sex hormones in postmenopausal women. *Obesity* **2006**, *14*, 1662–1677. [[CrossRef](#)] [[PubMed](#)]
88. Winuthayanon, W.; Lierz, S.L.; Delarosa, K.C.; Sampels, S.R.; Donoghue, L.J.; Hewitt, S.C.; Korach, K.S. Juxtacrine Activity of Estrogen Receptor alpha in Uterine Stromal Cells is Necessary for Estrogen-Induced Epithelial Cell Proliferation. *Sci. Rep.* **2017**, *7*, 8377. [[CrossRef](#)] [[PubMed](#)]
89. Noordhoek, I.; de Groot, A.F.; Cohen, D.; Liefers, G.J.; Portielje, J.E.A.; Kroep, J.R. Higher ER load is not associated with better outcome in stage 1-3 breast cancer: A descriptive overview of quantitative HR analysis in operable breast cancer. *Breast Cancer Res. Treat* **2019**, *176*, 27–36. [[CrossRef](#)] [[PubMed](#)]
90. Linde, N.; Casanova-Acebes, M.; Sosa, M.S.; Mortha, A.; Rahman, A.; Farias, E.; Harper, K.; Tardio, E.; Reyes Torres, I.; Jones, J.; et al. Macrophages orchestrate breast cancer early dissemination and metastasis. *Nat. Commun.* **2018**, *9*, 21. [[CrossRef](#)]
91. DeNardo, D.G.; Brennan, D.J.; Rexhepaj, E.; Ruffell, B.; Shiao, S.L.; Madden, S.F.; Gallagher, W.M.; Wadhvani, N.; Keil, S.D.; Junaid, S.A.; et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov.* **2011**, *1*, 54–67. [[CrossRef](#)]
92. Mantovani, A.; Marchesi, F.; Malesci, A.; Laghi, L.; Allavena, P. Tumour-associated macrophages as treatment targets in oncology. *Nat. Rev. Clin. Oncol.* **2017**, *14*, 399–416. [[CrossRef](#)]
93. Pepe, G.; Locati, M.; Della Torre, S.; Mornata, F.; Cignarella, A.; Maggi, A.; Vegeto, E. The estrogen-macrophage interplay in the homeostasis of the female reproductive tract. *Hum. Reprod. Update* **2018**, *24*, 652–672. [[CrossRef](#)] [[PubMed](#)]
94. Hillers-Ziemer, L.E.; McMahon, R.Q.; Hietpas, M.; Paderta, G.; LeBeau, J.; McCready, J.; Arendt, L.M. Obesity Promotes Cooperation of Cancer Stem-Like Cells and Macrophages to Enhance Mammary Tumor Angiogenesis. *Cancers* **2020**, *12*, 502. [[CrossRef](#)] [[PubMed](#)]
95. Brown, J.M.; Recht, L.; Strober, S. The Promise of Targeting Macrophages in Cancer Therapy. *Clin. Cancer Res.* **2017**, *23*, 3241–3250. [[CrossRef](#)] [[PubMed](#)]
96. Chamberlin, T.; Clack, M.; Silvers, C.; Kuziel, G.; Thompson, V.; Johnson, H.; Arendt, L.M. Targeting Obesity-Induced Macrophages during Preneoplastic Growth Promotes Mammary Epithelial Stem/Progenitor Activity, DNA Damage, and Tumor Formation. *Cancer Res.* **2020**, *80*, 4465–4475. [[CrossRef](#)] [[PubMed](#)]
97. Ginhoux, F.; Schultze, J.L.; Murray, P.J.; Ochando, J.; Biswas, S.K. New insights into the multidimensional concept of macrophage ontogeny, activation and function. *Nat. Immunol.* **2016**, *17*, 34–40. [[CrossRef](#)] [[PubMed](#)]
98. Weinstock, A.; Brown, E.J.; Garabedian, M.L.; Pena, S.; Sharma, M.; Lafaille, J.; Moore, K.J.; Fisher, E.A. Single-Cell RNA Sequencing of Visceral Adipose Tissue Leukocytes Reveals that Caloric Restriction Following Obesity Promotes the Accumulation of a Distinct Macrophage Population with Features of Phagocytic Cells. *Immunometabolism* **2019**, *1*, e190008. [[CrossRef](#)]
99. Khanna, M.; Parish, C.R. Heparanase: Historical Aspects and Future Perspectives. *Adv. Exp. Med. Biol.* **2020**, *1221*, 71–96. [[PubMed](#)]