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The relevance of EGFR, ErbB receptors and neuregulins in human adipocytes and adipose tissue in obesity

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ABSTRACT

Objective: To investigate the potential role of EGFR, ErbBs receptors and neuregulins in human adipose tissue physiology in obesity.
Methods: Gene expression analysis in human subcutaneous (SAT) and visceral (VAT) adipose tissue in three independent cohorts [two cross-sectional (N = 150, N = 87) and one longitudinal (n = 25)], and *in vitro* gene knockdown and overexpression experiments were performed.
Results: While both SAT and VAT *ERBB2* and *ERBB4* mRNA increased in obesity, SAT *EGFR* mRNA was negatively correlated with insulin resistance, but did not change in obesity. Of note, both SAT and VAT *EGFR* mRNA were significantly associated with adipogenesis and increased during human adipocyte differentiation. *In vitro* experiments revealed that *EGFR*, but not *ERBB2* and *ERBB4*, gene knockdown in preadipocytes and in fully differentiated human adipocytes resulted in decreased expression of adipogenic-related genes. *ERBB2* gene knockdown also reduced gene expression of fatty acid synthase in fully differentiated adipocytes. In addition, neuregulin 2 (*NRG2*) mRNA was associated with expression of adipogenic genes in human adipose tissue and adipocytes, and its overexpression increased expression of *EGFR* and relevant adipogenic genes.
Conclusions: This study demonstrates the association between adipose tissue ERBB2 and obesity, confirms the relevance of EGFR on human adipogenesis, and suggests a possible adipogenic role of NRG2.

1. Introduction

Obesity is a worldwide epidemic associated to increased risk of type 2 diabetes, cardiovascular diseases, and several types of cancer, and it is the result of a positive balance that causes an excessive expansion and dysfunction of white adipose tissue [1]. Obesity-associated adipose tissue dysfunction is characterized by reduced capacity to store excess of carbohydrates and lipids and generate new adipocytes and increased markers of cellular senescence and inflammation, and in consequence, is a relevant contributor to the progression of insulin resistance and other obesity-associated metabolic disturbances, such as hyperglycemia and dyslipidemia [2–4].

Searching new relevant factors in human adipose tissue physiology, a recent study reported that epidermal growth factor receptor (EGFR)

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expression in subcutaneous adipose tissue was decreased in obese participants with insulin resistance, and positively correlated with expression of some adipogenic genes [5]. In contrast, erbB2 receptor tyrosine kinase 2 (ErbB2) expression was not associated to obesity, insulin resistance and adipogenesis. The cohort size (only 32 women) is the main weakness of this study [5]. However, previous *in vitro* studies reported contradictory data in relation to the possible role of these receptors in adipogenesis [6–10].

Whereas some studies reported that EGFR and ErbB2 mRNA and protein levels decreased during 3T3-L1 adipogenesis [6] and the erbB2 blockage using tyrphostins stimulated 3T3-L1 adipocyte differentiation under adipogenic conditions [7], other studies in the same cells found increased levels of these receptors in adipocyte and during adipogenesis, pointing to a possible role in adipogenesis [8,9]. In human adipose-derived stem cells (ASC), EGFR and ErbB2 expression and activity were significantly attenuated when adipocyte differentiation was induced, and ErbB2 inhibition increased intracellular lipid accumulation, but the joint inhibition of both EGFR and ErbB2 partially prevented these effects [10].

Neuregulins, which include four members NRG1, NRG2, NRG3 and NRG4, are ligands for receptor tyrosine kinases of the ErbB family [11]. In mice, positive effects on NRG4-ErbB signaling in adipose tissue through the preservation of its vasculature [12,13] and the induction of thermogenesis [14,15] were reported. In humans, *NRG4* gene expression in both subcutaneous and visceral adipose tissue was positively correlated with markers of adipose tissue browning, but negatively with adipogenesis [16], and decreased in subjects with obesity [17]. *In vitro* experiments also demonstrated that Nrg4 administration in 3T3-L1 adipocytes induced expression of browning-related genes and glucose uptake, without any effects on adipogenesis [18]. To the best of our knowledge other neuregulins (such as neuregulin 1, 2 and 3) and their correlations with ERBBs receptors at the expression level have not been examined in adipose tissue.

To clarify the potential role of EGFR and ErbB2 on human adipogenesis and on human adipose tissue physiology and to study the potential role of other ErbBs receptors and neuregulins in this tissue, here we aimed to investigate expression of ErbB receptors and neuregulins in human subcutaneous and visceral adipose tissue according to obesity and insulin resistance in two cross-sectional cohorts and, longitudinally, after 2- years bariatric surgery-induced weight loss. In addition, the impact of siRNA-induced gene knockdown on EGFR and ErbB2 in human preadipocyte and adipocyte, as well as the effects caused by neuregulin 2 (NRG2) overexpression were also tested.

2. Materials and Methods

2.1. Subjects' recruitment for adipose tissue samples

2.1.1. Cross-sectional studies

In cohort 1 and cohort 2, visceral (VAT) and subcutaneous (SAT) adipose tissues from 150 and 87 participants with normal body weight and different degrees of obesity, with body mass index (BMI) within 20 and 68 kg/m^2, were analyzed. All participants were recruited at the Endocrinology Service of the Hospital of Girona "Dr Josep Trueta". All subjects were of Caucasian origin and reported that their body weight had been stable for at least 3 months before the study. Subjects were studied in the post-absorptive state. BMI was calculated as weight (in kg) divided by height (in m) squared. They had no systemic disease other than obesity and all were free of any infections in the previous month before the study. Liver diseases (specifically tumoral disease and HCV infection) and thyroid dysfunction were specifically excluded by biochemical work-up.

2.2. Interventional studies

In cohort 3, twenty-five Caucasian obese (BMI= 43.7 ± 4.6 kg/m^2, age=47 ± 9 years [mean ± SD]) subjects, who underwent bariatric surgery thought Roux-en-Y gastric bypass in Hospital of Girona "Dr Josep Trueta" were part of a previous study [19]. Inclusion criteria were age between 30 and 60 years, BMI > 35 kg/m^2 and ability to understand the study protocol. Exclusion criteria were use of medications able to interfere with insulin action and history of a chronic systemic disease. Adipose tissue samples from the SAT depot were obtained during bariatric surgery. Postoperative samples of SAT were obtained by subcutaneous biopsy at the mesogastric level after 2 years from surgery. Fasting blood samples were obtained at the same day of the biopsy.

All these studies were carried out in accordance with the recommendations of the ethical committee of the Hospital of Girona "Dr Josep Trueta". The protocol was approved by the ethical committee of the Hospital of Girona "Dr Josep Trueta". All subjects gave written informed consent in accordance with the Declaration of Helsinki, after the purpose of the study was explained to them. Samples and data from patients included in this study were partially provided by the *FATBANK* platform promoted by the *CIBEROBN* and coordinated by the *IDIBGI Biobank* (Biobank *IDIBGI*, B.0000872), integrated in the Spanish National Biobanks Network and they were processed following standard operating procedures with the appropriate approval of the Ethics, External Scientific and FATBANK Internal Scientific Committees.

2.3. Adipose tissue collection and handling

Adipose tissue samples were obtained from SAT and VAT depots during elective surgical procedures (cholecystectomy, surgery of abdominal hernia and gastric bypass surgery). Both SAT and VAT samples were collected from the abdomen, following standard procedures. Samples of adipose tissue were immediately transported to the laboratory (5–10 min). The handling of tissue was carried out under strictly aseptic conditions. Adipose tissue samples were washed in phosphate-buffered saline (PBS), cut off with forceps and scalpel into small pieces (100 mg), and immediately flash-frozen in liquid nitrogen before stored at − 80ºC. The isolation of adipocyte and stromal vascular fraction cells (SVF) was performed from 16 SAT and 20 VAT non-frozen adipose tissue samples. These samples were washed three to four times with PBS and suspended in an equal volume of PBS supplemented with 1% penicillin-streptomycin and 0.1% collagenase type I prewarmed to 37 °C. The tissue was placed in a shaking water bath at 37 °C with continuous agitation for 60 min and centrifuged for 5 min at 400 g at room temperature. The supernatant, containing mature adipocytes, was recollected. The pellet was identified as the SVF. Isolated mature adipocytes and SVF stored at − 80ºC for gene expression analysis.

2.4. Analytical methods

Serum glucose concentrations were measured in duplicate by the glucose oxidase method using a Beckman glucose analyser II (Beckman Instruments, Brea, California). Glycosylated haemoglobin (HbA1c) was measured by the high-performance liquid chromatography method (Bio-Rad, Muenchen, Germany, and autoanalyser Jokoh HS-10, respectively). Intra- and inter-assay coefficients of variation were less than 4% for all these tests. Serum insulin was measured in duplicate by RIA (Medgenix Diagnostics, Fleunes, Belgium). The intra-assay coefficient of variation was 5.2% at a concentration of 10 mU/l and 3.4% at 130 mU/l. The interassay coefficients of variation were 6.9% and 4.5% at 14 and 89 mU/l, respectively. HOMA-IR was calculated using the following formula: [Insulin (mU/l) x Glucose mmol/l]/22.5. Roche Hitachi Cobas c711 instrument (Roche, Barcelona, Spain) was used to do HDL cholesterol and total serum triglycerides determinations. HDL cholesterol was quantified by a homogeneous enzymatic colorimetric assay through the cholesterol esterase / cholesterol oxidase / peroxidase reaction (Cobas HDLC3). Serum fasting triglycerides were measured by an enzymatic, colorimetric method with glycerol phosphate oxidase and peroxidase (Cobas TRIGL). LDL cholesterol was calculated using the

Friedewald formula.

2.5. Experiments in mouse primary immortalised preadipocytes

Primary preadipocytes were isolated from WT, Erbb2 and Erbb4 Flox c57BL/6 mice. These animals were maintained by the animal facility from the Faculty of Biology of the Barcelona University. Before the extraction, mice were under anaesthetic treatment with isoflurane. After that, the animals were sacrificed and the inguinal adipose tissue from 4 to 8 weeks-old mice was extracted. The tissue was then placed in a plate with PBS and 1% (v/v) antibiotic-antimycotic. Then, in a type IIA cell culture cabin, the tissue was rinsed 3 times with PBS. Afterwards, mechanical lysis was performed using stile scissors. When the mixture was homogeneous, enzymatic digestion was carried out with the digestion solution. The enzymatic digestion was performed at 120 rpm in a shaker (l26 Shaker, New BrunswickTM) at 37 ºC for 15 min. After that, the digested mix was filtered with a 40 μm cell strainer. Collagenase activity was inhibited by adding fresh primary preadipocyte growth media. Finally, the cell suspension was centrifuged at 1600 rpm for 10 min and the supernatant was discarded. The pellet containing preadipocytes was then resuspended in primary preadipocyte growth media and the cells were seeded in 6-well plates. Specifically, the cells obtained from 3 mice were seeded per 6-well plate. Primary preadipocytes were kept at 37 ºC and 7% CO2 with primary preadipocyte growth media. The media was replaced every 2 days. Cells were rinsed with PBS during the first media change to clean cellular debris. Afterwards, primary preadipocytes were immortalised. Primary preadipocytes were immortalised by retroviral transfection with the MLV retrovirus. The viral genome contains the gene sequence of the thermosensitive large T antigen from the SV40 virus. In addition, the retroviral genome also incorporates the puromycin resistance gene to facilitate immortalised cell selection. The expression of the large T antigen in preadipocytes is double conditional to avoid interference with the adipogenic differentiation. First, the puromycin resistance and the expression of the large T antigen are under the promotor of the IFNγ. Thus, the IFNγ is supplemented in the growth media of immortalised preadipocytes, allowing the expression of the large T antigen during the proliferative state. In addition, the large T antigen contains a mutation that makes it thermosensitive at 37 ºC. Therefore, the large T antigen is only stable at temperatures below 37 ºC and thus, primary immortalised preadipocytes are kept at 33 ºC during the proliferation stage. To induce adipogenesis, the IFNγ is removed and the temperature is increased to 37 ºC, which allow the cells to enter to a non-proliferative state. Erbb2 Flox (iFlox Erbb2) and Erbb4 Flox (iFlox Erbb4) primary immortalised preadipocytes were transduced with Scr-GFP and Cre-GFP adenoviral particles to remove the Erbb2 and Erbb4 gene respectively. To this end, primary immortalised preadipocytes were transduced with 300 μl of serum-free growth media containing the adenoviral particles supplemented with 0.2% (w/v) BSA and 1% antibiotic-antimycotic. The MOI used to delete the gene expression of Erbb2 and Erbb4 was optimised in this study as it can be seen in Figures 45, 46. It was determined to use 250 MOI to delete the expression of these genes and preserve the adipogenic capabilities of the preadipocytes. The transduction had a length of 3 h and it was done at 37 ºC. Afterwards, the transduction media was replaced with primary immortalised preadipocyte growth media for 72 h. Primary immortalised preadipocytes were differentiated to adipocytes by changing the growth media with primary immortalised adipocyte differentiation media [FBS (10%), insulin (1 μg/ml), IBMX (0.5 mM), Dexamethasone (0.25 μM), Rosiglitazone (2.5 μM)] for 72 h. After that, the first differentiation media was changed for primary immortalised adipocyte media 2 [FBS (10%), insulin (1 μg/ml)] for 24 h. Finally, the second differentiation media was substituted with primary immortalised adipocyte media 3 [FBS (10%), insulin (0.5 μg/ml)] for 72 h.

2.6. Differentiation of human preadipocytes

Isolated human subcutaneous (sc) and visceral (v) preadipocytes (Zen-Bio Inc., Research Triangle Park, NC) were plated on T-75 cell culture flasks and cultured at 37 C and 5% CO2 in DMEM/nutrient mix F-12 medium (1:1, vol/vol) supplemented with 10 U/ml penicillin/streptomycin, 10% fetal bovine serum (FBS), 1% HEPES, and 1% glutamine (all from GIBCO, Invitrogen S.A, Barcelona, Spain). One week later, the expanded human sc and v preadipocytes were cultured (~40,000 cells/cm2) in 12-well plates with preadipocytes medium (Zen-Bio) composed of DMEM/nutrient mix F-12 medium (1:1, vol/vol), HEPES, FBS, penicillin, and streptomycin in a humidified 37 C incubator with 5% CO2. Twenty-four hours after plating, cells were checked for complete confluence (d 0), and differentiation was induced using differentiation medium (Zen-Bio) composed of preadipocytes medium, human insulin, dexamethasone, isobutylmethylxanthine, and PPARγ agonists (rosiglitazone). After 7 day (d7), differentiation medium was replaced with fresh adipocyte medium (Zen-Bio) composed of DMEM/nutrient mix F-12 medium (1:1, vol/vol), HEPES, FBS, biotin, pantothenate, human insulin, dexamethasone, penicillin, streptomycin, and amphotericin. Negative control (nondifferentiated cell) was performed with preadipocyte medium during all differentiation process. Fourteen days after the initiation of differentiation, cells appeared rounded with large lipid droplets apparent in the cytoplasm. Cells were then considered mature adipocytes, harvested, and stored at − 80ºC for RNA/protein purification. For time course experiment, Cells were harvested and stored at − 80ºC for RNA/protein purification at day 0, 5, 7, 9, 12 and 14. At the end of each experiment, cells were harvested, and pellets and supernatants were stored at − 80 ºC. All *in vitro* experiments were performed in three or four independent replicates.

2.7. Forward siRNA transfections in primary human preadipocytes and adipocytes

Primary human subcutaneous preadipocytes (Zen-Bio Inc., Research Triangle Park, NC) were forward transfected with siRNAs either during the differentiation process (at day 0 and day 4) or at the end of differentiation (at day 11). Briefly, the esiRNA (Sigma-Aldrich, St. Louis, MO) against ERBB2 and EGFR and Lipofectamine RNAiMAX (Life Technologies, Darmstadt, Germany) were diluted separately with Opti-MEM I Reduced Serum Medium (Life Technologies, Darmstadt, Germany) and mixed by pipetting afterwards. The esiRNA-RNAiMAX complexes were left to incubate for 20 min at room temperature and subsequently added on the top of the adherent cells drop-wise. The final concentration of Lipofectamine RNAiMAX and esiRNAs were 1.6 μl/cm2 and 70 nM, respectively, in 12-well cell culture plates, and the final amount of medium per well was 1 ml. Transfection conditions included silencing of either ERBB2 or EGFR. Adipocytes were harvested at day 7 and at day 14 of differentiation, meaning 72 h after transfection without changing cell culture medium. Transfection efficiency was assessed by real-time PCR and western-blot. The MISSION® esiRNAs (Sigma-Aldrich) used were human ERBB2 (EHU078751) and human EGFR (EHU076761). The MISSION® siRNA Universal Negative Control #1 (Sigma-Aldrich, SIC001) was used as control in all experiments.

2.8. Overexpression of NRG2 gene experiment

ORF expression clone for NRG2 (217EX-W0006-M90-GS) and negative empty control vector for pReceiver-M90 (217EX-NEG-M90) were obtained from Genecopoeia ((Tebu-bio, Spain). Human subcutaneous preadipocytes were transfected with either NRG2 overexpression plasmid or control plasmid, using Fugene® Transfection Reagent (Promega, WI, USA) at ratio 1:3 following manufacturer's instructions. Plasmid transfection was performed in different stages of adipocyte differentiation: 0–14 days, 0–7 days, 7–14 days. Culture medium replacement after transfection was not necessary. Overexpression

efficiency of NRG2 was checked by qPCR. Lipid droplet (LDs) area was quantified with Fiji software [20]. The area occupied by lipid droplets was selected and quantified using threshold plugin, after sharpening pixel intensity of lipid droplets. Values obtained were expressed as the lipid droplet count with respect to total image.

HEK293T cell line were treated using ORF expression clone for NRG2 or negative control using the same protocol as in adipocytes, and efficiency of transfection was checked both by qPCR and ELISA after 48 h of transfection. Intracellular NRG2 was measured using the Human Neuregulin-2 (NRG2) ELISA kit (Cat nº: CSB-EL016078HU, CUSABIO, Houston. TX, USA).

2.9. RNA expression

RNA purification, primer/probe sets, gene expression procedures and analyses were performed, as previously described [19]. Briefly, RNA purification was performed using RNeasy Lipid Tissue Mini Kit (QIAgen, Izasa SA, Barcelona, Spain) and the integrity was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression was assessed by real time PCR using a LightCycler® 480 Real-Time PCR System (Roche Diagnostics SL, Barcelona, Spain), using TaqMan® and SYBRgreen technology suitable for relative genetic expression quantification. The RT-PCR reaction was performed in a final volume of 12 µl. The cycle program consisted of an initial denaturing of 10 min at 95 °C then 40 cycles of 15 s denaturizing phase at 95 °C and 1 min annealing and extension phase at 60 °C. A threshold cycle (Ct value) was obtained for each amplification curve and then a ΔCt was first calculated by subtracting the Ct value for human cyclophilin A (*PPIA*) RNA from the Ct value for each sample. Fold changes compared with the endogenous control were then determined by calculating $2^{-\Delta Ct}$, so that gene expression results are expressed as expression ratio relative to *PPIA* gene expression according to the manufacturer's guidelines. TaqMan® primer/probe sets (Thermo Fisher Scientific, Waltham, MA, USA) used were as follows: Peptidylprolyl isomerase A (cyclophilin A) (4333763, *PPIA* as endogenous control), epidermal growth factor receptor (*EGFR,* Hs01076090_m1), erb-b2 receptor tyrosine kinase 2 (*ERBB2*, Hs01001580_m1), erb-b2 receptor tyrosine kinase 2 (*ERBB3*, Hs00176538_m1), erb-b2 receptor tyrosine kinase 4 (*ERBB4,* Hs00955525_m1), neuregulin 1 (*NRG1*, Hs01101538_m1), neuregulin 2 (*NRG2*, Hs00993399_m1), neuregulin 3 (*NRG3,* Hs01377907_m1), neuregulin 4 (*NRG4*, Hs00945534_m1), adiponectin (*ADIPOQ*, Hs00605917_m1), peroxisome proliferator-activated receptor gamma (*PPARG*, Hs00234592_m1), fatty acid synthase (*FASN*, Hs00188012_m1), acetyl-CoA carboxylase alpha (*ACACA*, Hs00167385_m1), solute carrier family 2 member 4 (*SLC2A4*, Hs00168966_m1), diacylglycerol O-acyltransferase 1 (*DGAT1*, Hs00201385_m1) Leptin (*LEP*, Hs00174877_m1), interleukin 6 (interferon, beta 2) (*IL6,* Hs00985639_m1), tumor necrosis factor (*TNF*, Hs00174128_m1), interleukin 8 (*IL8*, Hs00174103_m1), fatty acid binding protein 4, adipocyte (*FABP4*, Hs01086177_m1). Perilipin 1 (*PLIN1*) and cell death inducing DFFA like effector c (*CIDEC* or *FSP27*) were analysed by SYBRgreen technology using the following primer sets: *PLIN1* (forward: 5′-aagttgaagcttgaggagcgagg-3′ and reverse: 5′-gctcgcgatgggaacgctga-3′) and *CIDEC/FSP27* (forward: 5′-gaggtccaacg cagtccagctg-3′ and reverse: 5′-gtacgcactgacacatgcctggag-3′).

2.10. Statistical analyses

Statistical analyses were performed using SPSS 12.0 software. The relation between variables was analyzed by simple correlation (Spearman's test) and multiple regression analyses in a stepwise manner. One factor ANOVA with post-hoc Bonferroni test, paired t-test and unpaired t-test were used to compare expression of ERBB-related genes in human cohorts according to obesity and after bariatric surgery-induced weight loss. Nonparametric test (Mann Whitney test) was used to analyze *in vitro* experiments. Levels of statistical significance were set at $p < 0.05$.

3. Results

3.1. Increased SAT and VAT ERBB2 and ERBB4 gene expression in obesity

Both SAT and VAT *EGFR, ERBB2, ERBB3* and *ERBB4* gene expression were analyzed in two independent cohorts.

In cohort 1, SAT *ERBB2* mRNA levels were increased in obese participants, whereas no significant changes were found for the other genes (*EGFR, ERBB3* and *ERBB4*) (Table 1). SAT *ERBB2* mRNA was positively correlated with BMI, fat mass, fasting glucose, but negatively with age and HDL-cholesterol (Table 1). In multiple linear regression analysis, the associations between SAT *ERBB2* and fat mass, fasting glucose and HDL-cholesterol were lost after controlling for age and BMI. SAT *ERBB3* mRNA was negatively correlated to BMI and fat mass (Table 1). In VAT, increased *EGFR, ERBB2* and *ERBB4* mRNA levels were found in association to obesity (Table 1). Of note, both VAT *EGFR*, *ERBB2, ERBB3* and *ERBB4* mRNA were positively correlated with adiposity measures (BMI and fat mass) and hyperglycemia (fasting glucose) (Table 1). Again, that these associations were lost after adjusting for age and BMI.

Most of these findings were replicated in an independent cohort (cohort 2). In this cohort, both SAT and VAT *ERBB2* and VAT *ERBB4* mRNA were also increased in obese participants (Table 2). In SAT, *ERBB2* mRNA was positively correlated with BMI and fasting glucose and *ERBB4* mRNA with HDL-cholesterol (Table 2). In contrast, SAT *ERBB3* mRNA was negatively correlated with BMI, fasting glucose, HbA1c and fasting triglycerides, and *EGFR* with HbA1c (Table 2). In VAT, both *ERBB2* and *ERBB4* mRNA were positively correlated with BMI, fasting glucose and HbA1c (Table 2). In addition, VAT *ERBB4* mRNA was also positively correlated with fasting triglycerides and negatively with HDL-cholesterol (Table 2). Similar to in cohort 1, all these associations also were lost after controlling for age and BMI.

3.2. SAT EGFR gene expression was negatively correlated to insulin resistance and HbA1c

In a subgroup of participants from each cohort, insulin resistance was calculated by HOMA-IR in cohort 1 (n = 35. Table 1) and cohort 2 (n = 52, Table 2). In both cohort 1 and cohort 2, SAT *EGFR* mRNA was negatively correlated with HOMA-IR (Table 1 and Table 2). In line with these data, *EGFR* mRNA was negatively correlated with HbA1c (Table 2).

These data indicated that *ERBB2* and *ERBB4* mRNA were increased in obesity, without any associations with insulin resistance, hyperglycemia or dyslipemia. In addition and similar to previous study[5], SAT *EGFR* mRNA, which did not change according to obesity, was negatively correlated with insulin resistance.

Considering the impact of obesity and insulin resistance in adipose tissue physiology, next, we explored the possible role of ERBB family-related genes in both SAT and VAT, analysing gene expression markers of adipose tissue function (adipogenesis-, lipogenesis-, glucose uptake- and lipid droplet development-related genes) and dysfunction (leptin and inflammation).

3.3. Adipose tissue EGFR gene expression was associated to adipogenesis independently of obesity

Of note, both SAT and VAT *EGFR* mRNA were positively correlated with expression of some adipogenic genes (including *ADIPOQ*, *SLC2A4*, *FSP27, PLIN* and *PPARG*) in cohort 1 and cohort 2 (Fig. 1). After multivariate regression analysis, most of these associations were maintained after adjusting for age and BMI.

In cohort 1, VAT *EGFR* contributed to age- and BMI-adjusted *ADIPOQ* ($\beta = 0.34$, $t = 2.3$, $p = 0.03$), *FSP27* ($\beta = 0.29$, $t = 3.1$, $p = 0.003$), but not *LEP* ($\beta = 0.08$, $t = 0.6$, $p = 0.5$) mRNA variance. SAT *EGFR* contributed to age- and BMI-adjusted *ADIPOQ* ($\beta = 0.35$, $t = 3.6$,

Table 1
Anthropometric, clinical parameters and ERBB receptors according to obesity in cohort 1. Bivariate correlations (Spearman correlations) among ERBB receptors and metabolic parameters in all participants in cohort 1.

	Non-obese		**Obese**		**p**			
N	44		106					
Age (years)	47.4 ± 10.9		44.7 ± 10.1		0.1			
BMI (kg/m^2)	25.3 ± 3.3		45.3 ± 7.4		**< 0.0001**			
Fat mass (%)	32.9 ± 6.5		57.1 ± 10.4		**< 0.0001**			
Waist circumference (cm)	86.6 ± 10.6		124.9 ± 18.5		**< 0.0001**			
Fasting glucose (mg/dl)	90.4 ± 17.6		108.5 ± 41.3		**0.006**			
HOMA-IR	1.6 (1.3–5.5)		3.4 (1.5–5.4)		0.5			
(n = 35)	(n = 4)		(n = 31)					
HDL-cholesterol (mg/dl)	60.7 ± 22.5		56.8 ± 35.2		0.5			
Fasting triglycerides (mg/dl)	92.5 ± 42.1		127.7 ± 89.1		**0.02**			
SAT *EGFR* mRNA (RU)	0.054 ± 0.02		0.053 ± 0.01		0.9			
SAT *ERBB2* mRNA (RU)	0.014 ± 0.006		0.020 ± 0.007		**< 0.0001**			
SAT *ERBB3* mRNA (RU)	0.00045 ± 0.0003		0.00051 ± 0.0004		0.6			
SAT *ERBB4* mRNA (RU)	0.0046 ± 0.002		0.0048 ± 0.003		0.8			
SAT *NRG1* mRNA (RU)	0.00014 ± 0.0001		0.00024 ± 0.0002		**0.02**			
SAT *NRG2* mRNA (RU)	0.0064 ± 0.002		0.0062 ± 0.002		0.8			
VAT *EGFR* mRNA (RU)	0.042 ± 0.02		0.049 ± 0.01		**0.02**			
VAT *ERBB2* mRNA (RU)	0.017 ± 0.008		0.028 ± 0.01		**< 0.0001**			
VAT *ERBB3* mRNA (RU)	0.0019 ± 0.002		0.0022 ± 0.001		0.4			
VAT *ERBB4* mRNA (RU)	0.0035 ± 0.002		0.0065 ± 0.003		**< 0.0001**			
VAT *NRG1* mRNA (RU)	0.00046 ± 0.0005		0.00051 ± 0.0005		0.7			
VAT *NRG2* mRNA (RU)	0.0041 ± 0.002		0.0053 ± 0.004		0.06			
	***EGFR* (RU)**		***ERBB2* (RU)**		***ERBB3* (RU)**		***ERBB4* (RU)**	
SAT	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Age (years)	-0.15	0.07	-0.26	**0.001**	-0.06	0.5	0.12	0.1
BMI (kg/m^2)	-0.02	0.8	0.35	**< 0.0001**	-0.23	**0.004**	0.01	0.9
Fat mass (%)	-0.02	0.8	0.31	**< 0.0001**	-0.17	**0.04**	0.13	0.1
Fasting glucose (mg/dl)	-0.07	0.4	0.21	**0.01**	-0.02	0.8	0.05	0.5
HOMA-IR	-0.37	**0.03**	0.32	0.06	0.14	0.4	-0.06	0.7
HDL-cholesterol (mg/dl)	-0.06	0.5	-0.17	**0.04**	0.04	0.6	0.14	0.1
Fasting triglycerides (mg/dl)	-0.15	0.07	0.12	0.1	-0.07	0.4	-0.01	0.9
	***EGFR* (RU)**		***ERBB2* (RU)**		***ERBB3* (RU)**		***ERBB4* (RU)**	
VAT	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Age (years)	0.15	0.06	-0.04	0.6	-0.07	0.4	-0.11	0.2
BMI (kg/m^2)	0.12	0.1	0.43	**< 0.0001**	0.27	**0.001**	0.41	**< 0.0001**
Fat mass (%)	0.23	**0.005**	0.49	**< 0.0001**	0.31	**< 0.0001**	0.42	**< 0.0001**
Fasting glucose (mg/dl)	0.18	**0.03**	0.25	**0.003**	0.22	**0.007**	0.20	**0.01**
HOMA-IR	0.12	0.5	0.19	0.3	0.20	0.3	0.23	0.2
HDL-cholesterol (mg/dl)	-0.06	0.5	-0.12	0.1	-0.10	0.2	0.02	0.8
Fasting triglycerides (mg/dl)	0.09	0.3	0.11	0.2	-0.01	0.8	0.01	0.9

p < 0.0001), *FSP27* (β = 0.21, t = 2.1, p = 0.03) and *SLC2A4* (β = 0.24, t = 2.1, p = 0.04) mRNA variance.

In cohort 2, VAT *EGFR* contributed to age- and BMI-adjusted *ADIPOQ* (β = 0.58, t = 6.7, p < 0.0001), *SLC2A4* (β = 0.39, t = 3.3, p = 0.002), *FSP27* (β = 0.54, t = 4.4, p < 0.0001) and *PLIN* (β = 0.59, t = 4.7, p < 0.0001) mRNA variance. SAT *EGFR* contributed to age- and BMI-adjusted *ADIPOQ* (β = 0.45, t = 4.3, p < 0.0001), but not *PPARG* (β = 0.12, t = 0.8, p = 0.4) mRNA variance.

3.4. The association between expression of adipose tissue-related genes and ERBB2, ERBB3 and ERBB4 mRNA levels mostly depend on obesity

3.4.1. ERBB2 mRNA

In cohort 1, both SAT and VAT *ERBB2* mRNA mRNA were negatively correlated with some adipogenic gene expression, such as *SLC2A4*, *FASN*, *ACACA* and positively with *TNF* and *LEP* genes. Strikingly, *ERBB2* mRNA also were negatively correlated with proinflamatory cytokines (*IL6* and *IL8*) (Fig. 1A-B). Otherwise, after multivariate regression analysis, only the negative association between *ERBB2* mRNA and expression of lipogenesis-related genes were maintained in both SAT [*FASN* (β = −0.18, t = −1.9, p = 0.04) and *ACACA* (β = −0.30, t = −2.9, p = 0.004)] and VAT [*FASN* (β = −0.22, t = −2.8, p = 0.005), *ACACA* (β = −0.23, t = −2.5, p = 0.01)] after adjusting by age and BMI.

However, in cohort 2, the negative correlations between adipose tissue *ERBB2* mRNA and expression of lipogenesis-related genes were lost after adjusting by age and BMI.

3.4.2. ERBB3 mRNA

In cohort 1, SAT *ERBB3* was negatively correlated with *FSP27* and *LEP* mRNA, and VAT *ERBB3* negatively with *PPARG, SLC2A4, FASN, FSP27, IL6* and *IL8* mRNA levels (Fig. 1A-B). SAT *ERBB3* mRNA contributed to age- and BMI-adjusted *FSP27* (β = −0.28, t = −2.9, p = 0.004) and *LEP* (β = −0.29, t = −3.2, p = 0.002) mRNA variance. VAT *ERBB3* mRNA contributed to age- and BMI-adjusted *SLC2A4* (β = −0.21, t = −2.1, p = 0.04), *FASN* (β = −0.14, t = −2.1, p = 0.04), *PPARG* (β = −0.27, t = −1.9, p = 0.05), but not *FSP27* (β = 0.05, t = 0.5, p = 0.5), IL6 (β = −0.13, t = −1.3, p = 0.2) and IL8 (β = −0.17, t = −1.5, p = 0.1) mRNA variance.

In cohort 2, SAT *ERBB3* was positively correlated with *FASN* and *SLC2A4* mRNA, and VAT *ERBB3* positively with *PLIN*, but negatively with *ADIPOQ* (Fig. 1C-D). SAT *ERBB3* mRNA did not contribute to age- and BMI-adjusted *FASN* (β = −0.03, t = −0.33, p = 0.7) and *SLC2A4* (β = 0.01, t = 0.05, p = 0.9) mRNA variance. VAT *ERBB3* mRNA did not contribute to age- and BMI-adjusted *ADIPOQ* (β = −0.17, t = −1.6, p = 0.1) and *PLIN1* (β = 0.04, t = 0.25, p = 0.8) mRNA variance.

3.4.3. ERBB4 mRNA

In cohort 1, no significant associations between SAT *ERBB4* and adipogenic/inflammatory mRNAs were observed, whereas VAT *ERBB4* was negatively correlated with *SLC2A4, FASN, FSP27, IL6* and *IL8,* but positively with *TNF* mRNA (Fig. 1A-B). VAT *ERBB4* mRNA contributed to age- and BMI-adjusted *FASN* (β = −0.19, t = −2.5, p = 0.01), *IL8* (β = −0.40, t = −3.3, p = 0.002), *IL6* (β = −0.22, t = −1.9, p = 0.05), but not *SLC2A4* (β = 0.08, t = 0.61, p = 0.5), *FSP27* (β = −0.05,

Table 2
Anthropometric, clinical parameters and ERBB receptors according to obesity in cohort 2. Bivariate correlations (Spearman correlations) among ERBB receptors and metabolic parameters in all participants in cohort 2.

	Non-obese	Obese	p
N	28	59	
Age (years)	48.2 ± 7.9	47.5 ± 8.7	0.7
BMI (kg/m^2)	23.9 ± 3.3	44.4 ± 6.5	**< 0.0001**
Fasting glucose (mg/dl)	88.8 ± 7.7	105.8 ± 35.4	**0.01**
HOMA-IR (n = 52)	–	3.7 (1.9–5.1)	–
HbA1c (%)	5.4 ± 0.2	5.9 ± 1.1	**0.04**
HDL-cholesterol (mg/dl)	57.2 ± 18.6	47.6 ± 11.6	**0.004**
Fasting triglycerides (mg/dl)	99.3 ± 55.9	124.5 ± 53.3	**0.04**
SAT *EGFR* mRNA (RU)	0.055 ± 0.019	0.057 ± 0.019	0.5
SAT *ERBB2* mRNA (RU)	0.013 ± 0.004	0.022 ± 0.011	**< 0.0001**
SAT *ERBB3* mRNA (RU)	0.00054 ± 0.0003	0.00029 ± 0.0003	0.07
SAT *ERBB4* mRNA (RU)	0.0052 ± 0.003	0.0066 ± 0.004	0.07
SAT *NRG1* mRNA (RU)	0.00012 ± 0.0001	0.00025 ± 0.0002	0.2
SAT *NRG2* mRNA (RU)	0.0059 ± 0.002	0.0062 ± 0.002	0.7
VAT *EGFR* mRNA (RU)	0.042 ± 0.018	0.047 ± 0.017	0.3
VAT *ERBB2* mRNA (RU)	0.015 ± 0.006	0.028 ± 0.022	**0.004**
VAT *ERBB3* mRNA (RU)	0.0021 ± 0.002	0.0017 ± 0.001	0.5
VAT *ERBB4* mRNA (RU)	0.0027 ± 0.001	0.0069 ± 0.003	**< 0.0001**
VAT *NRG1* mRNA (RU)	0.00023 ± 0.0002	0.00042 ± 0.0004	0.06
VAT *NRG2* mRNA (RU)	0.0039 ± 0.002	0.0058 ± 0.002	**0.001**

	***EGFR* (RU)**		***ERBB2* (RU)**		***ERBB3* (RU)**		***ERBB4* (RU)**	
SAT	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Age (years)	-0.08	0.5	-0.12	0.3	-0.19	0.08	-0.04	0.7
BMI (kg/m^2)	0.07	0.5	0.55	**< 0.0001**	-0.54	**< 0.0001**	0.19	0.08
Fasting glucose (mg/dl)	-0.12	0.3	0.23	**0.04**	-0.39	**< 0.0001**	0.06	0.6
HOMA-IR	-0.31	**0.02**	-0.12	0.4	-0.39	**0.004**	0.01	0.9
HbA1c (%)	-0.26	**0.03**	0.17	0.1	-0.41	**< 0.0001**	0.08	0.5
HDL-cholesterol (mg/dl)	0.13	0.2	-0.22	0.05	0.17	0.1	0.25	**0.03**
Fasting triglycerides (mg/dl)	-0.09	0.4	0.33	**0.003**	-0.28	**0.01**	0.01	0.9
	***EGFR* (RU)**		***ERBB2* (RU)**		***ERBB3* (RU)**		***ERBB4* (RU)**	
VAT	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Age (years)	0.08	0.4	-0.04	0.7	-0.07	0.5	-0.16	0.1
BMI (kg/m^2)	-0.01	0.9	0.33	**0.002**	0.10	0.3	0.53	**< 0.0001**
Fasting glucose (mg/dl)	0.11	0.3	0.33	**0.002**	0.17	0.1	0.28	**0.01**
HOMA-IR	-0.14	0.3	-0.06	0.7	-0.03	0.8	-0.13	0.3
HbA1c (%)	0.15	0.2	0.23	**0.04**	0.14	0.2	0.21	0.05
HDL-cholesterol (mg/dl)	0.05	0.6	-0.07	0.5	0.01	0.9	-0.22	**0.04**
Fasting triglycerides (mg/dl)	-0.07	0.5	0.11	0.3	0.09	0.4	0.27	**0.01**

t = −0.57, p = 0.5) and *TNF* (β = −0.08, t = −0.8, p = 0.4) mRNA variance.

In cohort 2, SAT *ERBB4* was positively correlated with *PPARG, ADIPOQ, SLC2A4* and *PLIN1* mRNA, whereas VAT *ERBB4* negatively with *ADIPOQ* and *FASN* (Fig. 1C-D). SAT *ERBB4* mRNA contributed to age- and BMI-adjusted *PPARG* (β = 0.30, t = 2.2, p = 0.03), *ADIPOQ* (β = 0.39, t = 3.7, p < 0.0001), *SLC2A4* (β = 0.34, t = 2.6, p = 0.01), but not *PLIN* (β = 0.24, t = 1.6, p = 0.1) mRNA variance. Otherwise, the associations between VAT *ERBB4* and *FASN* (β = −0.08, t = −0.8, p = 0.4) or *ADIPOQ* (β = −0.09, t = −0.8, p = 0.4) mRNA were lost after controlling for age and BMI.

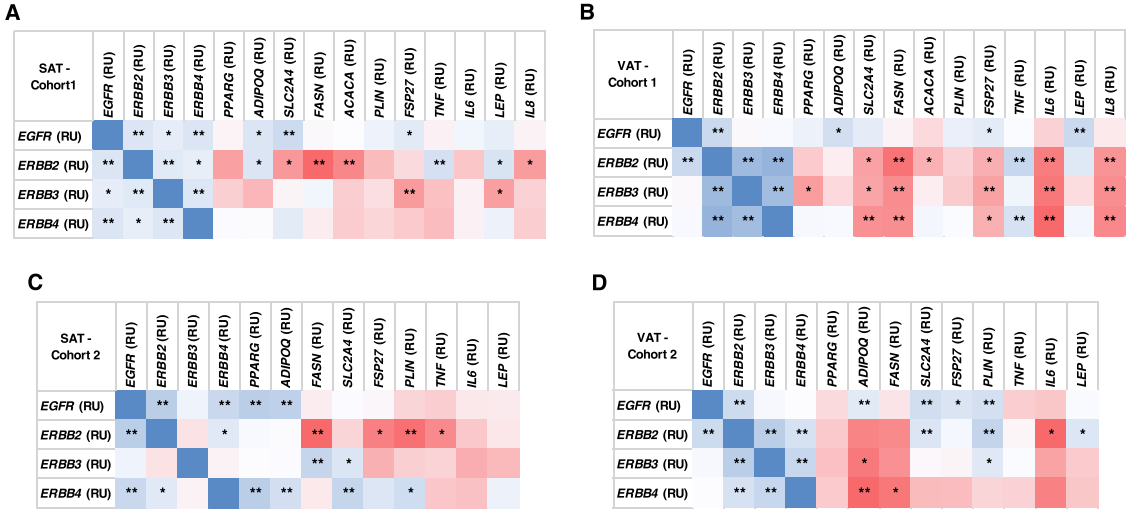

Fig. 1. Bivariate correlations (Spearman correlations) among expression of ERBB receptors-related genes and adipogenic and proinflammatory mRNA levels in SAT and VAT from cohort 1 **(A-B)** and cohort 2 **(C-D)**. The intensity of blue colour indicates the degree of positive correlations, and the intensity of red colour the same for negative correlations. *p < 0.05 and * *p < 0.01.

3.5. Impact of bariatric-surgery induced weight loss on ERBB receptors

Confirming the association between AT ERBB2 and ERBB4 and obesity, SAT *ERBB2* and *ERBB4* mRNA levels were significantly decreased after 2 years-bariatric surgery-induced weight loss in correlation with the percent change in BMI, whereas no significant changes were found in SAT *EGFR* and *ERBB3* mRNA levels (Fig. 2A-B).

The most consistent findings from these cross-sectional and longitudinal cohorts were that EGFR and ERBB2 were the most abundant ERBB receptors in human adipose tissue, being ERBB2 (and ERBB4) associated to obesity and EGFR associated to expression of adipogenesis-related genes.

3.6. ERBB receptors in adipose tissue cells

Of note in both SAT and VAT, *EGFR* mRNA were increased in adipocytes compared to SVF (Fig. 3A), In SAT, no significant differences in SVF or adipocyte *ERBB2* and *ERBB3* mRNA were found (Fig. 3B-C). In VAT, *ERBB2* and *ERBB3* mRNA were increased in SVF (Fig. 3B-C). SAT, but not VAT, *ERBB4* mRNA were increased in adipocytes (Fig. 3D). In addition, both *EGFR*, *ERBB2* and *ERBB4* increased during human subcutaneous and visceral adipocyte differentiation, whereas *ERBB3* tended to decrease (Fig. 3E-H).

3.7. Functional experiments in mouse primary preadipocytes

Taking into account the association between *ERBB2* and *ERBB4* gene expression and obesity, and to further investigate the relevance of these receptors in the generation of new adipocytes, experiments in mouse primary preadipocytes with *Erbb2* and *Erbb4* gene depletion were performed. These experiments revealed that *Erbb2* gene depletion, but not *Erbb4*, blunted adipocyte differentiation, inhibiting the intracellular lipid accumulation and expression of adipogenic genes (Figure S1A-C).

Considering that ERBB4 was expressed at low levels in human adipose tissue, the contradictory associations between *ERBB4* mRNA and expression of adipogenesis-related genes in each cohort, and that *Erbb4* depletion in mouse primary preadipocytes did not impact on these genes (Figure S1C), functional gene knockdown (KD) *in vitro* experiments in human cells were only focused in EGFR and ERBB2 genes.

3.8. Impact of EGFR and ERBB2 gene KD on early stage of human adipocyte differentiation

EGFR gene KD during early stage of adipocyte differentiation in human subcutaneous preadipocytes resulted in decreased adipogenic (*ADIPOQ, FABP4, DGAT1* and *FASN*) gene expression (Fig. 4A). Otherwise, *ERBB2* gene KD only decreased *DGAT1* mRNA levels (Fig. 4A).

3.9. Impact of EGFR and ERBB2 gene KD on fully differentiated human adipocytes

In fully differentiated human subcutaneous adipocytes, *EGFR* gene KD also led to decreased *ADIPOQ, FABP4, FASN* and *DGAT1* mRNA levels (Fig. 4B), and *ERBB2* gene KD decreased *FASN* mRNA, *DGAT1* and *FABP4* (Fig. 4B).

These experiments supported the relevance of EGFR in human adipogenesis and suggest that even though ERBB2 did not seem relevant during human adipocyte differentiation, this receptor might be required for adipocyte physiology.

3.10. NRG2 might mediate EGFR-associated adipogenic effects

Next, in the search for putative EGFR ligands that may enhance human adipose tissue adipogenesis, neuregulins were investigated. However, a previous study reported a negative association between VAT neuregulin 4 (*NRG4*) mRNA levels and expression of adipogenic genes in VAT, without any correlations in SAT[16]. Thus, in the present study other neuregulins (NRG1, NRG2 and NRG3) were analysed. In both SAT and VAT, very low *NRG1* mRNA levels were detected. Similar to *NRG4*, VAT *NRG1* mRNA was negatively correlated with adipogenic gene expression, including *ADIPOQ, FASN* and *SLC2*A4 (Figure S2A), but no significant correlations between *NRG1* and adipogenic genes were found in SAT. Of interest, *NRG2* was significantly increased compared to *NRG1* and *NRG4* mRNA levels (Fig. 5A-B) and positively correlated with adipogenesis-related gene expression (Fig. 5C-J). In line with these associations, during human subcutaneous and visceral adipocyte differentiation, *NRG2* mRNA was significantly increased at day 5, remaining induced until the end of the process (Figure S2B), whereas NRG1 mRNA was significantly attenuated (Figure S2C). NR*G3* mRNA were not

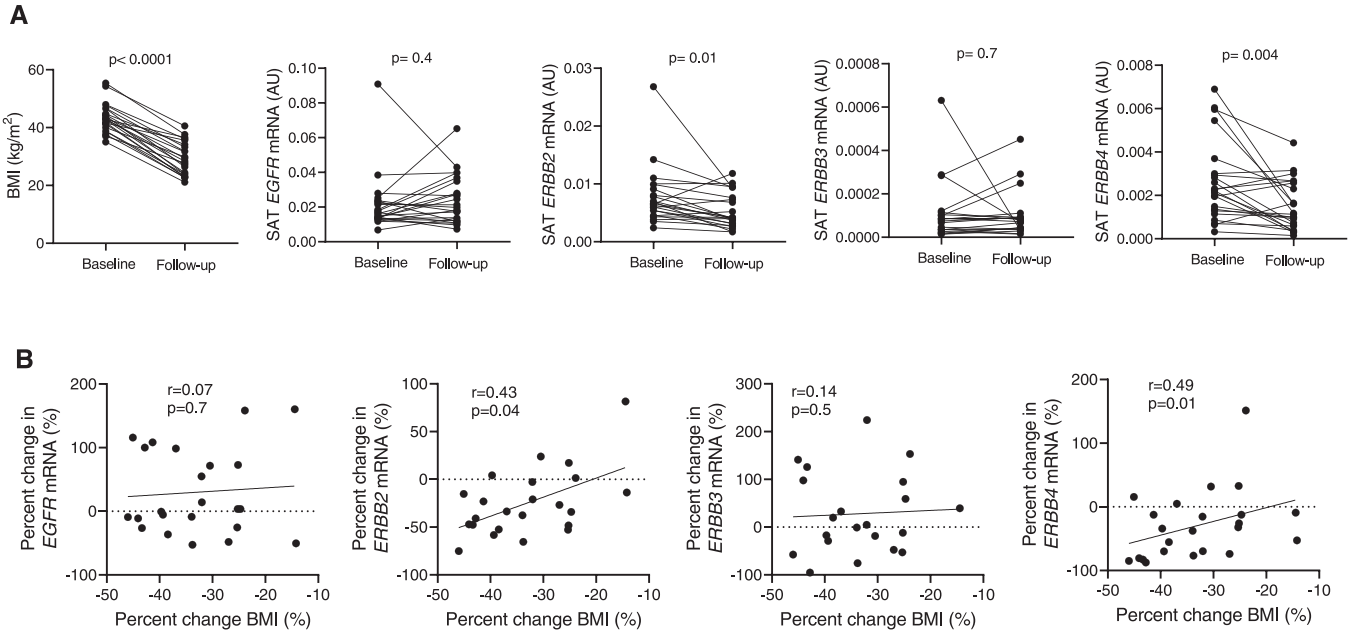

Fig. 2. A) Longitudinal changes in BMI and SAT *EGFR, ERBB2, ERBB3* and *ERBB4* mRNA levels after 2 year-bariatric surgery-induced weight loss intervention. **B)** Bivariate correlations (Spearman correlations) among the percent change in *EGFR, ERBB2, ERBB3* and *ERBB4* mRNA levels and BMI after weight los intervention.

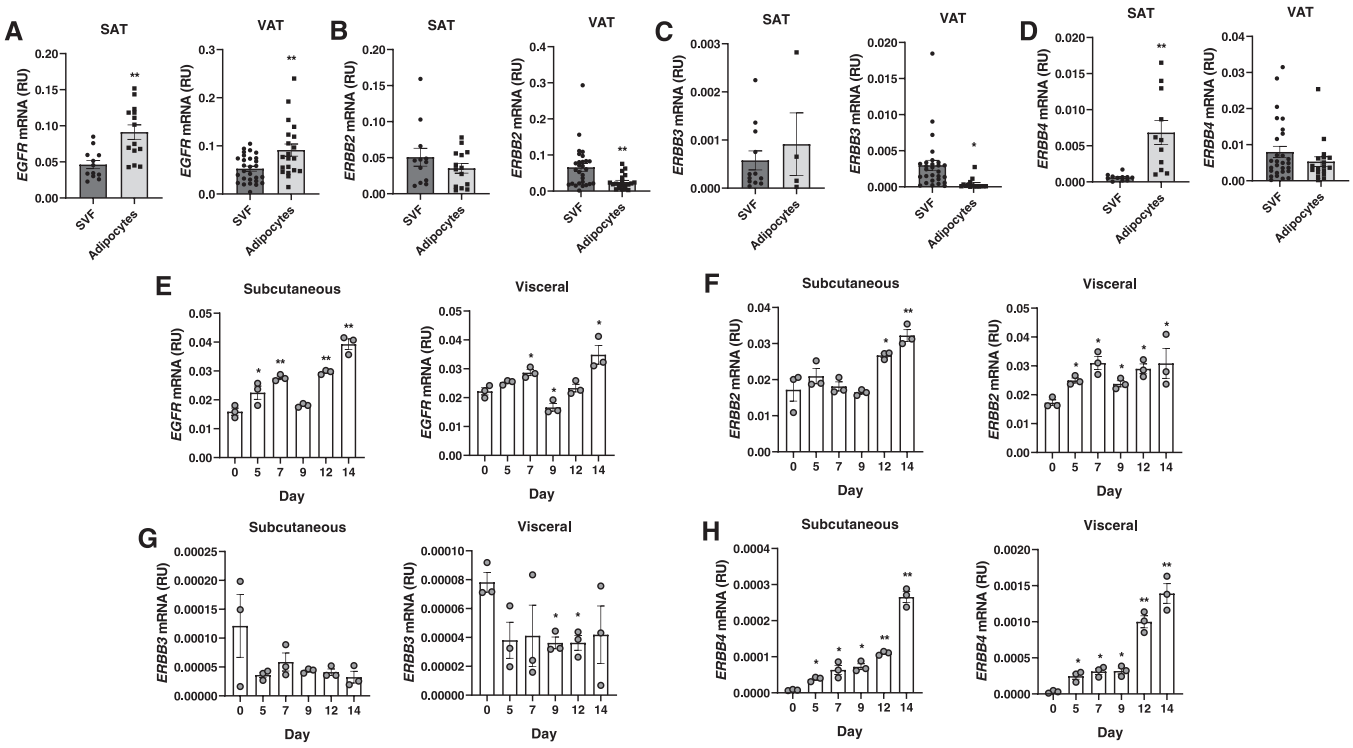

Fig. 3. A-D) SAT and VAT *EGFR* **(A)**, *ERBB2* **(B)**, *ERBB3* **(C)** and *ERBB4* **(D)** mRNA levels in adipose tissue cell fractions. *p < 0.05 and * *p < 0.01 compared to SVF. **E-H)** *EGFR* **(E)**, *ERBB2* **(F)**, *ERBB3* **(G)** and *ERBB4* **(H)** mRNA levels during adipocyte differentiation in subcutaneous and visceral preadipocytes. *p < 0.05 and * *p < 0.01 compared to day 0.

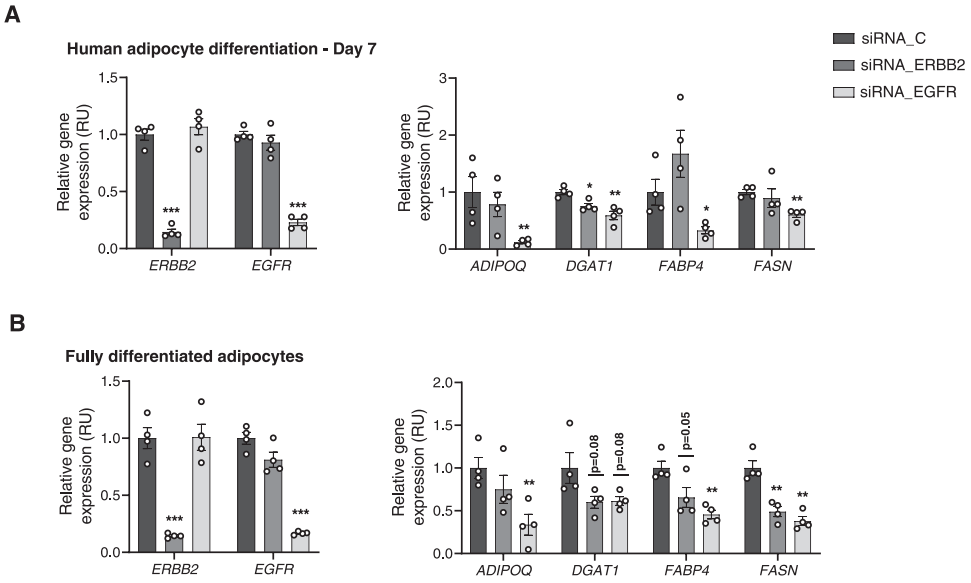

Fig. 4. A-B) Impact of *EGFR* and *ERBB2* gene knockdown on expression of adipogenic (*ADIPOQ, DGAT1, FABP4, FASN*) genes in humans subcutaneous preadipocytes during adipocyte differentiation **(A)** and in fully differentiated subcutaneous adipocytes **(B)**. *p < 0.05, * *p < 0.01 and * **p < 0.001 compared to siRNA_C. Black bars indicate siRNA_C, dark grey bars siRNA_ERBB2 and light grey bars siRNA_EGFR.

detected in human adipose tissue, preadipocytes or fully differentiated adipocytes. These data suggest that NRG2 might play a possible role in human adipocyte differentiation.

3.11. The impact of NRG2 gene overexpression on human adipocyte differentiation

To study the possible role of NRG2 induction in adipocyte differentiation, the impact of *NRG2* gene overexpression was evaluated at different stages of human adipocyte differentiation, including early stage (from day 0–7), late stage (from day 7–14) and during all the process (day 0–14). *NRG2* gene overexpression at day 0 slightly decreased expression of some adipogenic genes at day 7 (including *PPARG* and *FASN* genes; Figure S3A-B) and day 14 (including *ADIPOQ, SLC2A4, PPARG, DGAT1* and *FASN* genes; Figure S3C-D), without resulting in significant differences in lipid droplet counts (Figure S3E

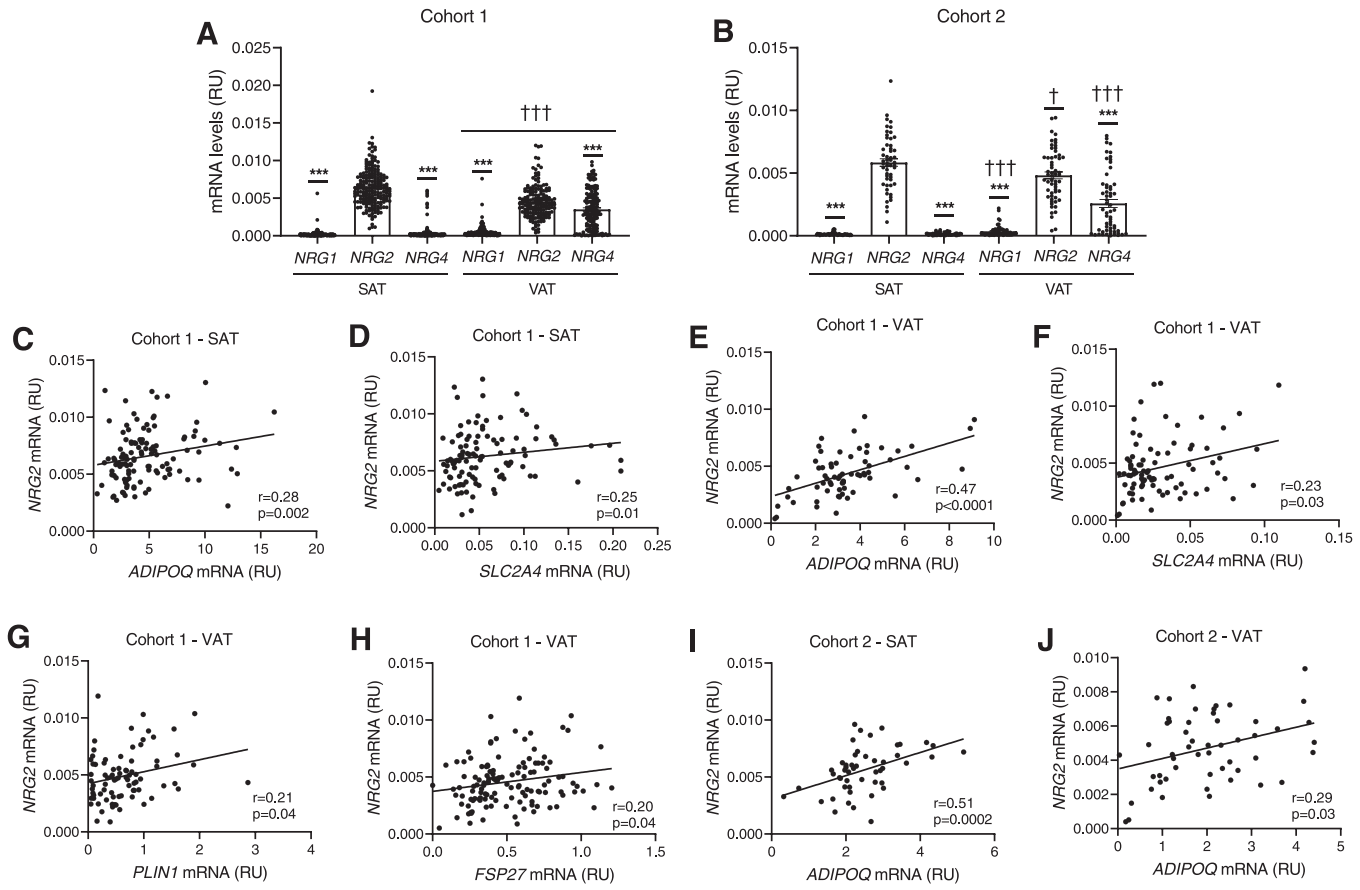

Fig. 5. A-B) SAT and VAT *NRG1, NRG2* and *NRG4* mRNA levels in cohort 1 **(A)** and cohort 2 **(B)**. * **p < 0.001 compared to *NRG2* mRNA levels; †< 0.05 and †††< 0.001 compared to SAT. **C-J)** Bivariate correlations (Spearman correlations) among SAT and VAT adipogenic and *NRG2* mRNA levels in cohort 1 and 2.

and Figure S4). However, the induction of *NRG2* gene overexpression at day 7 resulted in increased markers of adipocyte differentiation, including expression of relevant adipogenic genes (*CEBPA* and *FABP4*), and increased number of lipid droplets (Fig. 6A-C and Figure S5) at the end of adipocyte differentiation process. However, while transfected cells displayed a very high increase in *NRG2* mRNA levels, the improvement in adipogenesis was rather more restrained, leading us to question whether *NRG2* mRNA was properly translated to protein.

In HEK293 cell line, we confirmed the very significant increase in *NRG2* mRNA levels in transfected cells, whereas only slightly increased intracellular NRG2 protein, indicating that the increased *NRG2* mRNA levels were not proportional to intracellular NRG2 protein levels (Figure S6).

4. Discussion

Current study provides several evidences supporting an important role for EGFR in human adipogenesis:

i) In two independent cohorts, human adipose tissue *EGFR* gene expression was significantly correlated with expression of adipogenic genes in both SAT and VAT.
ii) In adipose tissue cell fraction, EGFR gene expression was significantly increased in adipocytes compared to SVF.
iii) In human subcutaneous and visceral preadipocytes, *EGFR* gene expression increased during first stage of adipocyte differentiation, achieving maximal levels at the end of the process.
iv) In human subcutaneous preadipocytes, *EGFR* gene knockdown resulted in decreased expression of adipogenic (*ADIPOQ, DGAT1, FABP4* and *FASN*) genes during adipocyte differentiation.
v) In fully differentiated human subcutaneous adipocytes, *EGFR* gene knockdown also resulted in decreased expression of adipogenic genes.

In line with these findings, a previous study in 32 women with different degrees of obesity and insulin resistance reported that subcutaneous adipose tissue EGFR protein levels were positively correlated with expression of some adipogenic genes, and that pharmacological inhibition of EGFR reduced expression of adipogenic genes in human preadipocytes after adipocyte differentiation induction [5]. Similar to this study [5], SAT *EGFR* gene expression was negatively correlate with insulin resistance ($HOMA_{IR}$). Taking into account the importance of adipose tissue function on insulin sensitivity [2–4], current data led us to suggest that adipose tissue EGFR might a useful therapeutic target to improve insulin resistance through the enhancing of adipose tissue adipogenesis. It is important to note that impaired adipogenesis led to decreased adiponectin production (an adipokine with insulin-sensitizing effects [21]), reduced cellular capacity to store excess energy by decreasing the rates of fatty acid uptake and esterification [22], and enhanced adipose tissue inflammation [23]. All these changes consequently promote an insulin resistance phenotype [24].

Another important finding of current study was the association between *ERBB2* mRNA and obesity. This association has been shown in two independent cross-sectional cohorts and longitudinally after bariatric surgery-induced weight loss. Consistent with these findings, previous studies reported increased serum HER2 levels, which were the soluble form of ERBB2 protein, in human obesity and insulin resistance [25,26]. Since serum HER2 levels reflects tumor ERBB2 overexpression in oncology patients [27], current data led us to postulate that the association between serum HER2 and obesity might come from increased

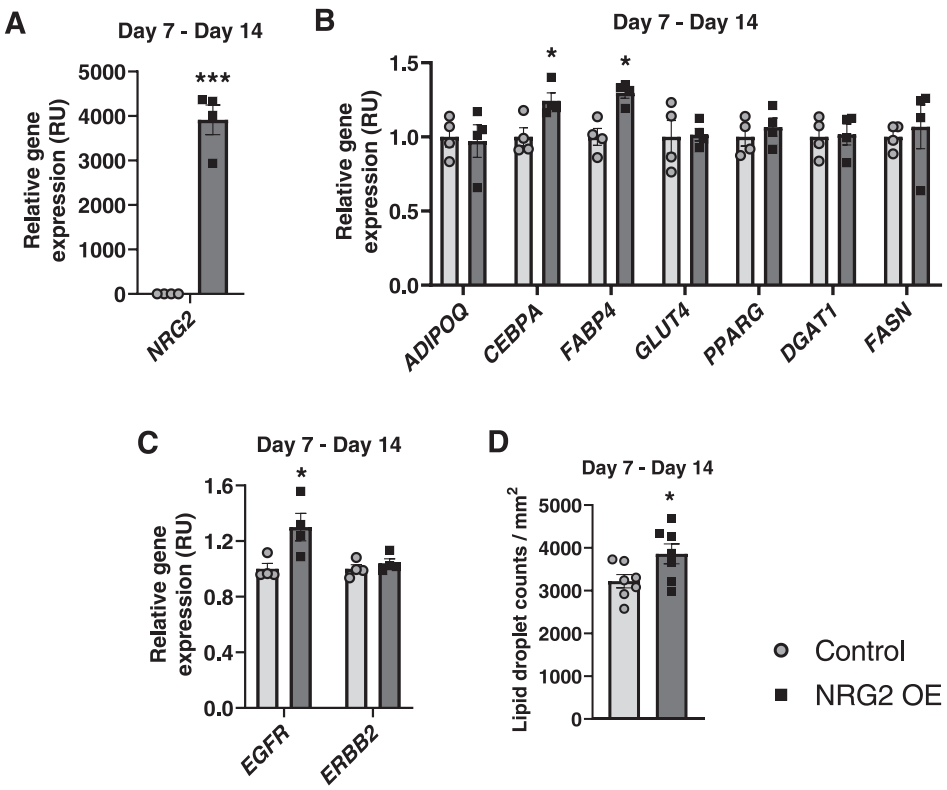

Fig. 6. A-D) Impact of day 7-induced *NRG2* gene overexpression on *NRG2* **(A)**, adipogenic (*ADIPOQ, CEBPA, FABP4, SLC2A4, PPARG, DGAT1, FASN*) **(B),** *EGFR* and *ERBB2* **(C)** gene expression, and on lipid droplet counts **(D)** at day 14. *$p < 0.05$ and * **$p < 0.001$ compared to control.

ERBB2 expression in adipose tissue. Adipose tissue *ERBB2* was also positively correlated with fasting glucose, and negatively with lipogenic genes, but most of these correlations were lost after adjusting by age and BMI. In fact, *ERBB2* gene knockdown did not impact on adipogenic genes (except for DGAT1) during human adipocyte differentiation, but in fully differentiated adipocytes it led to decreased mRNA levels of the major lipogenic enzyme fatty acid synthase (FASN). These data indicate that ERBB2 might support *de novo* lipogenesis in differentiated adipocytes, but it was not required for an optimal differentiation of adipocytes, as previously reported [5]. Strengthening these findings, previous studies demonstrated the bidirectional relationship between ERBB2 and lipogenesis in cancer cells [28], showing that the pharmacological or genetic inhibition of this receptor reduced FASN expression, and that the specific inhibition of FASN attenuated ERBB2 signaling [28–31]. However, it is important to note that the total depletion of Erbb2 gene in mouse primary preadipocytes blunted adipocyte differentiation, indicating that at least a minimal level of ErbB2 might be required to sustain adipose tissue adipogenesis and expansion.

Otherwise, even though *ERBB3* and *ERBB4* gene expression were associated with expression of some adipogenic and inflammatory genes, the loss of most of these associations after adjusting for age and BMI, the disparity between cohorts and the very low mRNA levels detected suggest that these receptors are of little relevance in adipose tissue physiology. In fact, Erbb4 depletion un mouse primary preadipocytes did not impact on adipogenic gene expression (current study). In line with this, previous studies also reported very low *ERBB3* and *ERBB4* mRNA levels in 3T3-L1 cells [6] and human adipose tissue [5]. The different associations observed in VAT from cohort 1 between expression of *ERBB4* and *IL6/IL8* (negative) and between *ERBB4* and *TNF* (positive) might be explained by their covariance with other metabolic factors. Interestingly, while the association between VAT *ERBB4* and *TNF* gene expression was no longer significant after adjusting by age and BMI, the negative correlation between VAT *ERBB4* and *IL6/IL8* expression remained statistically significant. Supporting these associations, ErbB4 deletion in mice resulted in increased adipose tissue inflammation [18], while the expression of NRG4, a specific ligand for ERBB4, was negatively correlated with *IL6* and *IL8* gene expression in VAT [16], and *Nrg4* gene knockdown in 3T3-L1 adipocytes also enhanced the expression of proinflammatory cytokines [32].

In addition, exploring neuregulins as some putative endogenous EGFR ligands in adipose tissue that promote adipogenic pathway, we found that NRG2 might be an optimal candidate. Compared to other detected neuregulins in adipose tissue (*NRG1* and *NRG4*), increased *NRG2* mRNA levels were found. SAT and VAT *NRG2* mRNA levels were positively correlated with adipogenic genes and increased during adipocyte differentiation, whereas NRG1 (current study) and NRG4 [16] negatively correlated with adipogenesis. To the best of our knowledge this is the first study reporting *NRG2* expression in adipose tissue and its association with adipogenesis. The overexpression of *NRG2* gene in the late stage of adipocyte differentiation (but not in preadipocytes) slightly increased expression of some adipogenesis-related genes, *CEBPA* and *FABP4,* and the number of intracellular lipid droplets, supporting a possible role of NRG2 on the maintenance of adipogenesis, but not in the induction of adipocyte differentiation.

Taken together, this study demonstrates the importance of EGFR and ERBB2 in human adipose tissue physiology, showing the association between adipose tissue ERBB2 and obesity, confirming the adipogenic role of EGFR and suggesting neuregulin 2 as a possible endogenous EGFR ligand with a hypothetical role in human adipogenesis.

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Author contributions statement

JMF-R and JMM-N participated in study design and analysis of data and wrote and edited the manuscript. JL, CM, FO, AL, NO-C and FD-S participated in acquisition of data. JA, MC, AG, and WR participated in interpretation of data. JL, CM, FO, AL, NO-C, FD-S, JA, MC, AG, and WR revised the manuscript critically for important intellectual content. All authors participated in final approval of the version to be published.

Conflict of interest statement

The authors declared no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113972.

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