

Adipocyte-derived extracellular vesicles sustain mitochondrial metabolism in breast cancer cells: New insights into the cross-talk between cancer cells and the tumor microenvironment

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Received January 20, 2025; Accepted July 14, 2025

DOI: 10.3892/ijo.2025.5806

Abstract. Adipocytes represent the most prominent component of breast tissue stroma and are recognized as significant contributors to the observed association between obesity and breast cancer (BC). It has been widely reported that dysfunctional adipose tissue in obesity has a profound effect on the biology of BC via the secretion of several bioactive molecules. Recently, extracellular vesicles (EVs), a heterogeneous group of membrane-enclosed structures, have been recognized as key players in adipocyte-BC cell communication. We previously demonstrated that adipocyte-derived EVs promoted BC proliferation, migration, invasion, stemness and traits of epithelial-to-mesenchymal transition through the activation of hypoxia inducible factor-1 α (HIF-1 α). The present study, to further understand the impact of EVs in breast adiponcosis, investigated the effects of adipocyte-derived EVs on the BC proteome. By employing liquid chromatography-tandem mass spectrometry and different bioinformatic tools (such as Proteomap, STRING, FunRich, Reactome and MsigDB), it

was found that adipocyte-derived EVs regulated the expression of multiple proteins implicated in metabolic processes. Adipocyte-derived EVs shifted cell metabolism towards oxidative phosphorylation in estrogen receptor-positive (ER+) BC cell lines, including MCF-7, ZR-75-1 and BT-474 BC cells, through an increased mitochondrial activity along with an enhanced ATP production. These findings were extended by treating BC cells with EVs isolated from the serum of patients with BC classified as normal weight (NW-EVs) and overweight or obese (OW/Ob-EVs). Treatment of BC cells with OW/Ob-EVs resulted in a significant increase of mitochondrial activity and ATP production compared with NW-EVs. Of note, inhibition of HIF-1 α expression/activity reversed the effects of both adipocyte-derived EVs and OW/Ob-EVs on BC cell metabolism. In conclusion, the present study underscored the pivotal role of EVs in the BC-obesity link, highlighting their involvement in driving metabolic reprogramming in ER+ BC cells through HIF-1 α .

Introduction

Intimate intercellular communication between breast cancer (BC) cells and other cells in the surrounding microenvironment exerts a significant influence on a number of key processes, including primary tumor growth, metastasis evolution and immune escape. In particular, the breast is a fat-rich organ and BC development and progression are strongly influenced by the intimate and highly vicious cycle between adipocytes, which act as endocrine cells, and tumor cells (1). The main methods of cell-to-cell communication are direct contacts involving adhesion molecules and indirect contacts through classical paracrine signaling mediated by secreted molecules (2,3). Beyond the secretion of various soluble factors (for example, leptin and adiponectin) (4-13), adipocyte-derived EVs, a

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Key words: extracellular vesicles, breast cancer, obesity, metabolism, oxidative phosphorylation, hypoxia-inducible factor 1

heterogeneous group of membrane-enclosed structures, have emerged as an essential part of the adipocyte secretome (14), acting as key mediators of intercellular communication and contributing to autocrine, paracrine and endocrine communication (15). Indeed, EVs are capable of transferring a multitude of biological molecules, including proteins, nucleic acids and lipids, which can subsequently alter the biology of the recipient cells. In this situation, it has been demonstrated that EVs derived from 3T3-L1 adipocytes, a widely used *in vitro* model for white adipocytes, sustain proliferation, migration and invasion and response to therapy in several cancer cell lines through multiple mechanisms (16-21). For instance, melanoma cell lines treated with adipocyte-derived EVs exhibit more aggressive features, including elongated morphology and actin-rich membrane profusion along with an increased cell migration *in vitro* and lung colonization *in vivo* (18). Furthermore, it has been also demonstrated that 3T3-L1-EV adipocytes promote lung cancer cell invasion *in vitro* and *in vivo* through an increased activity of matrix metalloproteinase 9 (MMP-9) (16). In another study, EVs derived from 3T3-L1 adipocytes were reported to: i) Promote the proliferation of prostate cancer cells, ii) sustain their migratory capabilities and iii) confer resistance to docetaxel. This is achieved through a mechanism involving the Akt/hypoxia-inducible factor 1 α (HIF-1 α) axis, which is accompanied by enhanced glycolysis and ATP production (17). The involvement of the HIF-1 α /von Hippel-Lindau axis in modulating the protumoral effects of adipocyte-derived EVs has been also reported in hepatocellular carcinoma (19). In line with this evidence, our research group also found that 3T3-L1 adipocyte-derived EVs support BC cell proliferation, growth, migration and invasion as well as their metastatic potential via the induction of HIF-1 α levels and activity (20).

The present study employed proteomic analysis to gain deeper insights into the role of EVs in breast adiponcosis, providing novel evidence on the role of adipocyte-derived EVs in sustaining mitochondrial metabolism in BC cells through HIF-1 α .

Materials and methods

Antibodies and reagents. Dexamethasone, 3-Isobutyl-1-Methylxanthine and bovine insulin were purchased from Merck KGaA. KC7F2 (cat. no. S7946) from Selleck Chemicals LLC. Tumor susceptibility gene 101 (Tsg101; cat. no. MA1-23296) was purchased from Thermo Fisher Scientific, Inc. ALIX (cat. no. ab186429) and total OXPHOS (cat. no. ab110411) cocktail was purchased from Abcam. CD81 (cat. no. sc-166029); Heat shock protein 90 (HSP90; cat. no. sc-7947); Calnexin (cat. no. sc-11397), HIF-1 α (cat. no. sc-13515), TOM20 (cat. no. sc-17764), β -Actin (cat. no. sc-47778) and GAPDH (cat. no. sc-166545) were acquired from Santa Cruz Biotechnology, Inc.

Cell cultures. Human MCF-7 (cat. no. HTB-22), ZR-75-1 (cat. no. CRL-1500) and BT-474 (cat. no. HTB-20) BC epithelial cells and murine 3T3-L1 (cat. no. CRL-1500) pre-adipocytes were acquired from American-Type-Culture-Collection (ATCC) and cultured in accordance with supplier specifications. MCF-7 and ZR-75-1 cells were cultured in EMEM

medium and RPMI-1640 (Thermo Fisher Scientific, Inc.), respectively, containing 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.). BT-474 cells were cultured in ATCC Hybri-Care Medium supplemented with 10% FBS, 1% penicillin-streptomycin. Every six months, cells were authenticated by single tandem repeat analysis at our Sequencing Core; morphology, doubling times and estrogen sensitivity and tested for mycoplasma negativity (MycoAlert; Lonza Group Ltd.).

Isolation of extracellular vesicles (EVs)

Isolation of 3T3-L1 adipocyte-derived EVs. EVs were isolated from murine 3T3-L1 and 3T3-L1 adipocytes (3T3-L1A) conditioned media. Briefly, 3T3-L1 and 3T3-L1A cells were cultured in serum-free medium for 24 h and EVs were obtained from the resulting conditioned medium using differential ultracentrifugation (20).

Isolation of normal weight (NW) and overweight/obese (OW/Ob) EVs. EVs from serum samples of NW and OW/Ob female patients with BC were isolated by the ExoQuick isolation agent (System Bioscience, Palo Alto, CA, USA), following the manufacturer's instructions (22). A total of 45 biological samples, with an age range of 30-73 years for NW group and 36-83 years for OW/Ob groups, were collected, between May 2016 and February 2020, at the Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II. The samples were stored at -80°C until use. The study protocol was reviewed and approved (approval no. 107/05) by the Ethics Committee of the University of Naples Federico II (Department of Advanced Biomedical Sciences).

Isolated EVs were stored at -80°C and characterized by transmission electron microscopy (TEM), Nanoparticle tracking analysis (NTA) and immunoblotting. For TEM analysis, the EV-containing aliquots were diluted in sterile NaCl 0.9% and then added onto formvar-carbon coated grids (Electron Microscopy Sciences, cat. no. FCF400-Cu). The grids were blotted dry at room temperature. The imaging process was conducted using a Jeol JEM 1400 Plus transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 80 kV. NTA was performed as previously reported (20). As storage at -80°C maintains EV stability, with minimal changes in size, concentration and protein content (23,24), these EVs were used in the respective experiments reported in the current study.

Gene silencing transfection. Cells were transfected with small interfering (si)HIF-1 α silencer validated siRNA (cat. no. 4390824; ID s6541) and with silencer select negative control (NC; cat. no. 4390843) (Ambion; Thermo Fisher Scientific, Inc.), using Lipofectamine® RNAi/MAX reagent (Thermo Fisher Scientific, Inc.) according to the manual instructions. Specifically, 35x10⁴ MCF-7 BC cells were transfected with 50 pmol of both validated (si)HIF-1 α silencer siRNA and silencer select negative control and plated in 6-well plates. After 24 h, transfected cells were plated for mitotracker analysis (35x10⁴ cells in 6-well plates) and for seahorse analysis (10⁴ cells in XFe-96 well plates). Assays were then performed as described.

Immunoblot analysis. Immunoblot were performed as previously described (25). Specifically, cell extracts or EV lysates were obtained using RIPA buffer (Sigma-Aldrich), supplemented with protease inhibitor. 50 μ g of proteins, previously quantified by Pierce™ BCA Protein Assay Kit (cat. no. 23227; Thermo Fishers Scientific, Inc.) were resolved on 10% SDS polyacrylamide gel and transferred to nitrocellulose membranes by using power blotter XL system (Thermo Fisher Scientific, Inc.). Nitrocellulose membranes were blocked with 5% milk in tris-buffered saline for 1 h at room temperature, which was followed by incubation overnight at 4°C in bovine serum albumin 5% with primary antibodies. Specifically, anti-Tsg101 (cat. no. MA1-23296; dilution 1:1,000); anti-ALIX (cat. no. ab186429; dilution 1:1,000); total OXPHOS cocktail (cat. no. ab110411; dilution 1:1,000); CD81 (cat. no. sc-166029; dilution 1:500); HSP90 (cat. no. sc-7947; dilution 1:500); Calnexin (cat. no. sc-11397; dilution 1:1,000), HIF-1 α (cat. no. sc-13515; dilution 1:200); TOM20 (cat. no. sc-17764; dilution 1:1,000), β -Actin (cat. no. sc-47778; dilution 1:10,000) and GAPDH (cat. no. sc-166545; dilution 1:10,000) primary antibodies were used. IRDye 600 and 800CW Goat anti-Mouse or anti-Rabbit (LI-COR, Inc.) IgG secondary antibodies were used at 1:15,000 dilution. Images were acquired using Odyssey FC (LI-COR, Inc.) and ImageJ software was used to quantify the band of interest (v.1.52a, NIH, USA).

Proteomic analysis

Filter-aided sample protocol (FASP) digestion. The cells were lysed with RIPA buffer and the protein digestion procedure was subsequently performed according to the FASP method (26) using 10 kDa molecular cut-off microcon filters. According to the digestion protocol, 50 μ g of cell lysate was mixed with 10% SDS, 500 mM DTT, 1 M Tris HCl, pH 8.0 and incubated at 650 rpm for 10 min at 95°C. After incubation, the samples were allowed to reach to room temperature and loaded onto the FASP filter. The protocol is characterized by several distinct steps including two washes with 200 μ l of 8M UREA (8 M UA, 100 mM Tris-HCl buffer pH 8.0) and subsequent centrifugation at 14,000 x g for 20 min at room temperature, followed by the addition of 50 μ l of 50 mM Iodioacetamide (IAA) and centrifugation at 6,000 x g for 20 min at room temperature. After the alkylation reaction, two washes with 8M UREA and two washes with 50 mM TEAB were performed. Enzymatic digestion was then performed overnight at 37°C by adding 500 ng of proteomics grade trypsin (MilliporeSigma) in 60 μ l of 50 mM TEAB buffer at pH 8.5.

SCX (strong cation exchange) purification. The next day H₂O (140 μ l) was added and the samples were centrifuged at 14,000 x g for 20 min at room temperature to collect 180-200 μ l of digest. Then, 40 μ l (corresponding to 10 μ g of digested peptides) of each extract were purified by StageTip SCX as described by Rappsilber *et al* (27), to remove detergent residues. Before proceeding with SCX purification, tryptic digests were acidified with 360 μ l of Wash B [80% acetonitrile/0.5% formic acid (v/v)], to reduce the salt concentration. Acidified tryptic peptides were then loaded onto StageTips containing approximately 100 μ g of a cation exchange resin (Millipore Extraction Disks; MilliporeSigma) that had before been conditioned with Wash A [20% acetonitrile/0.5% formic acid (v/v)]

and Wash B. After two washes with 50 μ l of Solution W2 and 50 μ l of Solution W1, respectively, the peptides were eluted by the addition of 10 μ l of 500 mM ammonium acetate-20% acetonitrile and were then diluted by adding 90 μ l of 0.1% formic acid. Then, 1 μ l of the purified sample was used for preliminary liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Following the preliminary injections, the final injections were performed. In particular, for each biological replicate, three LC-MS/MS injections were performed (technical replicate).

LC-MS/MS analysis. For peptide separation, the present study employed an easy nLC-1000 chromatographic instrument coupled to an Exploris 480 mass spectrometer (Thermo Fisher Scientific, Inc.). For LC-MS/MS analysis, samples were separated using a 14 cm, 75 μ m i.d. column, packed in-house with 3 μ m C18 silica particles (Dr. Maisch HPLC GmbH). The peptides were eluted from the column with a binary gradient of 140 min and a flow rate of 300 nl/min. The following mobile phases were used: Mobile phase A [2% acetonitrile/0.1% formic acid (v/v)] and mobile phase B [80% acetonitrile/0.1% formic acid (v/v)]. The peptide separation were obtained at a flow rate of 300 nl/min as follows: From 0% B to 3% B in 1 sec, from 3% B to 8% B in 40 min and from 42% B to 100% B in 13 min; the column was cleaned for 5 min with 100% B. The samples were analyzed in Data Independent Acquisition (DIA) mode. The DIA method included 60 windows (45 windows with an isolation window of 8 m/z, 10 windows with an isolation of 16 m/z and 5 windows with an isolation of 28 m/z). Full MS scans were performed in the range from 350 m/z up to 1,000 m/z with 60000 of resolution (AGC target Custom; maximum injection time 50 and collision energy 25).

Statistical analysis of proteome data. The RAW files were analyzed using the Spectronaut software (version 13.0; Biognosys AG). The search was performed with Direct DIA mode. Protein quantification was performed using the following parameters: Active precursor filtering (it required the precursor to be quantified in at least 20% of the runs); imputation by run wise imputing, Major Group Top N (Max 10-Min 1). Missing values at the protein quant level were 2006, averaging at ~111 proteins per sample (2.5%) and were handled in Spectronaut using the following parameters: Active precursor filtering (it required the precursor to be quantified in at least 20% of the runs); imputation by run wise imputing. The resulting matrix, which contained no missing values since already imputed in Spectronaut, was then imported into Perseus (version 2.0.6.0; Max-Planck-Gesellschaft) to perform the necessary statistical analysis (28). The data were transformed using a logarithmic scale (log₂). Protein quantities from technical replicates (separate LC-MS/MS injections) were combined using the median value. Finally, the unpaired two-sample t-test was used to assess the statistical significance of protein abundances. Correction for multiple hypothesis testing was permutation-based (FDR <0.05). The principal component analysis (PCA) plot was generated by using the R packages (version 4.4.2; <https://www.R-project.org/>), 'Factoextra' (version 1.0.7, <https://CRAN.R-project.org/package=factoextra>) and 'ggplot2' (version 3.5.2; <https://ggplot2.tidyverse.org>); 95%

confidence limits within the PCA plot were calculated using the function 'fviz_pca_ind' in the 'Factoextra' package.

Analysis of protein annotations. The resulting protein list was subjected to different annotation tools for further in-depth analysis. For functional makeup of the identified proteins Bionic Visualizations Proteomaps was used (<https://www.proteomaps.net>). All de-regulated proteins (39 upregulated and 59 downregulated) were used for the analyses.

Protein-protein interaction (PPI) network construction. The PPI network of the candidate proteins was generated using STRING version 12.0 (https://string-db.org/cgi/input?sessionId=bp7GGoXn66Fl&input_page_show_search=on).

Functional enrichment analysis. De-regulated proteins identified in MCF-7 cells treated with 3T3-L1A-EVs were subjected to Gene Ontology (GO) and biological pathway enrichment analysis using FunRich tool (<http://www.funrich.org>) against human FunRich background database. $P \leq 0.05$ was considered to indicate a statistically significant difference and the GO results were ranked by P-value. The top 10 significant pathways in the de-regulated proteins were exhibited as bar charts. The same bioinformatics tool was employed to construct a Venn diagram, with the objective of evaluating the potential overlap between the identified de-regulated proteins and EV proteins that have been previously annotated in Vesiclepedia (version 5.1, <http://www.microvesicles.org/>) and Exocarta (version 3.1, <http://www.exocarta.org/>) databases.

Gene set enrichment analysis (GSEA). GSEA was performed using the Molecular Signatures Database (MSigDB; <https://www.gsea-msigdb.org/gsea/msigdb>) to examine the gene expression profiles of de-regulated proteins identified in MCF-7 cells treated with 3T3-L1A-EVs. The analysis included overlaps with GO categories: GO:BP (Biological Process), GO:CC (Cellular Component) and GO:MF (Molecular Function). Results were visualized using the ggplot2 package in R.

Pathway enrichment analysis. The analysis of signaling pathways in which de-regulated proteins identified in MCF-7 cells treated with 3T3-L1A-EVs are involved was performed using the REACTOME free online database (<https://reactome.org/>). The resulting data were visualized in R using the ggplot2 package.

Seahorse XFe96 metabolic profile analysis. By using XFe-96 well cell culture plates MCF-7, ZR-75-1 and BT-474 cells were seeded in 150 μ l of regular growth medium for 24 h (10^4 cells/well). The next day, 2 μ g/ml of 3T3-L1A-EVs were added to Exo-depleted medium (Thermo Fisher Scientific, Inc.) for 48 h. The real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were assessed by using mito stress and glycolysis stress assays, respectively (Agilent Technologies, Inc.). The day prior to assay a sensor cartridge was hydrated using Seahorse XF Calibrant at 37°C for 1 h in a non-CO₂ incubator. On the day of the experiment, cells were in prewarmed XF assay media (10 mM glucose, 1 mM pyruvate, 2 mM L-glutamine to assess OCR levels; 2 mM L-glutamine to measure ECAR levels and adjusted to

pH 7.4) and cells were maintained at 37°C for 1 h in a CO₂-free incubator. Then, 1.5 μ M Oligomycin, 0.5 μ M FCCP and 0.5 μ M Rotenone/0.5 μ M Antimycin A mix were sequentially injected to measure mitochondrial activity. To assess glycolytic activity 10 mM glucose, 1 μ M Oligomycin and 50 mM M2-deoxyglucose (2-DG) were sequentially injected. The data were then normalized using the sulforhodamine B (SRB) assay.

Sulforhodamine B (SRB) assay. Trichloroacetic acid (TCA; 10%) was added to cells (1 h at 4°C), then SRB dye was used (30 min; Acid Red 52; Merck KGaA) and the cells washed two times with 1% acetic acid. The cells were left to air dry for a minimum of 3 h. Finally, 10 mM Tris pH 8.8 solution was added and the absorbance measured at a wavelength of 565 nm (Multiskan SkyHigh; Thermo Fisher Scientific, Inc.).

Determination of cellular ATP levels. MCF-7 cells were plated in 1.5 ml of Exo-depleted medium into 6 well-plates for 24 h (35×10^4 cells/well) and the next day treated or not with the same amount (2 μ g/ml) of EVs for 48 h. Intracellular ATP levels were determined by using an ATP Assay Kit (cat. no. ab83355; Abcam) and optical density (570 nm) was measured using a microplate reader (Multiskan SkyHigh; Thermo Fisher Scientific, Inc.). ATP levels were normalized to cell number.

Assessment of mitochondrial mass and mitochondrial membrane potential. Cells were plated in 1.5 ml of regular growth medium into 6 well-plates (35×10^4 cells/well) and the next day were treated or not with 2 μ g/ml of 3T3-L1A-EVs for 48 h in Exo-depleted medium. At the end of the treatment, cells were trypsinized and equally divided into two separate tubes. Each aliquot was then incubated with a different probe [MitoTracker® Deep Red FM or MitoTracker® Orange CMTMRos (Thermo Fisher scientific, Inc.) 10 nM in PBS for 30-60 min at 37°C] to assess mitochondrial mass and membrane potential, respectively, by FACS analysis (CytoFLEX Beckman, Beckman Coulter, Inc.). For data analysis, manual gating was used to remove debris and doublets and analysis was performed at constant events (10^4) in each sample (Fig. S1). Data analysis was performed using CytExpert Beckman Coulter software (version 2.4, Beckman Coulter, Inc.).

Statistical analysis. Statistical analysis was performed with unpaired Student's t test for the comparison between two groups and one-way ANOVA with Tukey's multiple comparisons post hoc test for the comparison between multiple variables by using GraphPad Prism 8 (GraphPad Software, Inc.; Dotmatics) and the results were reported as the mean $\pm$ SD or $\pm$ SEM, as indicated, of at least 3 independent experiments, each performed in triplicate. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

3T3-L1A-derived EVs affect the proteomic profile of BC cells. In order to evaluate the effect of EVs on the proteomic profile of BC cells, the present study used stored and fully characterized

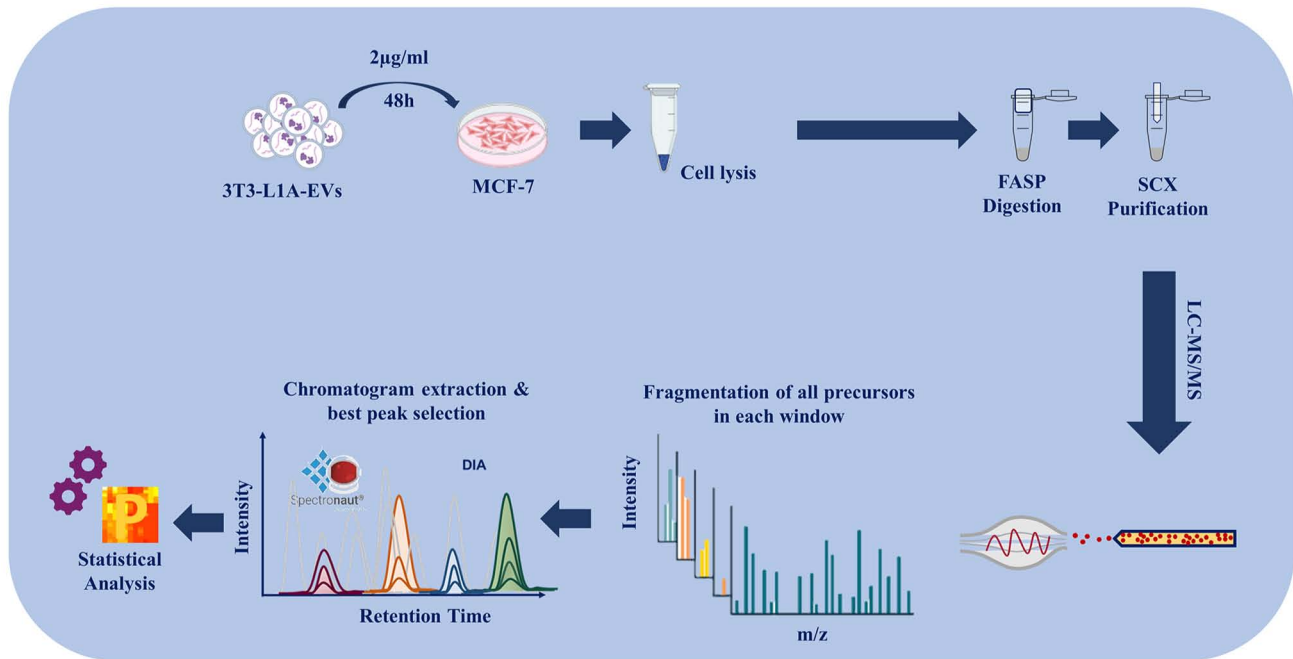


Figure 1. Schematic representation of the complete workflow to analyze changes in the proteome profile of MCF-7 BC cells treated with or without EVs-derived from 3T3-L1A (2 µg/ml) for 48 h. The figure was created using BioRender (<https://biorender.com>). EVs, extracellular vesicles; 3T3-L1A, murine 3T3-L1 adipocytes; MCF-7, estrogen receptor-positive breast cancer cells; FASP, filter-aided sample protocol; SCX, strong cation exchange; LC-MS/MS; liquid chromatography-tandem mass spectrometry; DIA, data independent acquisition.

EVs, which had previously been isolated from the conditioned media derived from 3T3-L1A cells, the most commonly used *in vitro* model for white adipocytes (20). EVs were characterized by transmission electron microscopy (TEM), Nanoparticle tracking analysis (NTA) and immunoblotting. Specifically, TEM analysis confirmed that EVs exhibited the characteristic oval or cup-shaped morphology, enclosed within a lipid bilayer membrane (Fig. S2A) and the NTA analysis revealed that the mode diameter remained consistently below 200 nm in all isolated EVs (Fig. S2B). Immunoblotting showed an enrichment of the EV hallmarks, including Alix, CD81, Tsg101 and HSP90, while the negative marker Calnexin was not detected in all isolated EVs (Fig. S2C). Estrogen receptor-positive (ER+) MCF-7 BC cells were treated for 48 h with 2 µg/ml EVs and then cells were subjected to LC-MS/MS, by using FASP protocol and DIA. Raw data were analyzed by using Spectronaut and Perseus (statistical analysis) software. A schematic diagram of the experimental design of the study is shown in Fig. 1.

PCA indicated good reproducibility of the technical replicates and injections demonstrating the different two-dimensional distribution between the three replicates of each sample (untreated and treated samples). Furthermore, the PCA revealed that the EV-treated cells clustered separately from the untreated cells, highlighting a significant difference in the proteomes of the two different conditions (Fig. 2A). LC-MS/MS analysis allowed us to identify 4,411 proteins (FDR <0.05). Prior to imputation, only very few proteins were exclusively identified and quantified in either one of the two conditions; in particular, syntenin-1, ubiquitin carboxyl-terminal hydrolase 4 and vinexin were exclusively identified in control MCF-7 cells, whereas skin-specific protein 32 was exclusively detected in 3 analyses of 3T3-L1A-EV-treated MCF-7 cells. Among the

proteins revealed by LC-MS/MS analysis, 98 proteins were found to be differentially expressed in 3T3-L1A-EV-treated MCF-7 cells, with a $P \leq 0.05$ (Table I). Of these, 39 proteins were upregulated and 59 proteins downregulated, as reported in the volcano plot (Fig. 2B). The hierarchical cluster analysis confirmed the alteration in protein levels between MCF-7 cells treated with 3T3-L1A-EVs and untreated cells (Fig. 2C). In addition, when the de-regulated proteins were compared with those identified in the Vesiclepedia and Exocarta databases, it was found that 40 of them could be attributed to vesicular origin (Fig. 2D). Furthermore, the association of the 98 differentially expressed proteins in 3T3-L1A-EVs-treated MCF-7 cells were investigated with the ‘extracellular exosome’ gene ontology (GO:0070062). It is notable that a total of 13 proteins (gene names: *PLG*, *APOD*, *PRKCA*, *CLIC1*, *SAMM50*, *HLA-B*, *GPRC5A*, *GSTM2*, *PPIC*, *AASDHPPT*, *NEDD8*, *LAMTOR1* and *VTN*) were found to be associated with the ‘extracellular exosome’ gene ontology (Fig. 2E).

To investigate the biological significance of the 98 de-regulated proteins revealed by proteomic analysis, the proteome of 3T3-L1A EV-treated MCF-7 cells was summarized into a proteomap (29) (<https://www.proteomaps.net>), wherein each protein is represented by a polygon which size is proportional to the protein's abundance. Taking into account the protein fold regulation, the present study gathered functional information clustered in five categories: Genetic information processing (blue), metabolism (orange), environmental information processing (cyan), cellular processes (red) and organismal systems (pink) (Fig. 3A). At the broadest level, most proteins identified in EV-treated MCF-7 cells have been assigned to the following biological processes: Genetic information processing and metabolism, especially in terms of biosynthesis and energy metabolism. In the category of metabolism, the de-regulated

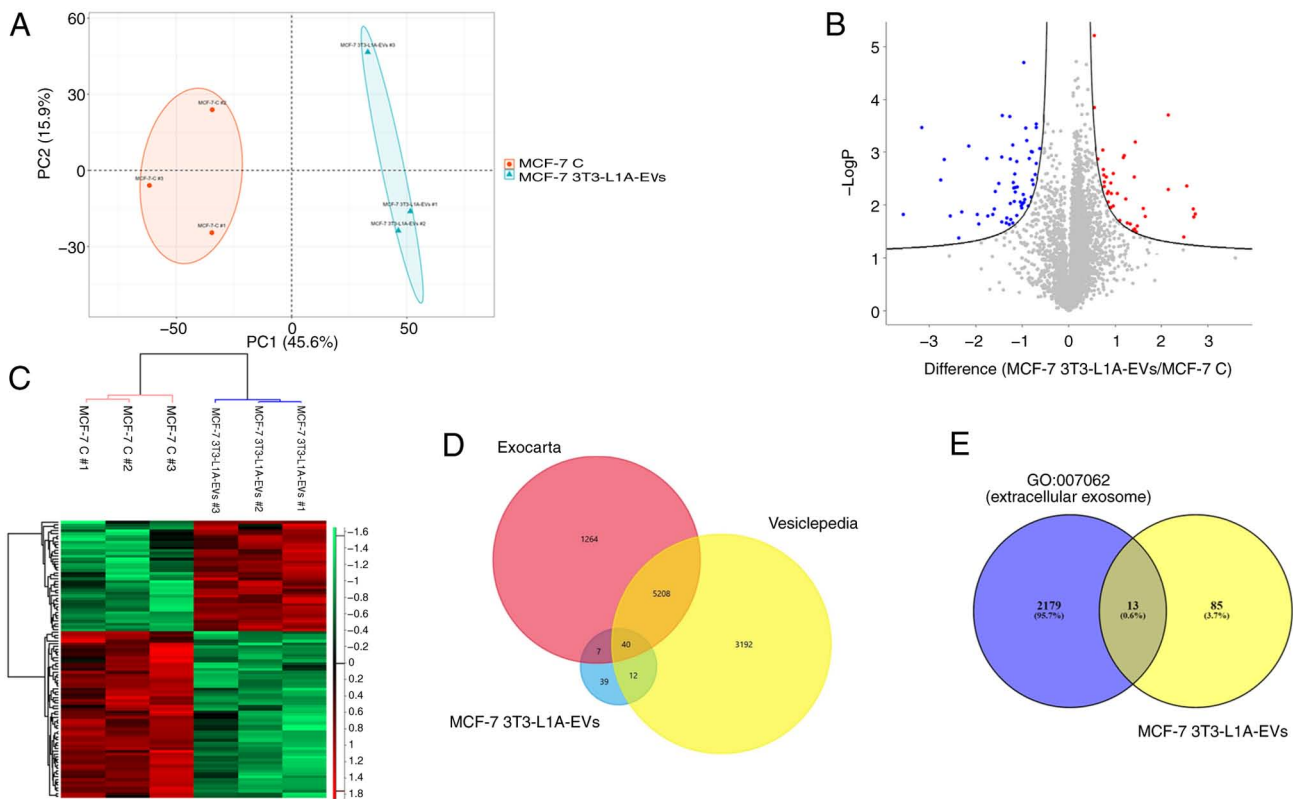


Figure 2. Proteomic analysis of MCF-7 BC cells treated with EVs isolated from mature 3T3-L1 adipocytes. (A) The PCA plot shows the control group (red spheres) and 3T3-L1A-EV-treated group (blue spheres) using R package (version 4.4.2). (B) Volcano plot displaying all identified proteins from MCF-7 BC cells treated with 3T3-L1A-EVs (red dots for upregulated proteins; blue dots for downregulated proteins). (C) Heatmap illustrating the differentially expressed proteins in control and EV-treated MCF-7 cells identified by LC-MS/MS. The color scale shows the relative amount of each protein across the six samples, with red indicating upregulation and green indicating downregulation; black represents the median expression level. (D) Proteins identified in MCF-7 BC cells treated with 3T3-L1A-EVs were subjected to a Venn diagram for comparison with proteins reported in the Vesiclepedia and Exocarta databases. (E) Proteins identified in MCF-7 BC cells treated with 3T3-L1A-EVs were subjected to a Venn diagram for comparison with proteins reported in the 'extracellular exosome' gene ontology (GO:007062). EVs, extracellular vesicles; PCA, principal component analysis; MCF-7, estrogen receptor-positive breast cancer cells; 3T3-L1A, murine 3T3-L1 adipocytes.

proteins were involved into the oxidative phosphorylation, lipid/steroid metabolism, glycan metabolism, cofactor biosynthesis, amino acid metabolisms and pentose phosphate metabolism (Fig. 3A). The interactions of the 98 de-regulated proteins identified by proteomic analysis were analyzed using the STRING software (https://string-db.org/cgi/input?sessionId=bAzNH23SoAwP&input_page_show_search=on). The established network shown in Fig. 3B contained 98 nodes and *MRLP4*, *MTIF2* and *NDUFAB1* had more degree of connection. Subsequently, a functional enrichment analysis was conducted, which corroborated the aforementioned findings. The 98 de-regulated proteins were found to be involved in metabolic processes and in mitochondria. In particular, cellular metabolic process (58 proteins; FDR: 0.0015), cellular component organization or biogenesis (52 proteins; FDR 0.0018), cellular nitrogen compound metabolic process (37 proteins; FDR 0.0046) and metabolic process (64 proteins; FDR 0.0021) were among the first five biological processes (Fig. 3C). In addition, mitochondrion (18 proteins; FDR 0.0124) was the top subcellular localization (Fig. 3D). In the Reactome Pathway analysis (<https://reactome.org/>), the present study found 'Metabolism of RNA', 'SUMOylation', 'SUMO E3 ligases SUMOylate target proteins', 'Interferon alpha/beta signaling', 'Mitochondrial translation' and 'Mitochondrial

translation initiation' among the top five enrichment pathways ranked by P-value (Fig. 3E). Moreover, among the cellular component GOs, 'mitochondrion' resulted the top enriched GO in the Molecular Signatures Database analysis (MSigDB; <https://www.gsea-msigdb.org/gsea/msigdb/>; Fig. 3F), in term of overlap and P-value. In order to conduct a more thorough investigation into the function of the 98 de-regulated proteins identified in MCF-7 cells treated with 3T3-L1A-EVs, the present study also conducted an evaluation of their potential association with other GOs, including 'ATP biosynthetic process', 'ADP metabolic process', 'response to hypoxia' and 'glycolytic process'. No relevant associations were found, except for NADH-ubiquinone oxidoreductase chain 2, which showed an association with 'ATP biosynthetic process' and 'response to hypoxia' and for Acyl carrier protein, mitochondrial, which was associated with 'ATP biosynthetic process' (data not shown).

The present study also used FunRich (<http://www.funrich.org>) platform to obtain GO enrichments of the all de-regulated proteins. According to the cellular component, the de-regulated proteins were clustered in 'mitochondrion' (21.2%), in 'nucleolus' (17.6%) and in 'cytoplasm' (52.9%; Fig. S3A). In the context of 'biological process', the investigated proteins were markedly enriched in GOs such as 'protein modification'

Table I. The 98 de-regulated proteins identified in the comparison MCF-7 3T3-L1A-EV treated cells vs. untreated cells.

Gene name	Fold change	Protein descriptions
ATPIF1	6.6	ATPase inhibitor, mitochondrial
PLG	6.5	Plasminogen
USMG5	6.5	Up-regulated during skeletal muscle growth protein 5
RABIF	5.8	Guanine nucleotide exchange factor MSS4
ANTXR2	5.6	Isoform 4 of Anthrax toxin receptor 2
COX17	4.4	Cytochrome c oxidase copper chaperone
FBXO7	4.4	F-box only protein 7
AGPAT1	3.1	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha
POLR2G	3.1	DNA-directed RNA polymerase II subunit RPB7
STAG2	2.8	Cohesin subunit SA-2; Isoform 2 of Cohesin subunit SA-2
YARS2	2.7	Tyrosine-tRNA ligase, mitochondrial
MAP4K5	2.7	Mitogen-activated protein kinase kinase kinase 5
AFAP1	2.7	Actin filament-associated protein 1
PRKCA	2.7	Protein kinase C alpha type
BTF3	2.6	Isoform 2 of Transcription factor BTF3
TUBGCP2	2.5	Gamma-tubulin complex component 2
MRPL4	2.4	39S ribosomal protein L4, mitochondrial
NSUN4	2.4	5-methylcytosine rRNA methyltransferase NSUN4
GEMIN6	2.3	Gem-associated protein 6
PPM1H	2.3	Protein phosphatase 1H
TMEM38B	2.2	Trimeric intracellular cation channel type B
IRF2BP1	2.1	Interferon regulatory factor 2-binding protein 1
MPDU1	1.9	Mannose-P-dolichol utilization defect 1 protein
TBC1D23	1.9	TBC1 domain family member 23; Isoform 2 of TBC1 domain family member 23
ACOT8	1.9	Acyl-coenzyme A thioesterase 8
KRI1	1.9	Protein KRI1 homolog
WDR6	1.9	WD repeat-containing protein 6
PATL1	1.8	Protein PAT1 homolog 1; Isoform 2 of Protein PAT1 homolog 1
HLA-B	1.8	HLA class I histocompatibility antigen, B-14 alpha chain
FHL3	1.8	Four and a half LIM domains protein 3
CMC1	1.8	COX assembly mitochondrial protein homolog
ALG8	1.7	Probable dolichyl pyrophosphate Glc1Man9GlcNAc2 alpha-1,3-glucosyltransferase;
XIAP	1.7	E3 ubiquitin-protein ligase XIAP
TAPBP	1.7	Tapasin; Isoform 2 of Tapasin; Isoform 3 of Tapasin
DDX50	1.7	ATP-dependent RNA helicase DDX50
IFI6	1.7	Interferon α -inducible protein 6
PPP1R10	1.5	Serine/threonine-protein phosphatase 1 regulatory subunit 10
SUMO1	1.5	Small ubiquitin-related modifier 1;
SCRN3	1.5	Secernin-3; Isoform 2 of Secernin-3
PPIC	0.6	Peptidyl-prolyl cis-trans isomerase C
NOP14	0.6	Nucleolar protein 14
CLIC1	0.6	Chloride intracellular channel protein 1
PFDN6	0.6	Prefoldin subunit 6
HNRNPR	0.6	Heterogeneous nuclear ribonucleoprotein R
RABGGTB	0.6	Geranylgeranyl transferase type-2 subunit beta
JMJD6	0.6	Bifunctional arginine demethylase and lysyl-hydroxylase JMJD6
RPP30	0.6	Ribonuclease P protein subunit p30; Isoform 2 of Ribonuclease P protein subunit p30
MRPL57	0.6	Ribosomal protein 63, mitochondrial
COIL	0.6	Coilin
CRBN	0.6	Protein cereblon; Isoform 2 of Protein cereblon
SAMM50	0.5	Sorting and assembly machinery component 50 homolog
CHRAC1	0.5	Chromatin accessibility complex protein 1

Table I. Continued.

Gene name	Fold change	Protein descriptions
CCDC175	0.5	Coiled-coil domain-containing protein 175
NEDD8	0.5	NEDD8 Ubiquitin Like Modifier
LAMTOR1	0.5	Regulator complex protein LAMTOR1
TOP2B	0.5	DNA topoisomerase 2- β ; Isoform β -1 of DNA topoisomerase 2- β
MTIF2	0.5	Translation initiation factor IF-2, mitochondrial
SEMA4C	0.5	Semaphorin-4C
ZNF687	0.5	Zinc finger protein 687; Isoform 2 of Zinc finger protein 687
DERA	0.5	Deoxyribose-phosphate aldolase
CNOT3	0.5	CCR4-NOT transcription complex subunit 3
DEGS1	0.5	Sphingolipid delta(4)-desaturase DES1
ENOPH1	0.5	Enolase-phosphatase E1
GOLM1	0.5	Golgi membrane protein 1; Isoform 2 of Golgi membrane protein 1
RPL7L1	0.5	60S ribosomal protein L7-like 1
LAMTOR5	0.5	Regulator complex protein LAMTOR5
GID8	0.4	Glucose-induced degradation protein 8 homolog
WDR3	0.4	WD repeat-containing protein 3
CBX1	0.4	Chromobox protein homolog 1
TPM1	0.4	Isoform 10 of Tropomyosin alpha-1 chain; Isoform 4 of Tropomyosin alpha-1 chain
GSKIP	0.4	GSK3-beta interaction protein
RNPS1	0.4	RNA-binding protein with serine-rich domain 1
UBQLN2	0.4	Ubiquilin-2
GPRC5A	0.4	Retinoic acid-induced protein 3
APOD	0.4	Apolipoprotein D
TAF10	0.4	Transcription initiation factor TFIID subunit 10
EIF1	0.4	Eukaryotic translation initiation factor 1
GSTM2	0.4	Glutathione S-transferase Mu 2; Isoform 2 of Glutathione S-transferase Mu 2
AASDHPPT	0.4	L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase
NUP62	0.4	Nuclear pore glycoprotein p62
NDUFAB1	0.4	Acyl carrier protein, mitochondrial
ESYT2	0.4	Isoform 6 of Extended synaptotagmin-2
ARL1	0.4	ADP-ribosylation factor-like protein 1; Isoform 2 of ADP-ribosylation factor-like protein 1
ESYT1	0.3	Isoform 2 of Extended synaptotagmin-1
LMBR1L	0.3	Protein LMBR1L
TAMM41	0.3	Phosphatidate cytidylyltransferase, mitochondrial
L3HYPDH	0.3	Trans-L-3-hydroxyproline dehydratase
HAS1	0.3	Hyaluronan synthase 1
DAAM1	0.3	Disheveled-associated activator of morphogenesis 1
NECAB1	0.3	N-terminal EF-hand calcium-binding protein 1
GTF2A1	0.2	Transcription initiation factor IIA subunit 1
TMEM128	0.2	Transmembrane protein 128
AURKA	0.2	Aurora kinase A
MT-ND2	0.2	NADH-ubiquinone oxidoreductase chain 2
C1QBP	0.2	Complement component 1 Q subcomponent-binding protein, mitochondrial
TAGLN3	0.1	Transgelin-3
VTN	0.1	Vitronectin
ABRACL	0.1	Costars family protein ABRACL

(2.1%), ‘anti-apoptosis’ (2.1%), ‘lipid metabolism’ (2.1%) and protein metabolism’ (13.4%; Fig. S3B). Moreover, among the GO ‘molecular functions’ the de-regulated proteins were

mainly related to ‘chaperone activity’ (4.1%), a family of proteins known to be regulators of metabolic processes (30) (Fig. S3C).

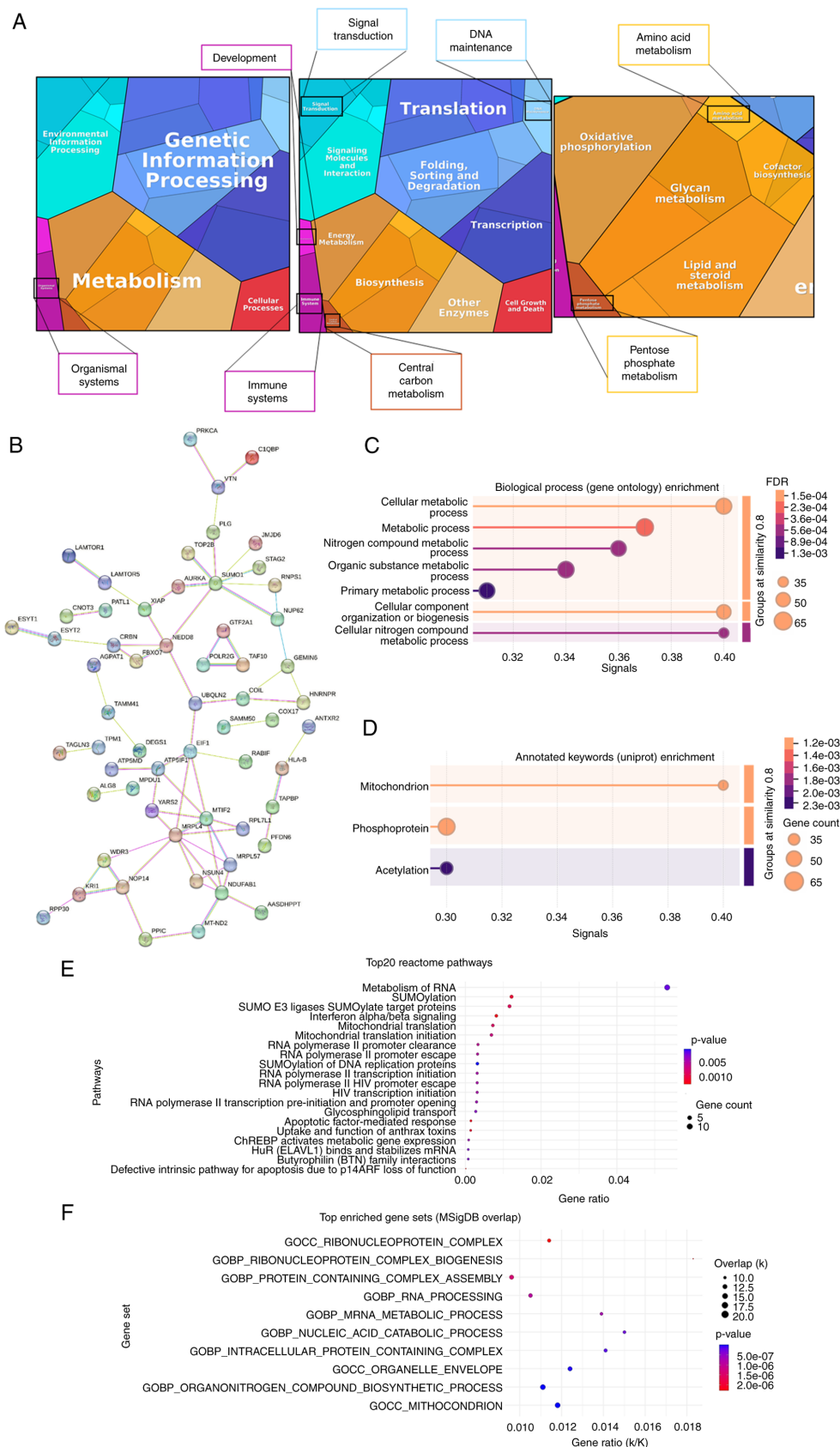


Figure 3. Functional enrichment analysis of the identified de-regulated proteins. (A) By using Proteomaps different levels of information are shown and each polygon represents a single Kyoto Encyclopedia of Genes and Genomes pathway clustered in five categories with different colors: Environmental information processing (cyan), genetic information processing (blue), metabolism (orange), cellular processes (red) and organismal systems (pink). The larger areas have more assigned proteins. (B) Interaction network derived from STRING database. Only proteins with an interaction are shown, as the confidence of the interaction was set to medium (0.4) and gave total 98 nodes; 70 edges with an average node degree of 1.44 and PPI (protein-protein interaction) enrichment P-value of 0.00225. Graphical representation of STRING Gene Ontology (GO) analysis of the GO terms (C) 'Biological Process' and (D) 'Annotated Keyword'. Bubble plot showing the 20 most markedly enriched pathways in the (E) REACTOME pathways and (F) MSigDB databases. BP, biological process; CC, cellular compartment.

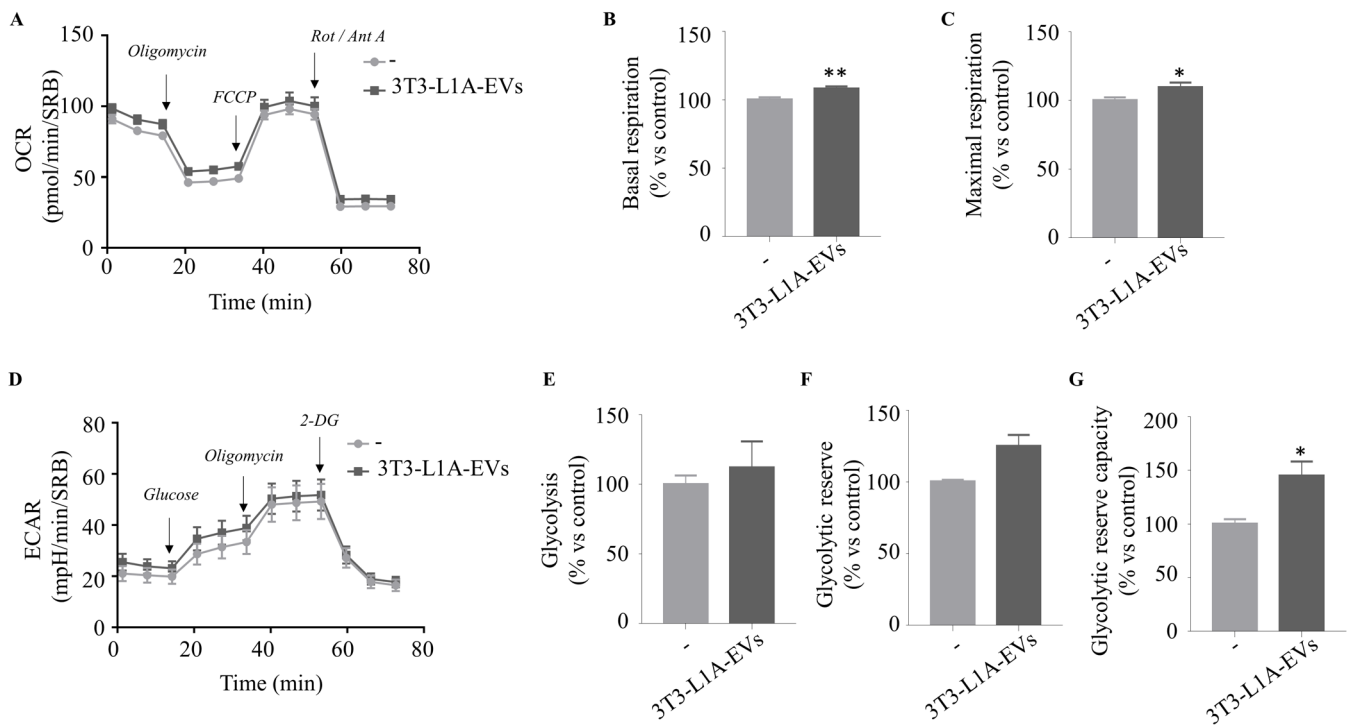


Figure 4. 3T3-L1A-EVs support metabolism of MCF-7 BC cells. The seahorse XF-e96 analyzer was used to assess the mitochondrial and glycolytic metabolic activity of MCF-7 cells untreated (-) or treated with 3T3-L1A-EVs (2 μ g/ml) for 48 h. (A) OCR flux, histograms of different mitochondrial respiratory parameters such as (B) basal and (C) maximal respiration are shown. (D) ECAR flux and relative histograms of (E) glycolysis, (F) glycolytic reserve and (G) glycolytic reserve capacity. Data were analyzed using Student's *t* test and are represented as mean $\pm$ SEM of five biological replicates from three independent experiments and normalized to protein content (SRB assay). **P* $\leq$ 0.05; ***P* $\leq$ 0.01. 3T3-L1A, murine 3T3-L1 adipocytes; OCR, oxygen consumption rates; ECAR, extracellular acidification rates; SRB, sulforhodamine B; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Rot, rotenone; Ant, Antimycin A.

Therefore, the present study sought to ascertain whether 3T3-L1A-EVs might exert an influence on metabolism in BC cells.

3T3-L1A-EVs support oxidative phosphorylation of ER-positive BC cells. In order to validate the hypothesis that EVs isolated from 3T3-L1 adipocytes can affect the metabolism of BC cells, the present study investigated possible changes in the OCR and the ECAR by using the Seahorse Extracellular Flux (XF96) Analyzer. OCR is a well-established indicator of mitochondrial respiration rate because oxygen consumption by respiration is a fundamental function of the mitochondria, mainly used in the process of ATP production. Thus, the present study performed Mito Stress Test that measures OCR levels in real time by sequential injections of oligomycin, an inhibitor of ATP synthase (complex V), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), an uncoupling molecule that disrupts the mitochondrial membrane potential and a mixture of rotenone and antimycin A, inhibitors of complex I and III, respectively (Fig. 4A). 3T3-L1A-EVs markedly increased basal (Fig. 4B) and maximal respiration (Fig. 4C) of MCF-7 cells compared with the control group. Meanwhile, by sequentially injecting a saturating concentration of glucose, oligomycin and 2-DG, an analogue of the glucose that inhibits glycolysis, ECAR, the release of protons into the extracellular milieu generated by anaerobic glycolysis, was estimated (Fig. 4D). The data showed that 3T3-L1A-EV treatment did not affect glycolysis (Fig. 4E) or glycolytic reserve (Fig. 4F), but they markedly increased the glycolytic

reserve capacity (Fig. 4G), which is the difference between glycolytic capacity and glycolysis rate. It is noteworthy that the glycolytic reserve is indicative of the capacity of cells to enhance ATP production via glycolysis in response to stress or other physiologically demanding circumstances. It was found that pre-adipocyte-derived EVs (3T3-L1-EVs) had no detectable effects on mitochondrial activity in MCF-7 cells (Fig. S4), further confirming previous data that 3T3-L1-EVs failed to promote malignant features in BC cells (20) and supporting the notion that functional changes in tumor cells are specifically driven by EVs from differentiated adipocytes.

To further support these findings, we also used two additional ER+ BC cell lines as ZR-75-1 (Fig. 5A-C) and BT-474 (Fig. 5D-F) and found that 3T3-L1A-EVs affected mitochondrial respiration of both experimental models. Therefore, EVs isolated from mature adipocytes may promote the mitochondrial metabolism of BC cells, leading to an increase in energy production in different cellular background.

As it is widely known that ATP production through oxidative phosphorylation is markedly greater than that produced by glycolysis alone, the present study also investigated the ATP amounts in BC cells treated with 3T3-L1A-EVs. Notably, 3T3-L1A-EVs enhanced the ATP production in MCF-7 BC cells (Fig. 6A). Furthermore, the present study investigated the mitochondrial phenotype of MCF-7 cells treated with 3T3-L1A-EVs employing FACS analysis by using two different dyes able to discriminate mitochondrial mass (MitoTracker Deep Red) and mitochondrial membrane potential (MitoTracker Orange). There was a slight reduction

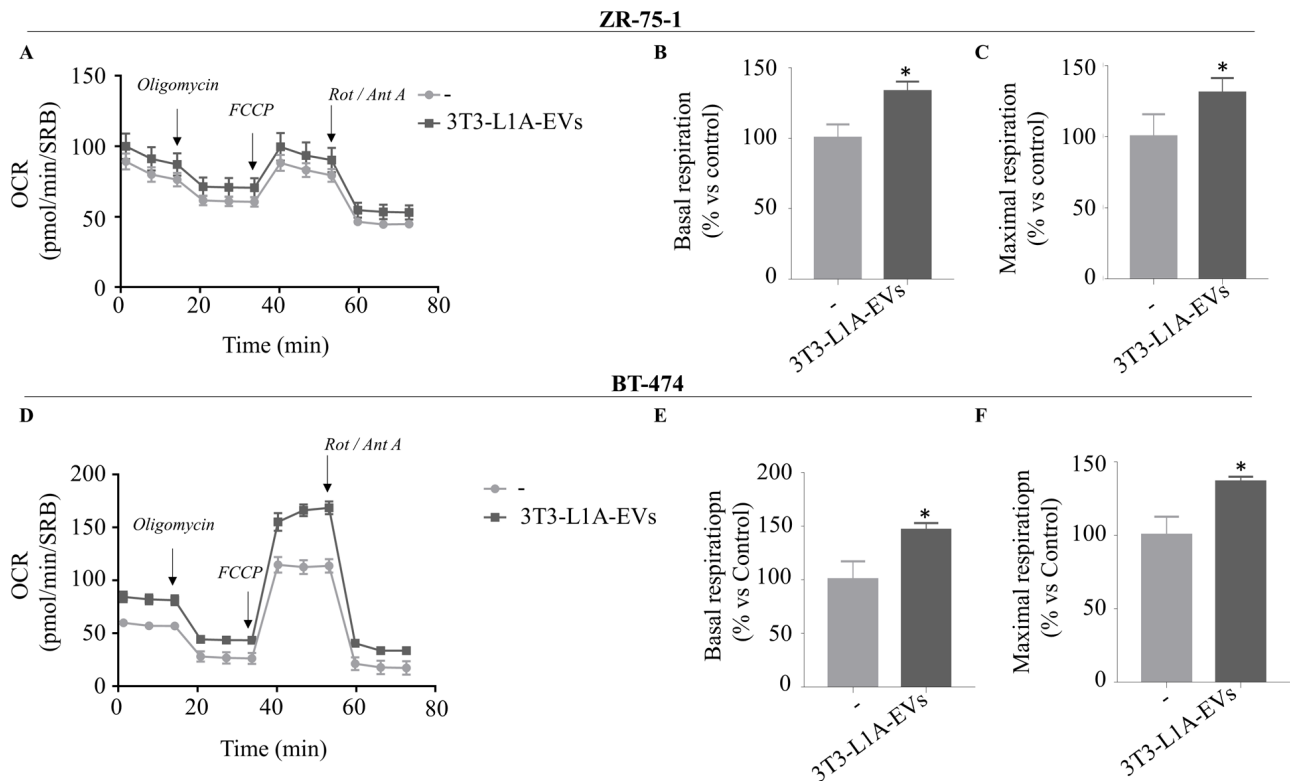


Figure 5. 3T3-L1A-EVs sustain mitochondrial metabolism of ZR-75-1 and BT-474 BC cells. The seahorse XF-e96 analyzer was used to assess the mitochondrial metabolic profile of ZR-75-1 and BT-474 cells untreated (-) or treated with 3T3-L1A-EVs (2 μ g/ml) for 48 h. (A and D) OCR flux, histograms of different mitochondrial respiratory parameters such as (B and E) basal and (C and F) maximal respiration are shown. Data were analyzed using Student's t test and are represented as mean $\pm$ SEM of five biological replicates from two independent experiments and normalized to protein content (SRB assay). * P $\leq$ 0.05. OCR, oxygen consumption rate; SRB, sulforhodamine B; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Rot, rotenone; Ant, Antimycin A.

of mitochondrial mass along with a significant increase in mitochondrial membrane potential in MCF-7 cells treated with 3T3-L1A-EVs compared with the control group (Fig. 6B and C). Consequently, the ratio of mitochondrial membrane potential/mitochondrial mass confirmed elevated mitochondrial activity in MCF-7 EVs-treated cells compared with the control group (Fig. 6D). In addition, an increase in mitochondrial markers from Complexes I to V was observed in EV-treated MCF-7 cells compared to control (Fig. 6E).

The aforementioned events confirmed the role of 3T3-L1A-EVs in regulating cell energy production by sustaining mitochondrial function in BC cells.

HIF-1 α is a potential mediator of the effects of 3T3-L1A-EVs on BC cell metabolism. We have previously demonstrated that HIF-1 α plays a role in mediating the effects of 3T3-L1A-EVs on BC cell biology (20). Indeed, treatment of BC cells with 3T3-L1A EVs resulted in increased mRNA and protein expression of HIF-1 α as well as in an upregulation of well-known HIF-1 α target genes (such as *VEGF*, *Ob*, *MMP-2*, *MMP-9* and *SERPINE1*). Accordingly, inhibition of HIF-1 α activity using KC7F2, able to downregulate HIF-1 α protein synthesis and its transcriptional activity, or using a specific siHIF-1 α , resulted in the reversal of the promoting effects of 3T3-L1A-EVs on BC cell motility, invasion and metastasis (20). In light of these findings, the present study sought to investigate the potential involvement of HIF-1 α in mediating the metabolic effects of 3T3-L1-EVs. As a

first step, the specific HIF-1 α inhibitor KC7F2 was used at 20 μ M, a concentration previously shown to be non-toxic both *in vitro* and *in vivo* (20). It was found that the inhibition of HIF-1 α activity, using KC7F2, reversed the effects of 3T3-L1A-EVs in increasing mitochondrial respiration in MCF-7 BC cells, which exhibited levels comparable to those observed in the control group (Fig. 7A-C). Data were confirmed by knocking down HIF-1 α expression in MCF-7 BC cells (Fig. 8A-D). Specifically, HIF-1 α silencing (Fig. 8A) counteracted 3T3-L1A-EV-mediated effect on basal (Fig. 8C) and maximal (Fig. 8D) respiration. The aforementioned data were further corroborated through an analysis of the ATP levels conducted under the same experimental conditions. Particularly, the specific blockade of HIF-1 α activity (Fig. 7D and E) as well as its reduced expression (Fig. 8E and F) counteracted the ATP production and the mitochondrial activity observed in MCF-7 cells when exposed to 3T3-L1A-EVs, further supporting the potential involvement of HIF-1 α in mediating the effects of 3T3-L1A-EVs on BC cell metabolism.

EVs isolated from serum of obese patients with BC promote mitochondrial metabolism and energy production in MCF-7 cells via HIF-1 α . Finally, in order to reproduce the findings within the context of obesity, the present study used EVs that had been isolated from the serum of NW and OW/Ob patients with BC and characterized by TEM, NTA and immunoblotting as shown in Fig. S2D-F. It was found that EVs derived

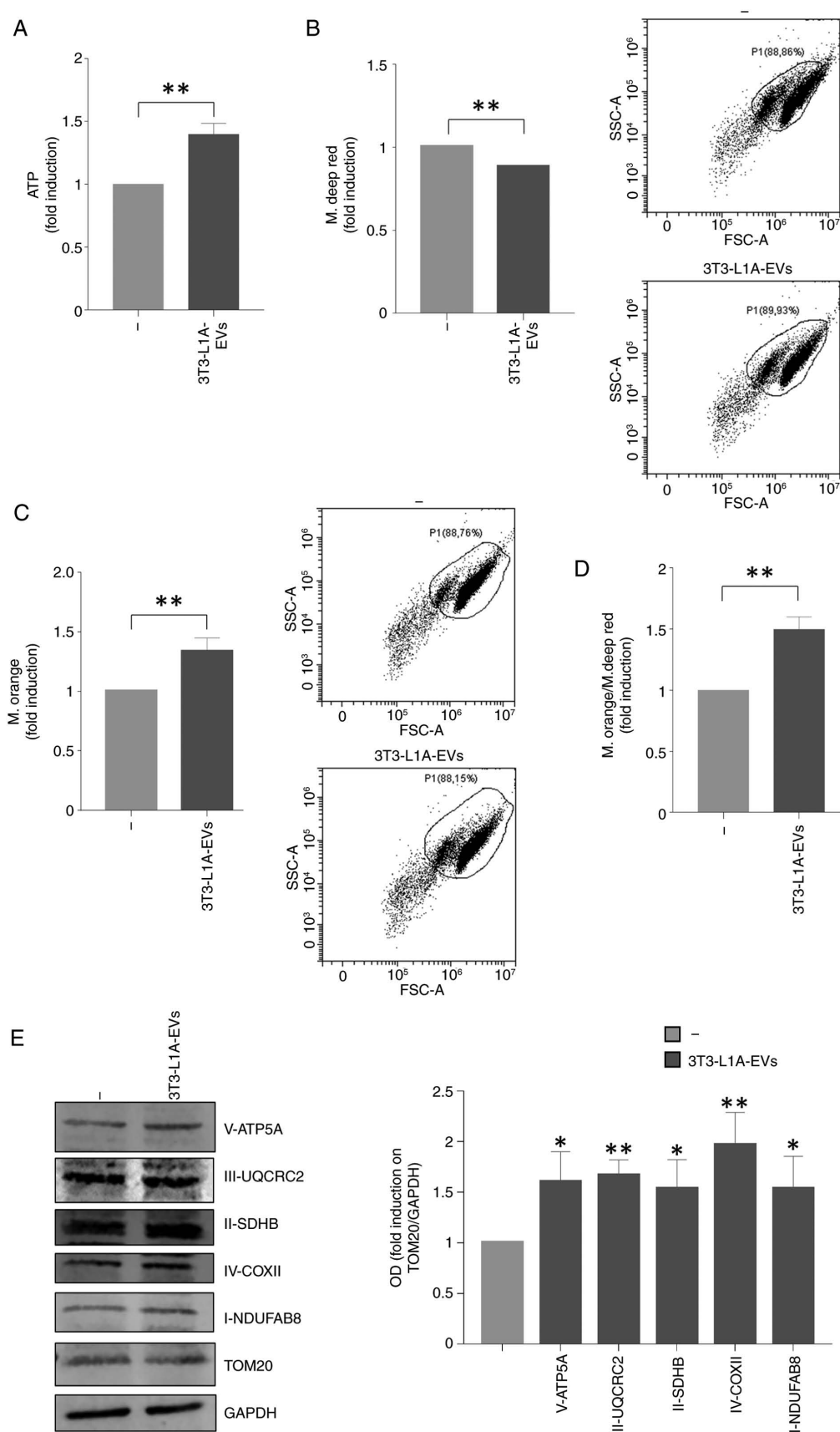


Figure 6. 3T3L-1A-EVs affect mitochondrial function and ATP production in MCF-7 cells. (A) ATP production in cells treated as indicated. Mitochondrial mass (B) and mitochondrial membrane potential (C) along with relative dot plots of gating strategy from FACS analysis. (D) Ratio between mitochondrial membrane potential and mitochondrial mass. (E) Mitochondrial marker expression (V-ATP5A, III-UQCRC2, II-SDHB, IV-COXII and I-NDUFAB8) in MCF-7 BC cells untreated (-) or treated with 3T3-L1A-EVs (2 μ g/ml) for 48 h. The outer mitochondrial membrane marker TOM20 and GAPDH were used as controls for equal loading and transfer. The histograms represent the mean $\pm$ SD of three separate experiments in which band intensities were evaluated in terms of OD and expressed as fold induction over untreated cells. Data were represented as mean $\pm$ SD of three biological replicates from three independent experiments. *P $\leq$ 0.05; **P $\leq$ 0.01. 3T3-L1A, murine 3T3-L1 adipocytes; EVs, extracellular vesicles; OD, optical density arbitrary units; M, mitotracker.

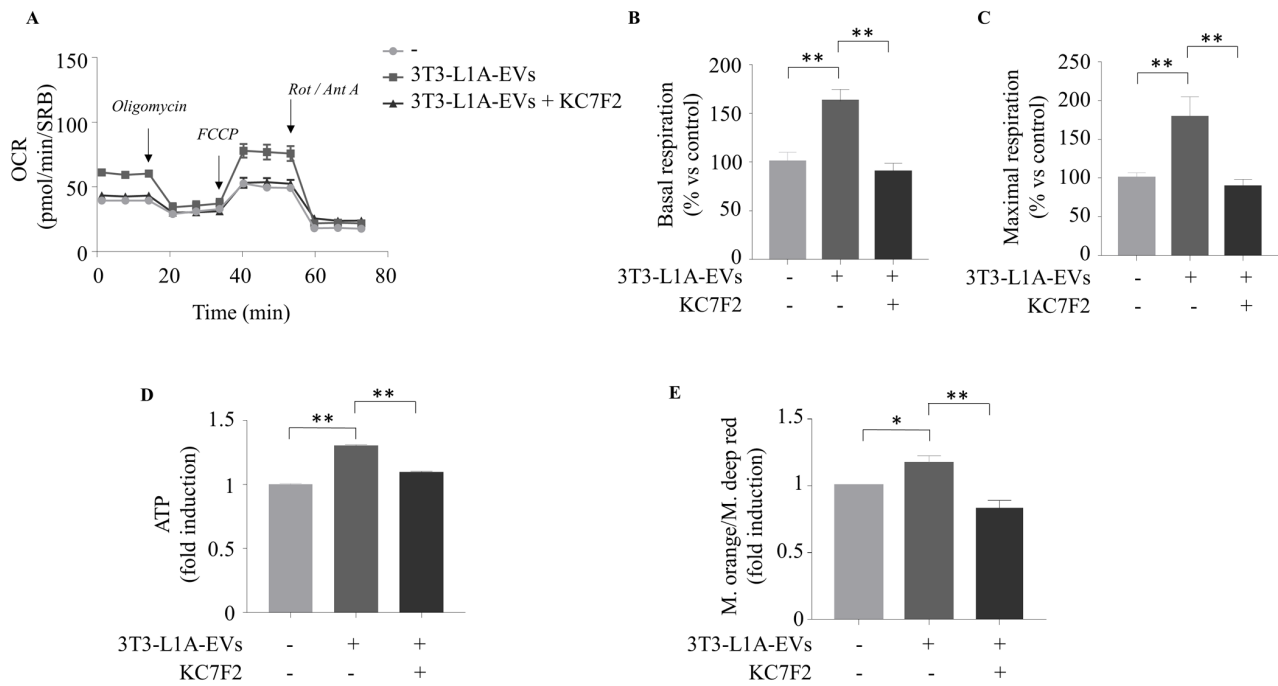


Figure 7. HIF-1 α specific blockade inhibits 3T3L-1A-EV effects on cell metabolism and energy production. The seahorse XF-e96 analyzer was used to assess the mitochondrial metabolic profile of MCF-7 cells untreated (-) or treated with 3T3-L1A-EVs (2 μ g/ml) for 48 h, in the presence or not of HIF-1 α specific inhibitor (KC7F2, 20 μ M). (A) OCR flux and histograms of different mitochondrial respiratory parameters (B) basal and (C) maximal respiration. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons post hoc test and represented as $\pm$ SEM of five biological replicates from three independent experiments and normalized to protein content (SRB assay). (D) ATP production; (E) ratio between mitochondrial membrane potential and mitochondrial mass of MCF-7 cells untreated (-) or treated with 3T3-L1A-EVs (2 μ g/ml) for 48 h, in the presence or not of HIF-1 α specific inhibitor (KC7F2 20 μ M). Data were analyzed using one-way ANOVA with Tukey's multiple comparisons post hoc test and represented as $\pm$ SD of three biological replicates from three independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$. 3T3-L1A, murine 3T3-L1 adipocytes; EVs, extracellular vesicles; OCR, oxygen consumption rates; SRB, sulforhodamine B; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Rot, rotenone; Ant, Antimycin A.

from OW/Ob patients increased the basal and maximal respiration of MCF-7 cells compared with the BC cells treated with NW-EVs (Fig. 9A-C). Accordingly, the OW/Ob-EVs enhanced the ATP production in BC cells (Fig. 9D). Of note, the HIF-1 α inhibitor KC7F2 abrogated the up-regulatory effects mediated by OW/Ob-EVs on mitochondrial respiration and energy production (Fig. 9A-D). Furthermore, the enhanced effects of OW/Ob-EVs on ATP production in MCF-7 cells were blocked by a specific siHIF-1 α (Fig. 9E).

All these data supported the role of HIF-1 α as a potential mediator of the stimulatory effects of EVs on BC cell metabolism.

Discussion

The complex and multifaceted interaction between adipocytes and BC cells within the tumor microenvironment (TME) exerts a profound influence on the development and progression of BC. However, this dialogue has been largely confined to the various factors secreted by adipose tissue, as well as adipokines, cytokines and growth factors (4-13,31-33). Recently, EVs have been identified as a component of the secretome of adipocytes and EV-mediated effects represent a pivotal aspect of the dynamic TME. Although, the majority of current studies focus on elucidating the cargo of EVs or identifying genomic alterations induced by EVs in cancer cells, there is still a gap in our knowledge regarding the potential effects of EVs in modulating the metabolic phenotype of BC cells.

The present study described for the first time how EVs derived from adipocytes may modify the proteomic profile of BC cells. The findings demonstrated that these vesicles are able to: i) Deregulate proteins associated with metabolic processes; ii) switch the metabolic profile towards mitochondrial respiration; and iii) enhance ATP production in BC cells. This occurs through a mechanism involving HIF-1 α .

It is now recognized that cells following the malignant transformation into neoplastic cells rewire cellular metabolism, essential to satisfy the demand of growth, proliferation and progression (34-36). The defined metabolic hallmark of tumor metabolism is aerobic glycolysis, accompanied by an increased rate of lactate production, phenomenon known as the 'Warburg effect' (37). Nevertheless, cancer cells are capable of autonomously reprogramming their metabolic pathways, enabling rapid adaptation to microenvironmental changes and the fulfilment of increased bioenergetic demand (38,39). Tumor-surrounding adipocytes facilitate metabolic reprogramming in cancer cells by increasing the availability of fatty acid and supporting beta-oxidation in cancer cells. This provides an excess of substrate for the production of ATP and the generation of lipid membranes (40-42). Studies have indicated that adipose tissue can influence the mitochondrial metabolism of cancer cells not only in close proximity through the secretion of substrates but also via EVs (43-46). Particularly, it has been demonstrated that the cargo of adipocyte-derived EVs may also contain metabolites that can markedly impact the metabolism of recipient cells, potentially contributing to

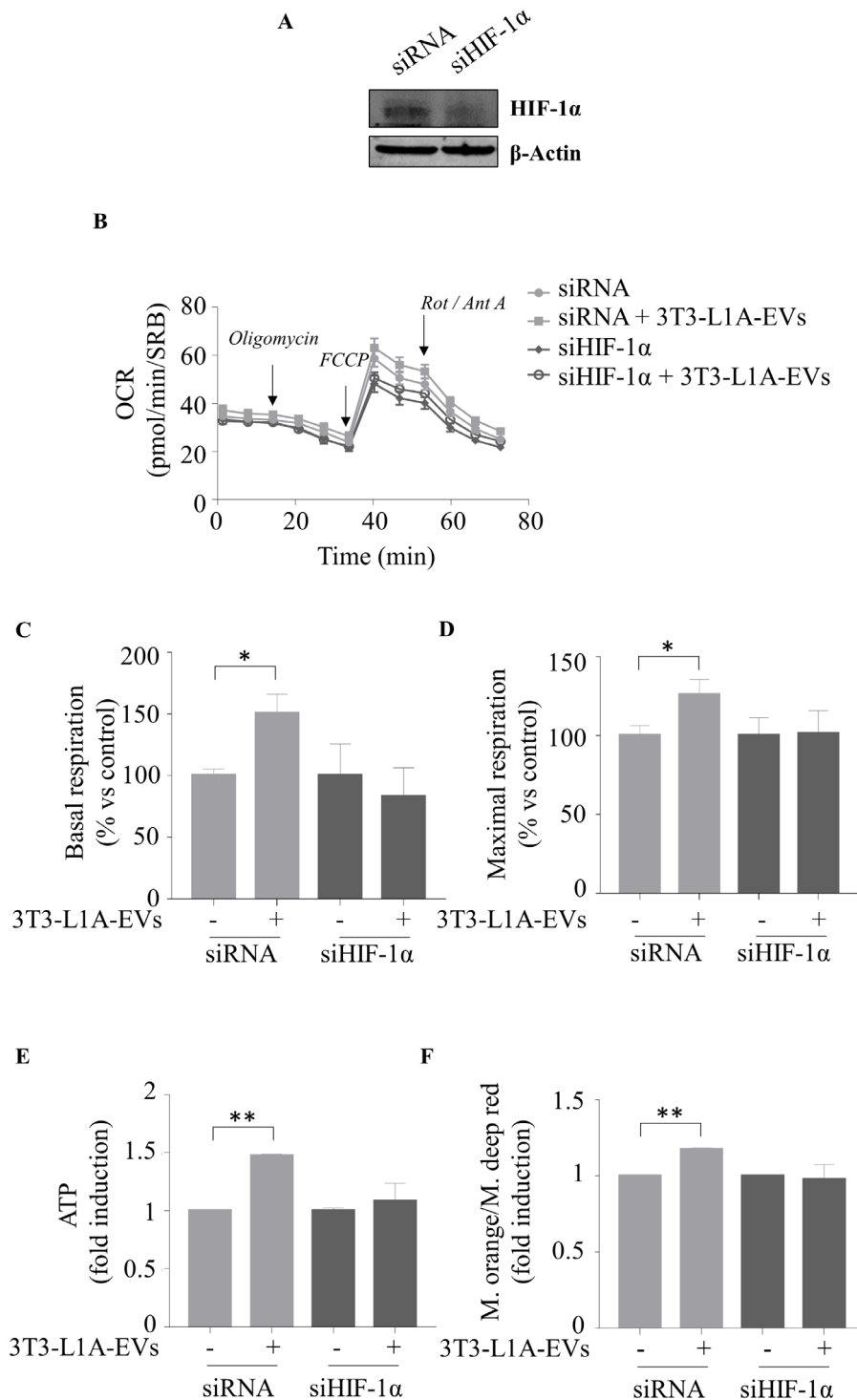


Figure 8. Silencing HIF-1 α expression inhibits 3T3L-1A-EV effects on cell metabolism and energy production. (A) Immunoblot analysis of HIF-1 α in whole cell lysate of MCF-7 cells transfected with negative control siRNA or siHIF-1 α for 48 h. β -Actin was used as a control of equal loading and transfer. The seahorse XF-e96 analyzer was used to assess the mitochondrial metabolic profile of MCF-7 cells untreated (-) or treated with 3T3-L1A-EVs (2 μ g/ml) for 48 h, previously transfected for 48 h with either siRNA or with siHIF-1 α . (B) OCR flux and histograms of different mitochondrial respiratory parameters such as (C) basal and (D) maximal respiration. Data were analyzed using Student's t test and represented as $\pm$ SEM of five biological replicates from two independent experiments and normalized to protein content (SRB assay). (E) ATP production; (F) ratio between mitochondrial membrane potential and mitochondrial mass of MCF-7 cells transfected for 48 h with either siRNA or with siHIF-1 α and then untreated (-) or treated with 3T3-L1A-EVs (2 μ g/ml) for 48 h. Data were analyzed using Student's t test and represented as $\pm$ SD of three biological replicates from two independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$. HIF-1 α , hypoxia inducible factor-1 α ; 3T3-L1A, murine 3T3-L1 adipocytes; EVs, extracellular vesicles; si, small interfering; OCR, oxygen consumption rates; SRB, sulforhodamine B; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Rot, rotenone; Ant, Antimycin A.

cancer progression (43-46). The present study demonstrated that adipocyte-derived EVs altered the proteome of BC cells. Notably, Proteomaps, STRING, REACTOME pathway,

MsigDB and FunRich analyses revealed that de-regulated proteins identified in the EV-treated cells were highly enriched in GOs mainly related to mitochondrion, protein metabolism,

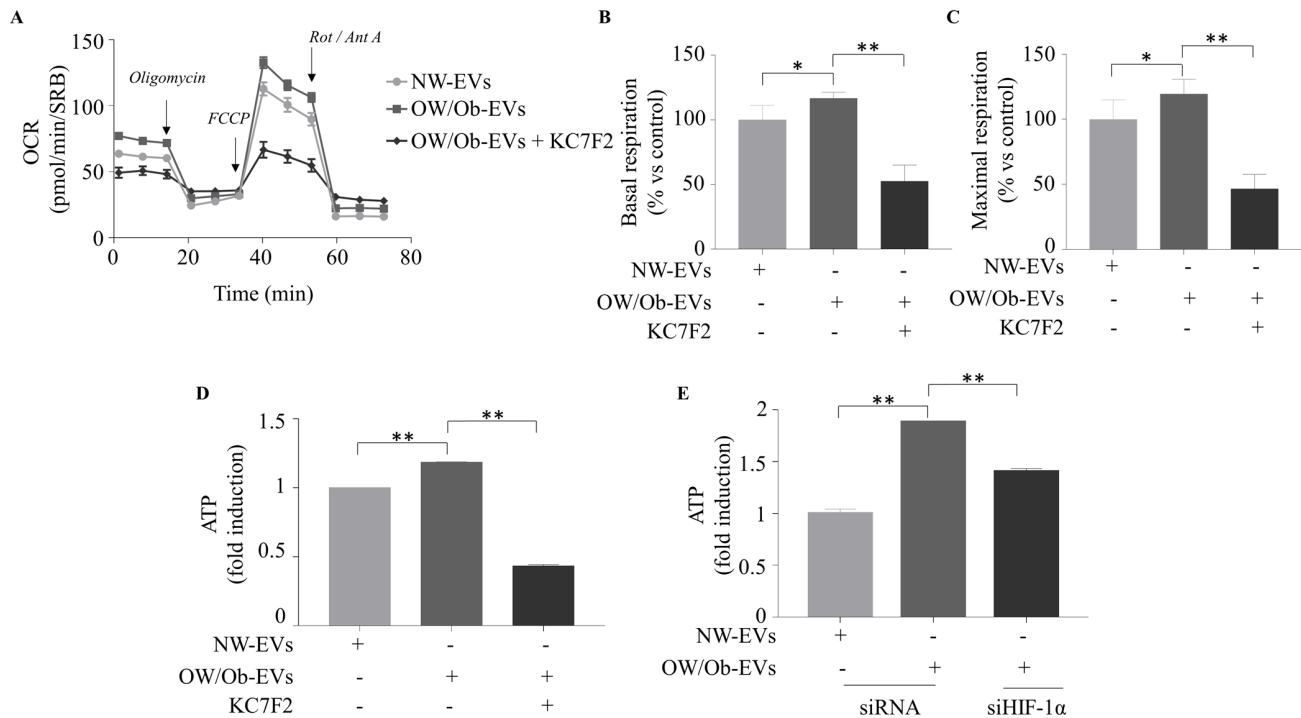


Figure 9. EVs derived from overweight/obese patients with BC enhance mitochondrial phosphorylation and ATP production in BC cells through HIF-1 α . The Seahorse XF-e96 analyzer was used to assess the mitochondrial metabolic profile of MCF-7 cells treated with EVs (2 μ g/ml) isolated from the serum of NW and OW/Ob patients with BC for 48 h, in the presence or not of HIF-1 α specific inhibitor (KC7F2; 20 μ M). (A) OCR flux and histograms of different mitochondrial respiratory parameters such as (B) basal (C) and maximal respiration are shown. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons post hoc test and represented as $\pm$ SEM of five biological replicates from two independent experiments and normalized to protein content (SRB assay). ATP production in MCF-7 cells treated as indicated (D) or transfected with siHIF-1 α (E). Data were analyzed using one-way ANOVA with Tukey's multiple comparisons post hoc test and represented as $\pm$ SD of three biological replicates from two independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$. EVs, extracellular vesicles; HIF-1 α , hypoxia inducible factor-1 α ; NW, normal weight; OW/Ob, overweight/obese; OCR, oxygen consumption rates; SRB, sulforhodamine B; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Rot, rotenone; Ant, Antimycin A.

energy pathways and oxidoreductase activity. Consequently, functional studies confirmed that adipocyte-derived EVs enhanced mitochondrial respiration, a trait now regarded as a hallmark of more aggressive cancers (47-49), in several ER-positive BC cells, including MCF-7, ZR-75 and BT-474 cells. By contrast, no changes in glycolysis were detected in the experimental conditions of the present study. Furthermore, in agreement with these previously reported data, the present study found that adipocyte-derived EVs enhanced ATP production as a consequence of increased mitochondrial activity, as evidenced by mitotracker analysis and the expression of several mitochondrial markers.

Regarding obesity, a well-established risk factor for BC, it has been extensively documented that the typical enlarged adipocytes secrete a greater quantity of EVs that differ in nature and size when compared with their NW counterparts. These EVs have profound implications in the pathogenesis of several malignancies, including breast, endometrial, prostate, colorectal and melanoma (44-46,50-54). In this frame, our research group identified a potential mechanism through which the obesity-associated hormone leptin sustains exosome generation and modifies the EV cargo in BC cells, thus supporting mitochondrial metabolism in recipient epithelial tumor cells and macrophages (52,55). Moreover, analysis of circulating EV-derived miRNAs in patients with BC revealed a differential miRNA profile within EVs in relation to a patient's body mass index and allow the identification of let-7

as a potential obesity-related miRNA whose downregulation may be associated with tumor progression (22). It has been reported that EVs derived from obese adipocytes influence the miRNome of BC cells by altering their metabolic processes (45). Clement *et al* (44) conducted a comparative proteomic analysis of EVs derived from adipocytes of lean and obese mice, identifying the presence of proteins involved in fatty acid oxidation (FAO). The horizontally transferred proteins, associated with FAO, mitochondrial respiration and ATP production, remodel melanoma metabolism and support aggressiveness. Furthermore, the modulation of lipid metabolism induced by vesicles derived from obese patients was also identified as a critical factor in the development of ferroptosis and chemoresistance in colorectal cancer cells (46). Consistent with this evidence, the results of the present study revealed that EVs isolated from the serum of OW/Ob patients with BC increased the oxidative phosphorylation and the ATP production in BC cells compared with those treated with EVs isolated from NW patients.

It has been widely reported that HIF-1 α regulates the expression of numerous genes involved in physiological and pathological processes, including cell metabolism. Indeed, HIF-1 α is a transcription factor that plays a pivotal role in modulating tumor metabolism in response to a low-oxygen environment. Specifically, under hypoxic conditions, HIF-1 α is responsible for regulating tumor cell metabolism towards glycolysis. This involves the overexpression of glucose

transporters (such as GLUT1 and GLUT 3) and glycolytic enzymes (such as hexokinase, phosphofructokinase, aldolase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, pyruvate kinase, lactate dehydrogenase and pyruvate dehydrogenase kinase), which ultimately results in an increased glucose uptake (56–60). In particular, HIF-1 α is also involved in the interactions between adipocytes and BC cells, primarily by promoting lipolysis and free-fatty acid release in adipocytes (61–63). This allows free fatty acid transfer from the adipocyte to the BC cells, which then initiate fatty acid metabolism in order to maintain high levels of ATP, thereby supporting the growth and progression of the tumor (41). Notably, since obesity is characterized by adipose tissue hypoxia, HIF-1 α expression and activity resulted increased in obese conditions, with a consequent release of pro-inflammatory signals (64–66). The present study demonstrated the direct involvement of HIF-1 α in the EV-enhanced effects on BC cell metabolism. In particular, the pharmacological inhibition of HIF-1 α by using the cystamine compound KC7F2 or genetic inhibition by employing a siHIF-1 α counteracts the stimulatory effects of adipocyte-derived EVs on the mitochondrial respiration and activity as well as in the ATP energy production in BC cells. HIF-1 α inhibition was also able to reverse the effects of OW/Ob-EVs on BC cell metabolic activity, further highlighting HIF-1 α as a potential target for therapeutic strategy in BC, especially in the context of obesity.

In conclusion, the present study represented a novel contribution to the growing body of evidence supporting the involvement of EVs in the obesity-BC link. Investigation into the function of adipocyte-derived EVs in educating BC cells towards a more aggressive phenotype may facilitate the discovery of novel biomarkers or innovative targets (for example, HIF-1 α) that might be involved in metabolic cancer vulnerability. Such discoveries could then be employed for the purposes of a personalized therapeutic approach in obese patients with BC.

Acknowledgements

Not applicable.

Funding

The present study was funded by BANDO PRIN 2017 grant no. 2017WNKSLR, PRIN 2022-European NextGenerationEU initiative under the Italian Ministry of University and Research-M4C2-I1.1 (grant no. 202239N8PR, CUP H53D23006360006) and PRIN PNRR 2022-European NextGenerationEU initiative under the Italian Ministry of University and Research as part of the PNRR-M4C2-I1.1 (grant no. P2022YAKJY, CUP H53D23010120001) to I. Barone; PRIN 2022, European NextGenerationEU initiative under the Italian Ministry of University and Research M4C2-I1.1 (grant no. 2022AA4FTJ, CUP H53D23006420006) to Giordano C; PRIN PNRR 2022-European NextGenerationEU initiative under the Italian Ministry of University and Research as part of the PNRR-M4C2-I1.1 (grant no. P2022FK2J8, CUP H53D23010420001) to L. Gelsomino; AIRC Investigator Grant no. 30782 to S. Catalano and grant no. 26246 to S. Andò. The present study was also funded by the National Plan for

PNRR Complementary Investments grant no. PNC0000003, AdvaNced Technologies for Human-centrEd Medicine (ANTHEM); POSRADIOAMICA project funded by the Italian Minister of Health (CUP: grant no. H53C22000650006); and POS CAL.HUB. RIA project funded by the Italian Minister of Health (grant no. CUP H53C22000800006).

Availability of data materials

The data generated in the present study may be requested from the corresponding author. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD059789.

Authors' contributions

LG, IB, CG and SC made substantial contributions to the conception or design of the work, and drafted the article; LG, IB and PDC contributed to the acquisition, analysis, or interpretation of data for the work; MF and PDC contributed to the acquisition, analysis, or interpretation of metabolism data; MG and MSM performed, analyzed and interpreted the proteomic data; FG, GDN and SP performed, analyzed and interpreted the flow-cytometry data; DB, SA, MG and GA participated to the acquisition, analysis, or interpretation of data from patients. All authors contributed to revising the work critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethical committee of University of Naples 'Federico II' (approval no. 107/05).

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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