

## Article

# Trastuzumab modulates the protein cargo of extracellular vesicles released by ERBB2<sup>+</sup> breast cancer cells

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**Abstract:** Cancers overexpressing the ERBB2 oncogene are aggressive and associated with a poor prognosis. Trastuzumab is a ERBB2 specific recombinant antibody employed for the treatment of these diseases since it blocks ERBB2 signaling causing growth arrest and survival inhibition. While the effects of Trastuzumab on ERBB2 cancer cells are well known, those on the extracellular vesicles released from these cells are scarce. This study focused on ERBB2<sup>+</sup> breast cancer cells and aimed to establish what type of EVs they release and whether Trastuzumab affects their morphology and molecular composition. To these aims, we performed immunoelectron microscopy, immunoblot, and high-resolution mass spectrometry analyses on EVs purified by differential centrifugation of culture supernatant. Here we show that EVs released from ERBB2<sup>+</sup> breast cancer cells are polymorphic in size and appearance, and that ERBB2 is preferentially associated with large (120 nm) EVs. Moreover, we report that Tz induces the expression of a specific glycosylated 50 kDa isoform of the CD63 tetraspanin and modulates the expression of 51 EVs proteins, including TOP1. As these proteins are functionally associated with organelle organization, cytokinesis, and response to lipids, we suggest that Tz may influence these cellular processes in target cells at distant sites via modified EVs.

**Keywords:** trastuzumab, HER2, extracellular vesicles, breast cancer, proteomic analysis, immunoelectron microscopy, TOP1, CD63, mitochondria.

## 1. Introduction

ERBB2 is a transmembrane protein that belongs to the ERBB family of growth factor receptors. At variance from other members of the family, ERBB2 has not a known ligand and therefore is classified as an orphan receptor. The ERBB2 structural conformation allows ligand-independent dimerization and signaling activity. ERBB2 overexpression, caused most often by gene amplification, may lead to oncogenic transformation in several human cell types, which in turn generate breast, ovarian, bladder, gastric and several other tumors. In fact, ERBB2 signaling promotes key cellular processes such as proliferation, survival, migration, invasion and angiogenesis, and therefore its overexpression in tumors is also associated with a poor prognosis [1,2]. For all these reasons,

targeting ERBB2 and blocking its signaling activity with a specific humanized antibody named trastuzumab (Tz), is still a milestone in the therapy of ERBB2 overexpressing (ERBB2<sup>+</sup>) tumors [3].

EVs can be classified by their size, biogenesis and cargoes, e.g. small EVs, including exosomes, and larger EVs such as shed microvesicles (MVs) and large oncosomes. Exosomes are a type of small EV with diameter of 50–150 nm and have been the most studied with respect to their involvement in EV function. Exosomes are formed as intraluminal vesicles (ILVs) in late endosomal organelles called multivesicular bodies (MVBs) and secreted after fusion of MVBs with the plasma membrane [4]. Tumor cells both *in vitro* and *in vivo* release in the environment EVs that have been implicated in intercellular communication with target cells at distant sites mediating a number of processes that promote tumor metastasis and progression, including microenvironment modulation, migration, invasion, angiogenesis, epigenetic modulation, immune evasion and drug resistance [5–7]. ERBB2<sup>+</sup> cancer cells release EVs displaying this orphan receptor on the membrane. This may contribute to resistance of ERBB2 tumors to Tz. In fact, it has been shown that EV carrying ERBB2 binds Tz and inhibits its antiproliferative activity on ERBB2 breast cancer cells, likely by a sequestering mechanism [8]. A similar mechanism of resistance to the therapeutic monoclonal Rituximab has been reported for EVs released from malignant lymphoma displaying the CD20 protein [9]. While there is a wealth of knowledge concerning the effects of Tz on ERBB2<sup>+</sup> cancer cells and on EVs biogenesis, structure and functions, less is known about possible modifications induced by Tz on EVs released by ERBB2<sup>+</sup> cells. To fill this gap of knowledge, we focused on cell line models of ERBB2<sup>+</sup> breast cancer (BCa) and used a multidisciplinary approach including transmission electron microscopy (TEM), biochemistry, and high-resolution mass spectrometry. Our results show that EVs released by ERBB2<sup>+</sup> cell lines fall in three morphological classes, ERBB2 is preferentially present in larger EVs, and that Tz modifies proteins carried by EVs with a possible impact on processes such as organelle organization, cytokinesis, and response to lipids in target cells.

## 2. Materials and Methods

### Cell lines and cell culture reagents.

The BC cell lines BT474, SKBR-3 and MDA-MD-361 were obtained from Banca Biologica and Cell Factory in IRCCS Ospedale Policlinico San Martino affiliated to the European Culture Collection's Organization. Culture media for routine cell expansion was DMEM high glucose supplemented with 1% glutamine, penicillin and streptomycin, and 10% heat-inactivated fetal bovine serum for BT474 and SKBR-3 or 20% for MDA-MD-361 (Euroclone S.p.A, Italy). EVs production medium was prepared as reported by They and collaborators [10]. Cultures were performed at 37°C in humidified 5% CO<sub>2</sub> atmosphere. Normal human IgGs (CLS Behring) and Tz (Genentech-Roche, South San Francisco, CA, USA) was dissolved with saline solution with 0.9% NaCl in a stock concentration of 21 mg/ml, donated by the Unità Farmaci Antitumorali of the IRCCS Ospedale Policlinico San Martino. Tz was used at a concentration of 10 µg/ml. Control cells were cultured with human IgGs at the same concentrations used for Tz.

### EVs purification.

After 72 hours of treatment, EVs purification from cell conditioned medium was performed as described previously [8]. Briefly, the day before treatment cells were detached with trypsin in phosphate buffered saline (PBS), counted and seeded at  $5.3 \times 10^4$  cells/cm<sup>2</sup>. At time 0 cells were washed 3 times with PBS before addition of EVs production medium containing human IgGs (control condition) or Tz, both at 10 µg/ml. After 72 hours the conditioned medium was subjected to two sequential centrifugations at 300g for 10 min

and at 2000g for 30 min followed by filtration through 0.22  $\mu\text{m}$  filters to eliminate cell debris. Filtered supernatants were centrifuged in polyallomer tubes at 100000g for 2 hours at 4°C using a SW28 or SW41 rotor and the Optima XPN-100 Ultracentrifuge (Beckman Coulter Inc., Fullerton, CA). The EVs-containing pellets were dissolved in PBS and concentrated at 100,000g for 60 min using a TLA 100.3 rotor and the TL100 Ultracentrifuge (Beckman Coulter Inc.). Pelleted EVs were dissolved in PBS.

### **Immunoblot and biochemical assays.**

Protein concentration in cell lysates and EVs preparations was established by using a colorimetric detection and quantitation assay based on bicinchoninic acid (BCA) (ThermoFisher Scientific, Waltham, MA, USA). When indicated cell lysates were digested with N-Glycosidase F (Roche Diagnostics, Monza, Italy) according to manufacturer's instruction. Proteins were subjected to electrophoresis on Bolt 4-12% Bis Tris Plus gels (ThermoFisher Scientific) and blotted on a PVDF membrane. Primary antibodies used in this study are listed in Table S1. Detection was performed using horseradish peroxidase-conjugated anti mouse or anti rabbit antibodies (ThermoFisher Scientific) and with ECL Detection Reagent from BioRad, according to manufacturer's protocol. ECL signals were imaged by the Nine Alliance, Uvitec Cambridge, gel documentation apparatus.

### **Mitochondrial morphological analysis by MitoTracker Red labeling.**

Cells were cultured in control conditions or in presence of Tz for 72 h and labeled with MitoTracker Red (ThermoFisher Scientific) according to the manufacturer' instructions. Cells were fixed and fluorescent images were acquired using Axio Imager A2M microscope (Carl Zeiss, Jena, Germany). Mitochondrial size and area were measured by using the object analyzer advanced tool of Huygens Professional version X11 (<http://svi.nl>) (Scientific Volume Imaging, The Netherlands).

### **TEM imaging of cells and EVs immunolabeling**

Cells were incubated with 5 O.D. of 5 nm gold-conjugated bovine serum albumin (BSA) (Utrecht, The Netherlands) for 2 hours at 37°C then washed twice in 0.1 M cacodylate buffer and fixed in 0.1 M cacodylate buffer, 2.5% glutaraldehyde (Electron Microscopy Science, Hatfield, PA, USA), for 1 hour at room temperature. Cells were postfixed in 1% osmium tetroxide for 2 hours and 1% aqueous uranyl acetate for 1 hour. Samples were then dehydrated through a graded ethanol series and flat embedded in resin (Poly-Bed; Polysciences, Inc., Warrington, PA) for 24 h at 60 °C. Ultrathin sections (50 nm) were cut parallel to the substrate and counterstained with 5% uranyl acetate in 50% ethanol.

EVs preparations resuspended in PBS were fixed with an equal volume of 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and then adsorbed for 10 min to formvar-carbon coated copper grids by floating the grids on 5  $\mu\text{l}$  drops on parafilm. Grids with bound vesicles were then rinsed in PBS and negatively stained with 2% uranyl acetate for 5 min at room temperature. Grids were embedded in 2.5% methylcellulose to improve preservation and air dried before imaging. For Immunolabeling of EVs, 5  $\mu\text{l}$  of concentrated solution of EVs were dropped on Formvar-coated copper grids and incubated for 20 min at room temperature. After washing in PBS for 5 min, the EVs were fixed in 1% Glutaraldehyde for 5 min. A second wash in PBS and two passages in 0.2% Glycine in PBS for 5 min each were performed to saturate non-specific reactive sites. After a passage in 1% BSA in PBS for 5 min the grids were incubated with a primary antibody in 1% BSA in PBS for 30 min at room temperature. Unbound antibody was removed by 4x washes in 1% BSA of 2 min each. Primary antibody was revealed by incubation with 10nm Protein A gold (PAG) for 20 min. Excess PAG was eliminated by four washes in 1% BSA of 2 min each which were followed by a further passage in 1% Glutaraldehyde for 5 min, a wash

in PBS for 5 min, and two washes in distilled water. Samples were counterstained with 2% Uranyl Acetate in 0.15 M oxalic acid for 5 min at room temperature. A final incubation in Methylcellulose 1.8% in Uranyl Acetate 4% for 5 min at room temperature was performed to enhance contrast and allow the formation of a protective layer on the samples. Electron micrographs were obtained with a Hitachi 7800 120Kv electron microscope (Hitachi, Tokyo, Japan) using a Megaview 3 digital camera and Radius software (EMSYS, Germany). Morphometry analysis of the size of EVs was measured on 10 randomly taken micrographs at 40.000x magnification for each condition and was calculated using the arbitrary line function embedded the measurement dialog box of Radius software (EMSYS, Germany). To visualize EVs size distribution, the results were plotted as box plot.

### Sample preparation and Mass Spectrometer Setup.

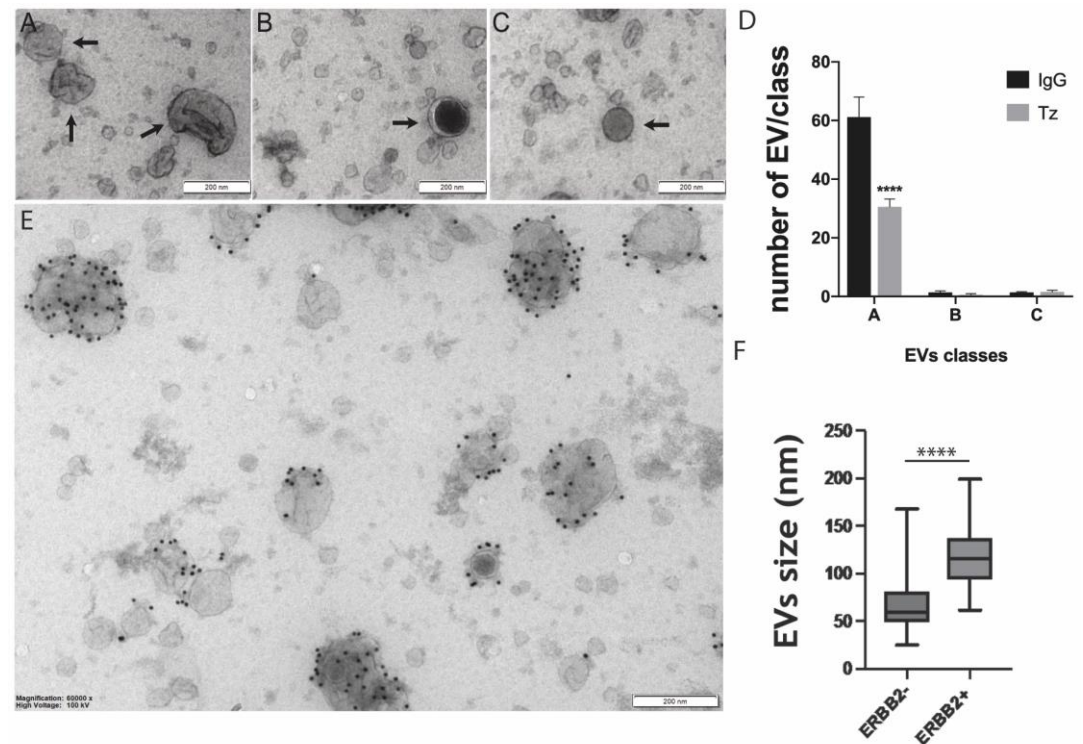
Samples were lysed, reduced and alkylated in 120  $\mu$ l 6M Guanidine, 10 mM TCEP, 4 mM CAA, 100 mM Tris pH 8. Then proteins were extracted with PAC method [11]. Briefly, 4  $\mu$ l of carboxyl coated magnetic beads were added to each sample and proteins were induced to aggregate on the beads with the addition of 70% ACN. The beads washed with 1 ml ACN and 1 ml 70% Ethanol were digested O.N. at 37°C with 0.7  $\mu$ g trypsin and 0.3  $\mu$ g LysC. Digested samples were loaded onto StageTips [12]. Resulting peptides were analyzed by a nano-UHPLC-MS/MS system using an Ultimate 3000 RSLC coupled to an Orbitrap Velos Pro mass spectrometer (Thermo Scientific Instrument) in positive ionization mode. Elution was performed with an EASY spray column (75  $\mu$ m x 50 cm, 2  $\mu$ m particle size, Thermo Scientific) at a flow rate of 250 nl/min with a 200 min non-linear gradient of 5-45% solution B (80% ACN, 20% H<sub>2</sub>O, 5% DMSO and 0.1% FA). MS scans were performed in the Orbitrap at a resolution of 60000 between 375 and 1,500 m/z using a maximal ion injection time of 50 ms. The automatic gain control was set to 1,000,000 ions. The analysis was done in data-dependent acquisition mode with alternating MS and MS/MS experiments. A maximum of 10 MS/MS experiments were triggered per MS scan. MS/MS spectra were acquired in the linear ion trap (rapid scan mode) after collision induced dissociation (CID) fragmentation at a collision energy of 35 % and an AGC target of 10,000. Dynamic Exclusion was set at 30 sec. Data processing was performed by MaxQuant [13] software version 1.6.10.43. A false discovery rate was set at 0.01 for the identification of proteins and a minimum of 6 amino acids was required for peptide identification. The Andromeda engine was used to search MS/MS spectra against the Uniprot human database (release UP000005640\_9606 April 2019). Algorithm MaxLFQ was chosen for the protein quantification with the activated option 'match between runs' to reduce the number of the missing proteins. The intensity values were extracted and statistically evaluated using the ProteinGroup Table and Perseus software version 1.6.8.0 [14]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [15] partner repository with the dataset identifier PXD024276. Reviewer account details, Username: reviewer\_pxd024276@ebi.ac.uk, Password: KGUuh69H

## 3. Results

### 3.1. SKBR-3 cells release three morphological classes of EVs.

To characterize EVs released in the culture media by the ERBB2<sup>+</sup> BCa cell line SKBR-3, we performed an ultrastructural analysis by TEM. The first and most abundant class (A) included cup-shaped EVs with size ranging 30-300 nm. The second one (B) with a 30-200 nm in diameter, showed a round shape with a dark electron dense core surrounded by a clear peripheral region. Lastly, the third class (C) included a more homogeneously lighter electron dense and round-shaped subpopulation of EVs characterized by a

diameter of 30-80 nm (Fig. 1A-D). We then performed a morphometry analysis to assess whether Tz modulates the release of the overall population and/or a specific subset of EVs. Results showed that Tz significantly decreased the number of class (A) cup-shaped subset of EVs (Fig.1D). Next, we assessed the association of ERBB2 with a particular subset of these EVs. We performed an immuno-electron microscopy analysis using an antibody that recognizes the N-terminal domain of this receptor (9G6). Results showed that ERBB2 is preferentially expressed by larger class (A) EVs with a median diameter of about 120 nm ( $P < 0.0001$ ) (Fig. 1E, F). The association of ERBB2 to large EVs is maintained after Tz treatment (not shown).

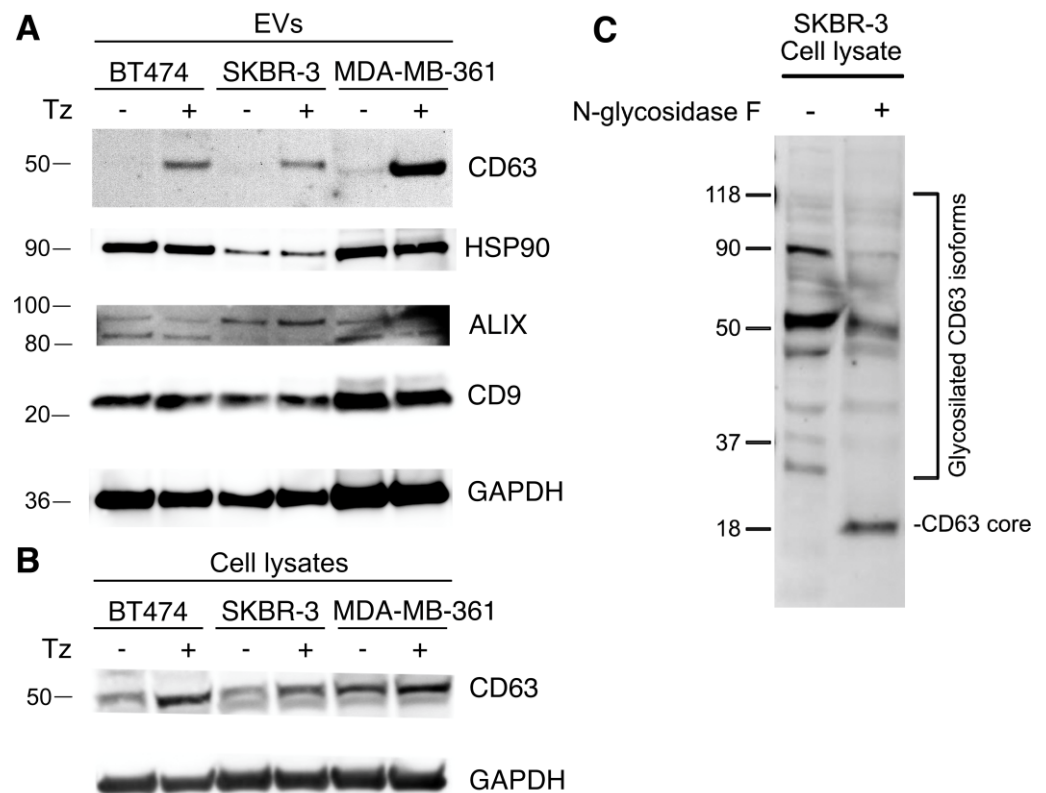


**Figure 1. Representative TEM images of EVs purified from conditioned media of SKBR-3 cells.** Three classes of EVs, which differ by size and ultrastructure can be identified: (A) included 30-300 nm cup-shaped EVs indicated by arrows; (B) 30-200 nm round EVs with a dark electron dense core and a clear peripheral region indicated by an arrow; (C) 30-80 nm round and homogeneously light electron dense EVs indicated by an arrow. (D) Grouped histogram showing the number of EVs falling into each class in Tz-treated compared to control SKBR3 cells counted on 10 random micrographs at 30,000x magnification. Bars represent the mean $\pm$ SEM. \*\*\*\*  $P < 0.0001$  (2-way Anova test). (E) ERBB2 specific immunogold staining of purified EVs. Gold particles conjugated to protein A are 10 nm and appear as black dots. (F) Box-and-whiskers box plot showing size distribution ERBB2<sup>+</sup> and ERBB2<sup>-</sup> EVs purified from SKBR-3 cells, \*\*\*\*  $P < 0.0001$  (Student's t-test). Magnification bars = 200 nm.

### 3.2. Tz treated ERBB2<sup>+</sup> BCa cells release EVs expressing the 50kDa isoform of the CD63 tetraspanin.

To better characterize the EVs released from SKBR-3 cells we performed immunoblot analysis with antibodies recognizing the HSP90, which stabilize the ERBB2 receptor, and several EVs markers such as PDCD6IP/Alix, CD9, and CD63. GAPDH, which is a constitutive expressed protein in EVs, was used as loading control. The immunoblot analysis showed that the EVs express the HSP90 chaperone along with CD9 and Alix and that Tz treatment did not induce major changes in the levels of these proteins. In contrast, a faint signal corresponding to a 50 kDa CD63 isoform was observed only in EVs purified from Tz treated SKBR-3. A similar Tz-dependent expression was displayed by EVs from BT474 and MDA-MB-361 cells (Fig. 2A). A Tz-dependent expression of the

CD63 50 kDa isoform reflected the results obtained in the cell lysates obtained from the three ERBB2+ BCa cell lines (Fig. 2B). We hypothesize that this 50 kDa isoform is the result of a specific glycosylation. To test this hypothesis, we performed a N-glycosidase F treatment of lysates of SKBR-3 treated with Tz followed by an immunoblot analysis with the CD63 antibody. Results showed that N-Glycosidase F caused a reduction of the intensity of the 50 kDa band and the appearance of an 18 kDa band, corresponding to the CD63 core protein, confirming our hypothesis (Fig. 2C).

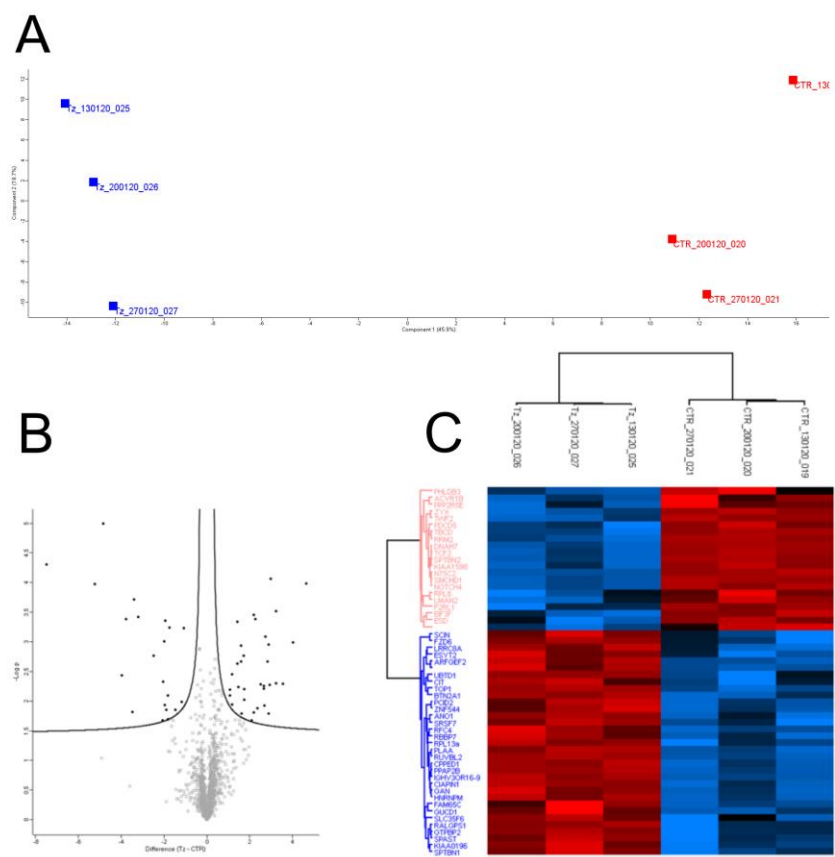


**Figure 2. Immunoblot analyses of ERBB2+ cancer cell lines, lysates and EVs purified from conditioned media.** Cells were either treated with human normal IgGs (Tz- condition) or with 10  $\mu\text{g}/\text{ml}$  of Tz (Tz+ condition) for 72 h. Migration of molecular mass standards expressed in kDa, are indicated on the left. (A) Immunoblot analysis of EVs purified from conditioned media of BT474, SKBR-3 and MDA-MB-361 cell lines. Antibody tested are indicated on the right. GAPDH was used as loading control. (B) Immunoblot analysis of the expression levels of the CD63 50 kDa isoform in BT474, SKBR-3 and MDA-MB-361 cell layers. GAPDH was used as loading control. (C) Immunoblot analysis of cell lysates treated with reaction buffer (N-Glycosidase F- condition) or with buffer plus enzyme (N-Glycosidase F + condition). Lysates were obtained from SKBR-3 cells treated with Tz. Signals from glycosylated CD36 isoforms and the 18 kDa CD63 core protein are indicated on the right.

### 3.3. Tz modulates the expression of proteins associated with EVs of SKBR-3 cells.

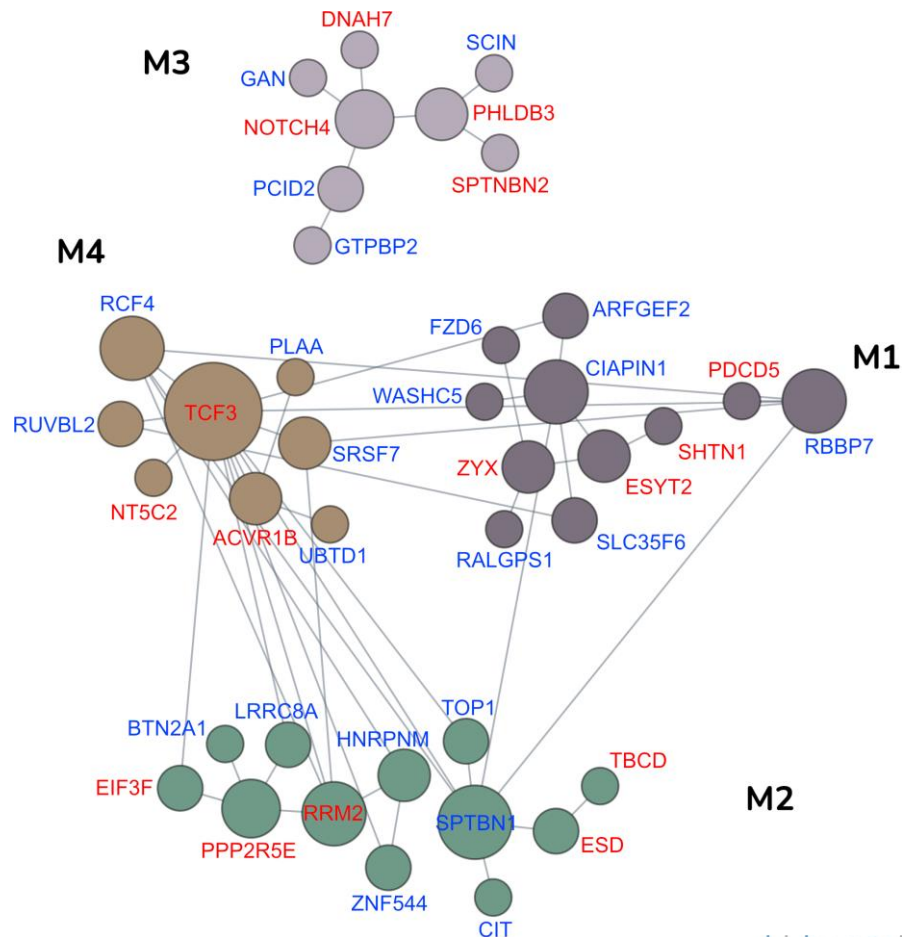
To gain insight on the protein associated with EVs produced by SKBR-3 cells under Tz treatment, we used a proteomic approach based on high-resolution mass spectrometry on EVs purified from the SKBR-3 cell line treated with Tz or with IgGs for 72 hours, in triplicate experiments. A range of 1478-1483 proteins were identified in the 3 control EVs samples while 1503-1520 proteins were identified in the 3 Tz treated EVs samples. To reveal differences or similarities between the two treatments, we performed principal

component analysis (PCA) and hierarchical clustering analysis. The PCA analysis showed that the EVs associated proteins are separated according to treatments in two groups (Fig. 3A) and the volcano plot in Figure 3B shows differentially expressed proteins. In particular, 32 proteins (including the IgG heavy chain of the Tz added in the treatment) were upregulated while 20 proteins were downregulated in EVs derived from Tz treated SKBR-3 compared to controls (t-test  $S_0 = 0.1$  and FDR = 0.05). A list of these 51 proteins (which obviously excluded the IgG heavy chain) regulated by Tz in EVs from SKBR-3 is provided (Table S2). An unsupervised hierarchical clustering analysis showed that the modulated proteins are clustered in two well defined groups, which is in agreement with the PCA analysis (Fig. 3C). To gain insights into possible functions associated to the two protein groups, we performed a bioinformatic analysis using the HumanBase functional module discovery tool available at <https://hb.flatironinstitute.org/>, which perform data driven predictions of gene functions [16–19].



**Figure 3. Principal component analysis, volcano plot and unsupervised hierarchical clustering analysis of proteins associated to EVs released from Tz treated or control SKBR-3 cells.** A) Two-dimensional scatter plot of the principal component analysis of EVs released from Tz treated (blue dots) or control IgGs treated SKBR-3 cells (red dots). (B) Volcano plot representation of differentially expressed proteins. (C) Unsupervised hierarchical-clustered heatmap of 52 proteins identified by Multiple-samples test ANOVA performed on the EVs purified from the SKBR-3 cell line. The amount of each protein in individual samples is represented by the color scheme in which red and blue indicate high and low expression of proteins, respectively. Three independent biological replicates of cells treated with Tz or IgGs (control, CTR) are shown. Proteins are clustered into 2 groups according to their expression value. In particular, 32 were up-regulated in EVs from Tz treated cells while 21 were upregulated in EVs from control cells.

In particular, this analysis was focused on data obtained from mammary epithelium and showed that the genes encoding for the 51 proteins regulated by Tz in EVs belongs to 4 distinct functional modules: M1 mitochondrial membrane organization, M2 mitotic cytokinesis, M3 negative regulation of organelle organization, and M4 cellular response to lipid (Q value < 0.01) (Fig. 4).

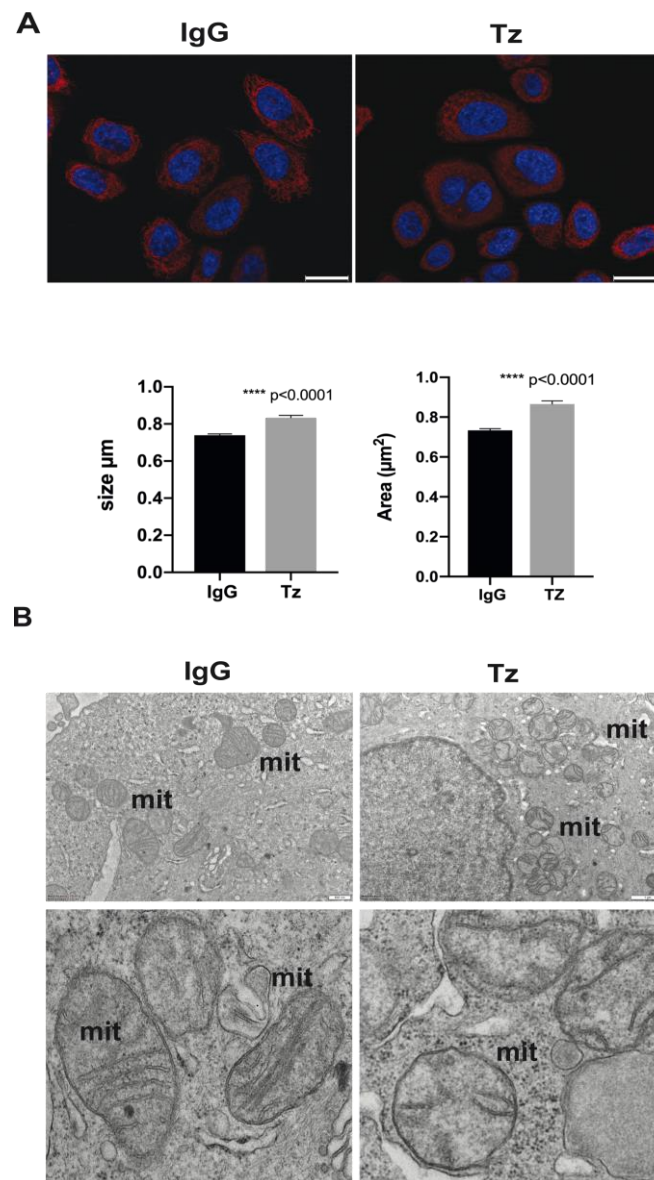


**Figure 4. Functional networks of Tz modulated proteins in EVs released from SKBR-3 cells.** The biological processes associated to the Tz modulated protein signature associated to EVs released from SKBR-3 cells proteins were characterized by projecting these signature proteins onto mammary epithelium specific functional networks at HumanBase (<https://hb.flatironinstitute.org>). These network modules represent genes and their interactions in biological processes and pathways active in mammary epithelium. The most representative biological processes enriched within each module are as follows: M1 mitochondrial membrane organization, M2 mitotic cytokinesis, M3 negative regulation of organelle organization, and M4 cellular response to lipid. The protein expression is represented by the color scheme in which blue and red protein symbols indicate high and low expression of the corresponding proteins in EVs from Tz treated cells, respectively.

### 3.4. Tz triggers mitochondrial alterations in SKBR-3 cells.

Because the most represented functional module found through our bioinformatic analysis of the EVs associated proteins modulated by Tz was related to mitochondrial

membrane organization we hypothesize that Tz could affect the mitochondrial structure in cancer target cells. In particular, we analyzed size and area of SKBR-3 mitochondria labeled with the specific fluorescent marker MitoTracker Red and found a statistically significant increase of both of these parameters upon Tz treatment (Fig. 5A). This data, indicative of mitochondrial alterations caused by Tz, prompted us to better characterize the mitochondrial phenotype in ERBB2<sup>+</sup> cells by performing TEM analysis of SKBR-3. Our results showed a reduction of mitochondrial cristae in Tz treated cells compared to controls (Fig. 5B).



**Figure 5. Fluorescence and TEM imaging of SKBR-3 cell mitochondria.** (A) Mitochondria stained with MitoTracker Red display increased size and area in cells treated with Tz compared to controls (IgG). \*\*\*\*p<0.0001. Bar=20 μm. (B) Mitochondria (mit) in Tz treated cells show a reduced number of cristae compared to controls (IgG).

#### 4. Discussion

A great research effort has been recently focused on EVs released from human malignancies because they may potentially contribute to early diagnosis of primary tumors

and in the detection of relapse after therapy. However, much information is needed on the effect of the anticancer therapy on the structure and composition of EVs released from treated tumors. This study focused on the effects of the drug Tz on EVs released from ERBB2<sup>+</sup> (BCa) cells using widely used cell lines derived from this tumor type and found that Tz indeed modulates the composition of proteins carried by EVs. Our TEM and immuno-EM results showed that EVs released from these cells fall in three morphological classes and also determined that ERBB2 is preferentially associated with larger cup-shaped EVs (class A). The presence of these vesicles along with the detection of the exosomal markers CD9, Alix and CD63 by immunoblot analysis strongly suggests the presence of an exosome subset in these EVs. We found that Tz significantly decreases the release of EVs but has no major impact on all exosomal markers with the notable exception of the 50 kDa CD63 isoform inclusion in the EVs, which was undetectable in those from control cells. Our biochemical data demonstrate that this is a specific glycosylated isoform, which is present also in the cell lysates but whose level of expression in the cells and especially its association to EVs is dependent upon Tz administration to the cells. To our knowledge this is the first report of a Tz specific effect on posttranslational modification and trafficking of a protein, which in this case is an exosomal marker. Interestingly, CD63 is a member of the tetraspanins superfamily and a key regulator of beta1 integrin signaling and it is involved in the regulation of membrane protein trafficking, reorganization of the actin cytoskeleton, cell adhesion, spreading and migration [20,21]. As all these processes are critical for cancer progression, our observation warrants further research on the molecular mechanisms that mediate this Tz effect and possible functional implications of the inclusion of CD63 50 kDa isoform in exosomes released by ERBB2<sup>+</sup> BCa cells. The fact that CD63 was found in mass spectrometry, but is not significantly modulated, may be due to the highly glycosylated structure of the protein. In fact glycopeptides have a lower ionization efficiency, due to an increase in negative charge and acidity, which causes an ion suppression effect compared to the higher number of unmodified peptides displaying a more intense ion current. This leads to a measurement less accurate which may justify the discrepancy with the immunoblot results. However, the analysis of our proteomic data revealed that the influence of the Tz treatment of ERBB2<sup>+</sup> cells on EVs composition is more extensive. In fact, we found that Tz modulates the abundance in these EVs of 51 proteins, which were associated by a network analysis to four biological processes: mitochondrial membrane organization, organelle organization, cytokinesis and response to lipid. It is interesting to notice that several studies reported that Tz treatment induces mitochondrial morphological alterations up to disruption of both external and internal membranes leading to mitochondrial dysfunction [22–24]. However, these studies were focused on myocardiocytes. Here we show that mitochondria of Tz treated ERBB2<sup>+</sup> BCa cells are enlarged and display a reduction of the number of cristae. Therefore, we hypothesize that these cells may sort in EVs some components of damaged or dysfunctional mitochondria, perhaps along with components of other damaged organelles, in an effort to dispose of them. Possible effects of these proteins in cells that may eventually collect these EVs *in vivo*, remain to be investigated. Perhaps more complex and more relevant for a possible role of EVs released from ERBB2<sup>+</sup> BCa Tz treated cells on distant target cells are those proteins which incorporation in EVs is modulated by Tz and that are related to response to lipid and cytokinesis. Among the latter TOP1, which was also found in EVs from murine BRCA1-deficient tumors [25], may be of particular interest to be evaluated in future studies as EVs-associated biomarker in monitoring breast cancer response to therapy for the following reasons: it functions as an oncogene [26] and it is frequently amplified in breast cancer [27], and lastly because it is a target for deruxtecan, which is effectively delivered to ERBB2<sup>+</sup> breast cancer cells by Tz as antibody drug conjugate (DS-8201a) [28].

## 5. Conclusions

In conclusion, our study shows that Tz has an impact on the protein cargo of EVs released in the extracellular environment by ERBB2+ BCa cells. This cargo reflects at least in part alterations induced by Tz on cell organelle organization and may influence several cellular processes linked to cancer progression in target cells at distant sites

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Table S1: Antibodies used in this study, Table S2: proteins regulated by Tz in EVs from SKBR3 cell.

**Author Contributions:** Conceptualization, Katia Cortese and Patrizio Castagnola; Data curation, Martina Bertolucci and Andrea Petretto; Formal analysis, Katia Cortese and Patrizio Castagnola; Funding acquisition, Katia Cortese and Patrizio Castagnola; Investigation, Silvia Marconi, Sara Santamaria, Sara Stigliani, Cinzia Aiello, Maria Cristina Gagliani, Grazia Bellese, Andrea Petretto, Katia Cortese and Patrizio Castagnola; Methodology, Silvia Marconi, Sara Santamaria, Martina Bertolucci, Sara Stigliani, Maria Cristina Gagliani and Andrea Petretto; Supervision, Katia Cortese and Patrizio Castagnola; Writing – original draft, Katia Cortese and Patrizio Castagnola; Writing – review & editing, Katia Cortese and Patrizio Castagnola. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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