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RESEARCH ARTICLE

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Cholesteryl ester transfer protein knock-down in conjunction with a cholesterol-depleting agent decreases tamoxifen resistance in breast cancer cells

Liang Gu | **Ruvesh Pascal Pillay** | **Ruth Aronson** | **Mandeep Kaur**

Department of School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa

Correspondence
Mandeep Kaur, School of Molecular and Cell Biology, Private Bag 3, WITS-2050, Johannesburg, South Africa.
Email: mandeep.kaur@wits.ac.za

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Abstract

The cholesterogenic phenotype, encompassing de novo biosynthesis and accumulation of cholesterol, aids cancer cell proliferation and survival. Previously, the role of cholesteryl ester (CE) transfer protein (CETP) has been implicated in breast cancer aggressiveness, but the molecular basis of this observation is not clearly understood, which this study aims to elucidate. *CETP* knock-down resulted in a >50% decrease in cell proliferation in both 'estrogen receptor-positive' (ER+; Michigan Cancer Foundation-7 (MCF7) breast cancer cells) and 'triple-negative' breast cancer (TNBC; MDA-MB-231) cell lines. Intriguingly, the abrogation of *CETP* together with the combination treatment of tamoxifen (5 μM) and acetyl plumbagin (a cholesterol-depleting agent) (5 μM) resulted in twofold to threefold increase in apoptosis in both cell lines. *CETP* knockdown also showed decreased intracellular CE levels, lipid raft and lipid droplets in both cell lines. In addition, RT^2 Profiler PCR array (Qiagen, Germany)-based gene expression analysis revealed an overall downregulation of genes associated in cholesterol biosynthesis, lipid signalling and drug resistance in MCF7 cells post-*CETP* knock-down. On the contrary, resistance in MDA-MB-231 cells was reduced through increased expression in cholesterol efflux genes and the expression of targetable surface receptors by endocrine therapy. The pilot xenograft mice study substantiated *CETP*'s role as a cancer survival gene as knock-down of *CETP* stunted the growth of TNBC tumour by 86%. The principal findings of this study potentiate CETP as a driver in breast cancer growth and aggressiveness and thus targeting CETP could limit drug resistance via the

Abbreviations: AP, acetyl plumbagin; BSA, bovine serum albumin; CE, cholesteryl esters; CETP, cholesteryl ester transfer protein; ER+, estrogen receptor positive; GEPIA, gene expression profiling interactive analysis; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDR, multidrug resistance; OD, optical density; RCT, reverse cholesterol transport; SREBF, sterol regulatory element binding protein transcription factor; SREBP, sterol regulatory element binding protein; TAM, tamoxifen; TNBC, triple-negative breast cancer.

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reduction in cholesterol accumulation in breast cancer cells, thereby reducing cancer aggressiveness.

KEYWORDS

breast cancer, CETP, cholesterol depletion, drug resistance, lipoprotein signalling pathways

1 | INTRODUCTION

Breast cancer has emerged as the second leading cause of cancer death in women worldwide and remains a global burden.[1] Despite the availability of several chemotherapies and interventions, drug resistance (acquired or de novo) in conjunction with the heterogeneous nature of breast cancer has rendered this disease increasingly difficult to treat. The majority of breast cancers are hormone responsive (~75%),[2] therefore, endocrine therapies, such as tamoxifen (TAM), have become the most popular means to prevent relapse of breast cancer. TAM has been administered for over 40 years for women with estrogen receptor-positive (ER+) breast cancer.[3] Although TAM is effective in its early stages of its use, over-time many patients develop TAM resistance and relapse.[4] TAM usage is shown to cause harmful side effects, including the development of endometrial cancer, blood clots and the early onset of menopausal symptoms.[5] In addition, endocrine therapies cannot be administered to patients diagnosed with triple-negative breast cancer (TNBC), a breast cancer type that lacks the hormone receptors targeted by endocrine therapies, which represents 15%–20% of the breast cancer cases.[6] Therefore, there is an urgent need to improve on existing therapies and/or develop novel treatments for patients with breast cancer to reduce the increasing morbidity and mortality rates.

Over the past decade, the role of cholesterol in cancer progression, more particularly in breast cancer, has been greatly emphasised. Several studies have supported the link between cholesterol and cancer growth. This includes increased cholesterol synthesis,[7,8] the accumulation of oxysterols in cancer,[9] the overexpression of receptors involved in cholesterol import,[10,11] cholesterol's role in the regulation of cell cycle,[12] metastasis[13,14] and steroidogenesis.[15] Cholesterol dyshomeostasis, encompassing intracellular cholesterol accumulation, allows for the abnormal activation of several cell proliferation and survival pathways,[16,17] a characteristic in several cancers.[18] Since cholesterol accumulation contributes to cancer aggressiveness and survival, cholesterol deprivation in cancer cells is anticipated to be a useful strategy in the treatment of breast cancer.[19–21] Several review articles have highlighted various drugs that targeted cholesterol metabolism as a potential anti-cancer strategy.[22–26] Cholesterol is a crucial component for normal cell functioning; therefore, finding a mechanism to deplete excess cholesterol in cancer cells while not halting cholesterol synthesis (the mechanism of statins) could arguably be a more feasible option in the treatment of breast cancer.[27] A study performed by Sharma et al. showed that metformin inhibited cell viability, migration, epithelial–mesenchymal transition and stemness in breast cancer via reduced intracellular cholesterol content.[28] Previously, we have shown that acetyl plumbagin (AP)[29] induces apoptosis in cancer cells by depletion of cholesterol and significantly decreases cholesteryl ester transfer protein (*CETP*) expression.[30] The role of CETP is twofold. Intracellularly, CETP facilitates the uptake of cholesteryl esters (CEs) and triglycerides from the endoplasmic reticulum into storage droplets of adipocytes,[31] which limits intracellular toxicity of free cholesterol. Extracellularly, CETP acts as the shuttle protein of CEs between high-density lipoprotein (HDL) and low-density lipoprotein (LDL) during the reverse cholesterol transport (RCT) process.[32] *CETP*-deficient cells have been shown to a have lower intracellular cholesterol levels compared with normal cells, which could be due to the accumulation of CEs at the synthesis site (endoplasmic reticulum).[30] When CE transport is impeded, there is inefficient removal of CEs from the endoplasmic reticulum, resulting in sterol abundance. This could lead to the downregulation of pathways that synthesise cholesterol and the upregulation of pathways involved in cholesterol efflux.[30] Moreover, in the absence of *CETP*, plasma LDL levels decrease while HDL levels are elevated, suggesting an enhanced clearance of cholesterol in the plasma.[30] Therefore, targeting *CETP* has been investigated as a possible therapeutic against cholesterol-related diseases, such as arteriosclerosis and coronary heart disease.[33] However, the association between *CETP* and cancer is not well-documented, especially at the molecular level when *CETP* is knocked down and combined with a cholesterol-depleting agent. Furthermore, the potential clinical relevance of *CETP* and the molecular basis of its contribution to cancer cell survival is non-existent—a gap, which this study seeks to address.

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2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Breast cancer cells MCF-7 (ER+) (European Collection of Authenticated Cell Culture; ECACC, UK) and MDA-MB-231 (TNBC) (American Type Culture Collection; ATCC, USA) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and 3:1 DMEM/Hams F12 media (Gibco: Life Technologies, UK), respectively. All media were supplemented with 10% fetal bovine serum (FBS) (Celtic Molecular Diagnostics, South Africa) and 1% Penicillin–Streptomycin (Sigma Aldrich, UK). Cells were cultured and maintained at 5% CO_2 in a 37°C incubator. Cells were treated with 5 and 10 μM of TAM (Sigma-Aldrich, UK) and AP (synthesised in-house) in single and combination treatments for 24 h.

2.2 | siRNA transfection

Overall, 300,000 cells were seeded for transfection. MCF-7 and MDA-MB-231 cells were transfected with 25 nM/37.5 nM of Silence® Select Pre-designed CETP/negative control small interfering RNA (siRNA), respectively (s2933, Ambion®, Life Technologies, USA) with 3 μL of a lipid-mediated DharmaFECT™ transfection reagent (DH T-2001-02, Dharmacon™, GE Healthcare, UK). Concentrations of siRNA and transfection reagents were determined accordingly using manufacturers' guidelines as the baseline (Horizon's DharmaFECT transfection protocol; https://horizondiscovery.com/-/media/Files/Horizon/resources/Protocols/basic-dharmafect-protocol.pdf). Cells were incubated for 72 h at 37°C. Successful transfection was confirmed with qPCR and western blotting.

2.3 | Cholesterol quantification assay

A cholesterol quantification assay was performed as previously described.[34] Briefly, this method involves the hydrolysis of CEs by cholesterol esterase, which forms cholesterol. Cholesterol is then oxidised by cholesterol oxidase that results in two products: (1) cholest-4-ene-3-one and (2) hydrogen peroxide (H_2O_2). H_2O_2 is detected with a highly specific colorimetric probe (ampliflu red). The horseradish peroxidase (HRP) catalyses the reaction between H_2O_2 and ampliflu red that binds in a 1:1 ratio. This reaction results in the development of a pink colour, which the optical density (OD) was measured at 570 nm using the Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ Software 2.0).

2.4 | Lipid staining: BODIPY, Vybrant™ Alexa Fluor™ 594 Lipid Raft Labelling and Filipin

Transfected, non-transfected and siRNA-negative control cells were seeded into six-well plates at 50,000 cells/well; 1 mM methyl beta-cyclodextrin (MβCD) was utilised as a positive control. Cells were fixed with 4% formalin (Sigma Aldrich, UK) for 10 min at room temperature. The reaction was stopped following three 1× PBS washes. Cell membranes were permeabilised using 1% Triton-X (Sigma Aldrich, UK) for BODIPY staining and Vybrant™ Alexa Fluor™ 594 Lipid Raft Labelling. Cells were incubated depending on the lipid stain as follows: 1 μL of 1 mg/mL BODIPY at 37°C for 30 min; 2 μL cholera-toxin subunit B (Vybrant™ Alexa Fluor™ 594 Lipid Raft Labelling Kit, V34405, Thermo Fisher Scientific, USA) at 37°C for 30 min then anti-cholera-toxin subunit B (1:200 dilution) for 30 min at 37°C; 0.05 mg/mL filipin (in PBS), at 37°C for 2 h. Subsequently, nuclei were either stained with NucRed™ Dead 647 (R37113, Thermo Fischer Scientific) for the filipin assay or 0.001 mg/mL DAPI (Sigma Aldrich, UK) in PBS for BODIPY and lipid raft labelling. Cells were visualised using the FLoid™ Cell Imaging Station (Thermo Fisher Scientific, USA).

2.5 | Growth inhibition and apoptosis assays

Overall, 5000 cells/well were seeded into 96-well plates and treated with the appropriate drugs over 24 h. Then, 40 μM Plumbagin (Sigma-Aldrich, UK) was used as a positive control for cell death. Following incubation, 5 μL of sterile MTT (5 mg/mL) (Sigma-Aldrich, UK) dissolved in PBS was incubated for 4 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) crystals were solubilised in 55 μL of solubilisation solution (10% sodium dodecyl sulfate (SDS), 10 mM HCl), overnight at 37°C. OD was measured at 570 nm using a Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ Software 2.0).

The effect of *CETP* knock-down on cell growth was determined by a cell counting assay. Overall, 300,000 cells/well were seeded into 6-well plates, an untreated and transfection control group was included. After 72 h, the cells were detached, and an equal volume of neutralised media was added to 0.4% Trypan Blue

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Solution (Thermo Fisher Scientific, USA). Cells were counted in triplicate using a Neubauer Hemocytometer.

An apoptosis assay was performed to characterise the effects of *CETP* knock-down and drug treatments on apoptosis. Media was removed from 96-well plates (5000 cells/well), and cells were stained with an APOPercentage™ (Biocolor, Carrickfergus, UK) dye as previously described.[35] OD was measured at 550 nm using a Multiskan GO Microplate Reader (Thermo Fisher Scientific, SkanIt™ Software 2.0).

2.6 | Kaplan–Meier survival plots

Kaplan–Meier survival plots generated *CETP* expression profiles using the Kaplan–Meier Plotter [breast cancer mRNA] analysis tool (https://kmplot.com/analysis/).[36] The plots represent overall survival in breast cancer patients with luminal A, luminal B, basal-like and HER+ breast cancer sub-types.

2.7 | RT^2 profiler™ PCR arrays

RT^2 Profiler™ PCR arrays (Qiagen, Germany) were used to identify the expression levels of genes involved in human cancer drug resistance, human lipoprotein signalling and cholesterol metabolism pathways; post-*CETP* knock-down. This array uses reverse transcription quantitative polymerase chain reaction (RT-qPCR) to identify expression of 84 different genes in one 96-well plate. Total extracted RNA was reverse transcribed into cDNA using the RT^2 First Strand Kit (Qiagen, Germany). Thereafter, complementary DNA (cDNA) was added to the RT^2 SYBR Green qPCR Mastermix (Qiagen, Germany) and samples of equal volume were loaded into array plates. Several controls were included in the RT^2 Profiler™ PCR arrays (Qiagen, Germany), these included: housekeeping genes, genomic DNA controls, replicate reverse transcription controls and replicate-positive PCR controls. The arrays were performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, South Africa) and analysed using the RT^2 Profiler™ PCR array guidelines. Gene expression was compared with the data obtained from Gene Expression Profiling Interactive Analysis (GEPIA) using multiple gene analysis tool (http://gepia.cancer-pku.cn/).[37]

2.8 | Heatmap and gene network map

Omicsnet[38] was utilised to predict interaction networks between the selected genes obtained from the RT^2 Profiler™ PCR array. Subsequently, Cytoscape[39] was used to create the gene network map including the expression profiles obtained from RT^2 Profiler™ PCR array.

2.9 | In vivo xenograft study

An in vivo pilot study was performed to observe the effect of *CETP* knock-down on tumour growth in MF-1 nude female mice ($n = 6$). The mice (aged between 5 and 8 weeks) were obtained from the University of Cape Town, South Africa, and housed at the Wits Research Animal Facility, Parktown Campus, Johannesburg, South Africa. The study was approved by the Wits Animal Ethics Screening Committee (AESC number: 2016/09/41/D). Mice were subcutaneously injected with[1] transfected MDA-MB-231 cells ($n = 3$) or[2] non-transfected MDA-MB-231 cells ($n = 3$) ($\pm 5 \times 10^6$ cells/mice) into the flank. All mice were euthanised on day 18 post-tumour detection as the tumours injected with non-transfected cells grew too large and started to impact negatively to their well-being. The formed tumours were harvested, then weighed and photographed. The tumour volume was calculated with the formula $V = a \times b^2/2$, where 'a' is the long diameter and 'b' is the short diameter of the tumour. For long-term storage, tumours were snap frozen in liquid nitrogen for RNA and protein extraction.

2.10 | Total RNA extraction and RT-qPCR

RNA was extracted using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Inqaba Biotec™, South Africa). TRIzol® reagent (300 μL/10^6 cells) (Ambion®, Life Technologies, USA) was added to cells and mixed thoroughly for 5 min at room temperature.[40,41] An equal volume of 99.9% ethanol (Thembane Chemicals, South Africa) was added to precipitate the nucleic acids. Subsequently, RNA extraction was carried out as per the manufacturer's protocol. Total RNA of 1 μg was used for cDNA synthesis using the RevertAidFirst Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) and executed according to the protocol. Thermal cycling was performed with the MultiGene™ OptiMax Thermal Cycler (Labnet International, UK). Subsequently, qPCR was performed using the SensiFAST™ SYBR® No-ROX kit. Primers were synthesised by Integrated DNA Technologies (IDT, Illinois, USA), the sequences are listed in Table SI2. A 3-step cycling parameter was set on the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad). The change in mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.[42]

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2.11 | Western blotting

Cells were harvested and lysed in RIPA lysis buffer (100 μL/10^6 cells). A BCA assay (Sigma Aldrich, UK) was performed to quantify the protein lysates, which were normalised against a bovine serum albumin (BSA) (Inqaba Biotec™, SA) standard curve. A final protein concentration of 10 mg/mL lysate was loaded into a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF) membrane, using a semi-dry Trans-Blot®Turbo™ Transfer System (Bio-Rad, USA). The membrane was blocked with 3% bovine serum albumin (BSA) for 1 h. Primary antibodies were incubated overnight at 4°C. Subsequently, the blots were probed with HRP-conjugated secondary antibody for 1 h (summarised in Table S1) and chemiluminescence was detected with Clarity™ Western ECL HRP substrate (1,705,061, Bio-Rad) and viewed on a ChemiDoc XRS+ System (Bio-Rad, USA).

2.12 | Immunofluorescent staining

CETP knock-down was visualised with immunofluorescent imaging. Overall, 20,000 cells were seeded into a 6-well plate; 25 μM Torcetrapib (Sigma Aldrich, UK) was used as positive control. Cells were fixed with 4% formalin (Sigma Aldrich, UK) at room temperature for 10 min. Subsequently, cells were permeabilised with 0.1% Triton™ X-100 (Sigma Aldrich, UK) in PBS and incubated at room temperature for 20 min and blocked with 0.05% BSA (Inqaba Biotec™, South Africa) for 20 min. Cells were then incubated with anti-CETP primary antibody overnight at 4°C. This was followed by the addition of goat anti-rabbit IgG secondary antibody, Texas red (T-2767, Thermo Fischer Scientific, USA) for 1 h in the dark, at 4°C. Nuclei were stained 0.0001 mg/mL DAPI (Sigma Aldrich, UK) for 5 min at room temperature. Images were captured on the FLoid™ Cell Imaging System (Thermo Fisher Scientific, USA). The immunofluorescence intensity was calculated using ImageJ1 software.[43]

2.13 | Statistical analysis

The statistical analyses were performed in Microsoft Office Excel© and GraphPad Prism version 8 (San Diego, CA, USA). Statistical significance was calculated using the Student's *t*-test and one-way analysis of variance (ANOVA) followed by a Bonferroni pairwise analysis.[44] A *p*-value of less than .05 is the standard estimation of measuring significance of the observations. A *Z*-factor[45] was also used for each 96-well plate, and assays having *Z*-factor above >0.6 were considered the acceptable range in which the technicality of the experiments was trustworthy.

3 | RESULTS

3.1 | CETP knock-down decreases intracellular cholesterol levels

CETP has a defined role in regulating intracellular cholesterol levels.[31] We observed that, on average, CE levels were decreased upon *CETP* knock-down in untreated MCF-7 and MDA-MB-231 cells by ∼54.87% and ∼21%, respectively (Figure 1A,B). This decrease in CE levels were, however, not statistically significant, and this is possibly due to the high standard deviations within samples. Similarly, there was a decrease in CE levels in non-transfected MCF-7 cells treated with AP (Figure 1A); however, AP did not exert the same effects in the non-transfected MDA-MB-231 cells (Figure 1B). Previous study demonstrated the ability of AP to downregulate CE levels in MCF-7 cells; however, it was not tested in MDA-MB-231 cells.[35] Interestingly, we observed a further decrease in cholesterol levels in the AP-treated group post-*CETP* knock-down in MCF-7 cells (Figure 1A) and more so in MDA-MB-231 cells (Figure 1B). These findings suggest an additive effect, wherein *CETP* inhibition enhances cholesterol depletion in cells treated with AP, reinforcing the potential role of CETP as a critical regulator of cellular cholesterol homeostasis under AP treatment.

The observation of *CETP* knock-down leading to decreased cellular cholesterol levels was confirmed through visualisation of cholesterol in various forms. Filipin staining of free cholesterol qualitatively showed total lipid reduction upon *CETP* knock-down in MCF-7 cells (Figure 1C) and MDA-MB-231 cells (Figure 1D). In addition, cholesterol accumulates in specified regions of the cell membrane, together with sphingolipids, forming micro-domains termed lipid rafts. Lipid rafts are central to key signalling pathways involved in apoptosis, cell cycle progression, as well as tumour development and metastasis.[25] Lipid rafts were reduced in both cell lines post-*CETP* knock-down (Figure 1E,F). This suggests that CETP plays a role in regulating the accumulation of cholesterol in the lipid rafts, which ultimately influences cancer proliferation and oncogenic signalling. As expected, lipid storage droplets were also reduced upon *CETP* knock-down in both cell lines (Figure 1G,H). Readily available cholesterol in the form of CEs, reduces the energy demand that would be required through de novo cholesterol biosynthesis. Breast cancer cells are found to

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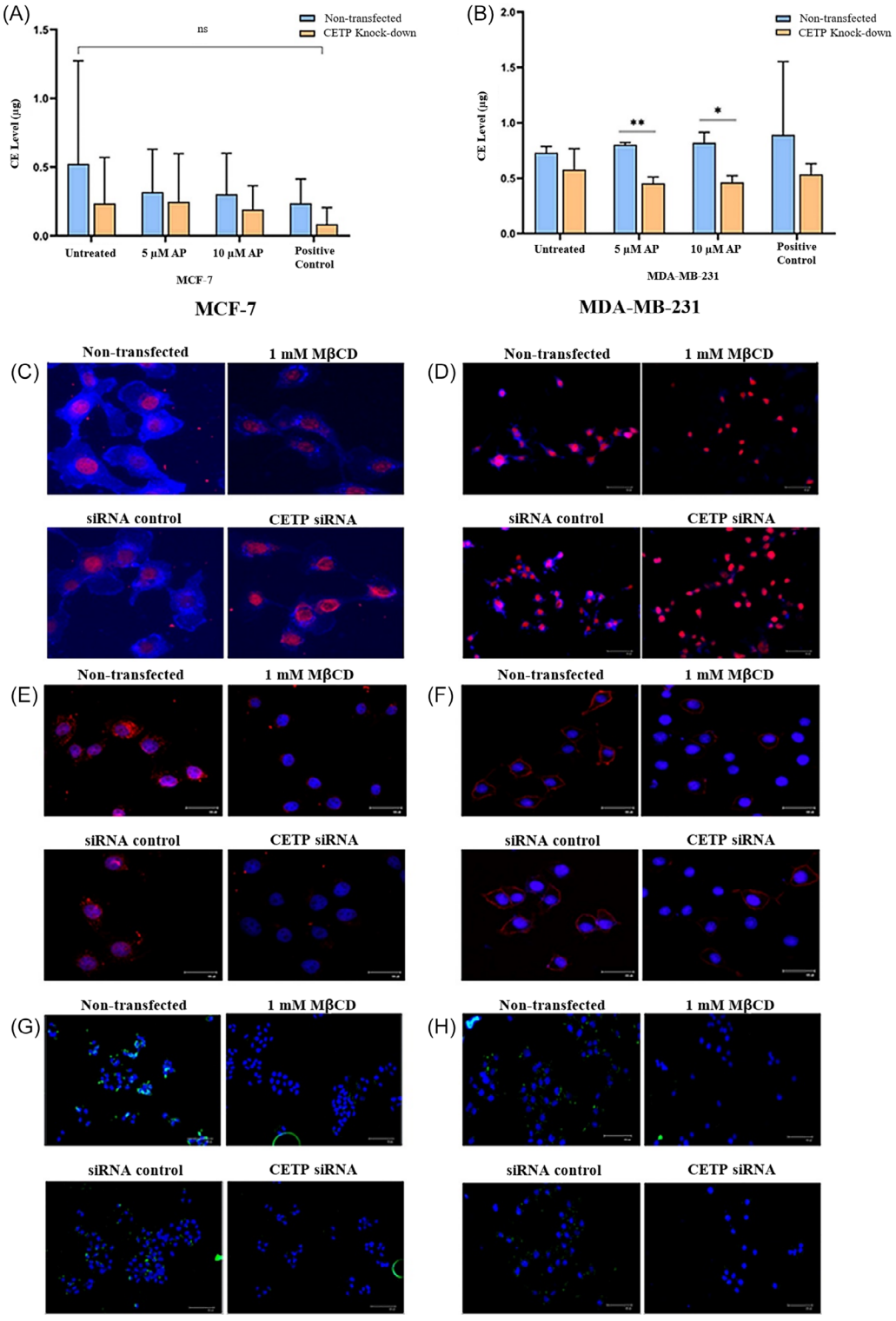


FIGURE 1 Cholesteryl ester (CE) transfer protein (CETP) knock-down decreases intracellular cholesterol levels in MCF-7 and MDA-MB-231 cells. Intracellular CE levels following CETP knock-down in (A) MCF-7 and (B) MDA-MB-231 cells. Plumbagin (PL) (40 µM) was used as positive control. Filipin staining (blue) following CETP knock-down of free cholesterol in (C) MCF-7 and (D) MDA-MB-231 cells. Nuclei were stained with NucRed™ Dead 647 (Red). Lipid raft staining (red) following CETP knock-down in (E) MCF-7 and (F) MDA-MB-231 cells. Nuclei were stained with DAPI (blue). BODIPY fluorescent stain (green) following CETP knock-down in (G) MCF-7 and (H) MDA-MB-231 cells. Nuclei were stained with DAPI (blue). All images were taken on the EVOS Floid Cell Imaging station and ×20 magnification, with scale bar of 1 cm = 100 µM. Methyl beta-cyclodextrin (MβCD) (1 mM; known cholesterol-depleting agent) acted as a positive control for cholesterol depletion. All treatments were incubated with the cells for 24 h. Data represents mean ± standard deviation (SD) ($n = 4$). A Student's *t*-test was employed where $^{*}p < .05$, $^{**}p < .01$ and ns = non-significant.

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have an abnormal accumulation of lipid droplets,[46–48] which subsequently act as reservoirs of cholesterol, to enable membrane biogenesis and modify lipid raft structure, which ultimately enhances cancer aggressiveness.[46] Therefore, the overall decrease in lipid droplets may contribute to a decrease in cancer aggressiveness. To investigate this hypothesis, the effect of *CETP* knock-down on cell viability and growth was examined.

3.2 | CETP is an essential gene for breast cancer cell survival

Our previous work[30] identified *CETP* as an essential cell survival marker. This was also demonstrated that an upregulation of *CETP* in breast cancer patients contributed to cancer cell growth and survival. The study demonstrated that by knocking down *CETP* combined with a cholesterol-depleting agent, such as AP, induced apoptosis by increasing caspase 3/7 activity.[30] Therefore, in the present study, we investigated the effects of *CETP* knock-down on breast cancer survival exposed to TAM and AP. As expected, *CETP* knock-down alone reduced cellular proliferation by more than 50% in both cell lines (Figure 2A,B). Despite recent findings where cholesterol depletion only affected proliferation of hormone-positive MCF-7 cells and not MDA-MB-231 cells, it is interesting to note that these breast cancer cells were deprived of cholesterol via lowered cholesterol media.[49] Although several other research has supported the fact that lowered cholesterol through treatment affected proliferation in both hormone-positive, as well as TNBC cells.[50–52] This could suggest that TNBC cells switch to a cholesterol synthesis-driven proliferation as opposed to cholesterol uptake when subjected to lowered cholesterol growing conditions, while treatment with chemotherapeutics that affect both uptake and synthesis would have a greater inhibitory effect. In our study, *CETP* knock-down also resulted in a decrease in cell viability along with apoptosis induction, and this was observed in both cell lines treated with TAM (5 μM and 10 μM) compared with the non-transfected cells (Figure SI3A,C). Low concentrations of AP did not induce significant apoptosis and cell inhibition (Figure SI3B,D). This was noted particularly in the MDA-MB-231 cell line, which only showed growth inhibition from 20 μM (Figure SI4B) due to its inherent resistance to chemotherapies. Interestingly, *CETP* knock-down with a combination treatment of TAM and AP showed 2–3 folds increase in cellular apoptosis in both cell lines (Figure 2C,D). In MCF-7 cells, percentage change in *CETP* knock-down-induced cellular apoptosis was 121.3% and 97.1% compared with TAM and AP single treatment at 5 μM, respectively. Similarly, when AP concentration increased to 10 μM, there was an increase in cellular apoptosis by 89.8%–96.3% compared with single treatments at 10 μM, respectively (Figure 1C). In MDA-MB-231 cells, *CETP* knock-down, along with a combination treatment of 5 μM TAM and 5 μM AP, increased cellular apoptosis by 22.9% compared with 5 μM TAM single treatment and 52.9% compared with 5 -μM AP single treatment (Figure 2D). The increase in AP concentration (10 μM) in the combination treatment resulted in an increase in cellular apoptosis by 43.64%–61.9% compared with single treatments, respectively. These results indicate that *CETP* inhibition consequently disrupts cellular cholesterol homeostasis, induces apoptosis and reduced TAM resistance in cells, potentially indicating a synergistic effect of TAM and AP in inducing apoptosis.

The effect of CETP on breast cancer survival was further explored through Kaplan–Meier Survival Plots on different breast cancer types based on cell surface receptor status. It was noted that high *CETP* expression decreases patient survival outcome in different luminal A, luminal B, basal-like and HER+ breast cancer sub-types (Figure 2E). Evidently, *CETP* seems to play a crucial role in patient survival outcome and can possibly be used as a breast cancer resistance marker in predicting treatment effectiveness.

3.3 | *CETP* knock-down induces a shift in gene expression in breast cancer resistance and lipoprotein signalling pathway dynamics

The strong link between *CETP* and cancer resistance (as observed in Figures SI3 and SI4) led us to measure the effect of *CETP* on gene expression in human breast cancer resistance and lipoprotein signalling pathways (Figure 3). RT^2 Profiler™ PCR arrays were performed, providing insights into the role of CETP in the molecular mechanisms of breast cancer progression. It was noted that *CETP* knock-down downregulated lipoprotein and cholesterol signalling in MCF-7 cells, whereas minimal effects were observed in transfected MDA-MB-231 cells (Figure 3 and Figure SI5). In addition, gene expression in breast cancer drug resistance was notably downregulated in MCF-7 cells and less significantly in the MDA-MB-231 cell line (Figure 3 and Figure SI5). These results could share insight into some possible explanations to the involvement of CETP in cellular survival.

Cellular cholesterol levels are largely maintained by the balance between cholesterol biosynthesis, export, uptake by lipoproteins and esterification.[54] Cholesterol mechanisms become deregulated to support the increase

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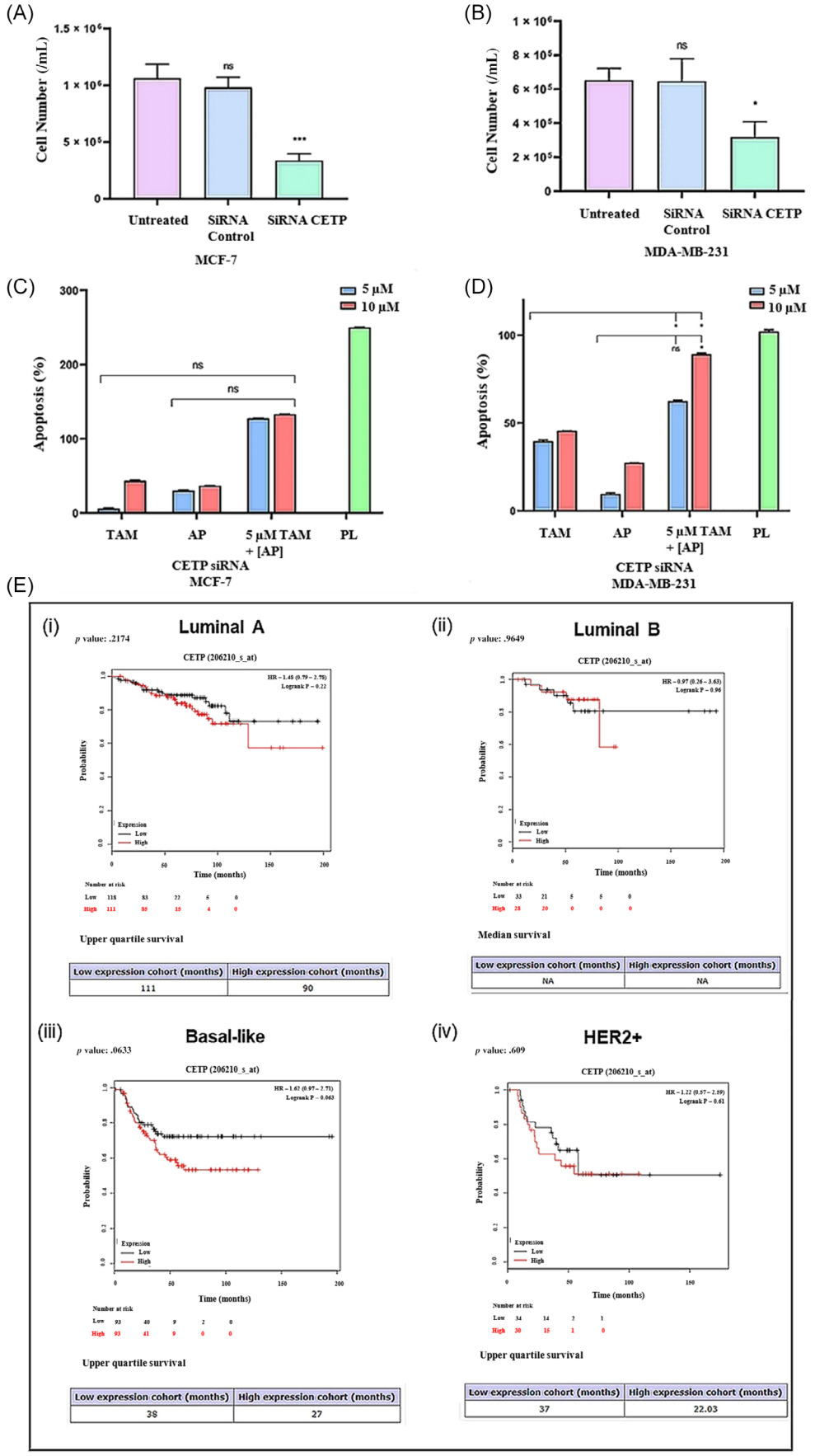


FIGURE 2 Legend on next page.

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in cell metabolic requirements such as hyperproliferation and migration in cancer cells.[25] In addition, in an intriguing study, it was also found that certain cancer cells are more dependent on the uptake of cholesterol rather than synthesis; however, it is unknown which pathway is preferential in different types of cancers.[55] Upon *CETP* knock-down, there was a reduction in expression of genes in ER+ MCF-7 cells involved in cholesterol biosynthesis (*MVD, MVK, HMGCR, FDPS, PRKAG2, PRKAA2*), metabolism (*SREBF1, INSIG1, INSIG2, APOL1, CYP11A1*), uptake (*LDLR, PCSK9, STAB1, LRPAP1*) and RCT (*LCAT, APOE, APOA1, APOA2, APOF*) (Figure 3A and Table SI1). Notably, the aberration of genes involved in cholesterol synthesis upon *CETP* knock-down in MCF-7 cells suggests that these cancer cells are heavily reliant on cholesterol for proliferation.[56] Thus, they are unable to generate the usual amount of cholesterol and hence reduced cancer cell proliferation. The downregulation of the RCT pathway is likely in response to the lack of intracellular cholesterol. Conversely, in TNBC MDA-MB-231 cells, *CETP* knock-down induced increase in gene expression in cholesterol metabolism (*SREBF1, SCAP, APOL1, CYP11A1, NR1H4*), uptake (*LDLR*, *PCSK9, STAB2, LRP18*) and RCT (*APOA2, APOF, APOA1*) (Figure 3A and Table SI1). Increased expression of *APOF* suggests that MDA-MB-231 cells favour HDL-mediated CE removal as opposed to HDL–LDL cholesterol removal, which is mediated by CETP.[57] This RCT upregulation would increase cholesterol efflux and, consequently, decrease drug resistance, as observed in non-small cell lung cancer[58] and glioblastoma.[59] These results confirm that CETP activity mediates cholesterol biosynthesis, uptake and cholesterol efflux.

We further supplemented the above data with preliminary Western blotting on ER and sterol regulatory element binding protein (SREBP) expression in MCF-7 cells pre- and post-*CETP* knock-down (Figure SI6), and it was found that *CETP* knock-down decreased ER expression, which was in concordance with reduced *ER* gene expression (Figure 3). Therefore, *CETP* knock-down is linked to reduced resistance in MCF-7 cells through the ablation of surface receptors. Interestingly, despite a decreased *SREBF* gene expression, *CETP* knock-down rather increased SREBP protein expression in MCF-7 cells (Figure 3 and Figure SI6). This could possibly suggest post-transcriptional and translational modifications, where the protein is being synthesised at a faster rate to compensate for the loss of the gene expression.[60] In addition, the rate of protein degradation is also speculated to have decreased to atone for the reduced cholesterol levels from *CETP* knock-down. Further research is required to confirm these speculations, and results on MDA-MB-231 cells would also shed light into a more in-depth understanding on the effects of *CETP* knock-down on these breast cancer cell lines.

The accumulation of intracellular cholesterol signals for cholesterol efflux through ABCA1 transporter membrane protein, where ApoA1 initiates cholesterol esterification via LCAT in HDLs to prevent re-entering of the cholesterol into peripheral cells.[61] CETP then mediates the transfer of these CEs from HDL to LDLs for excretion by the liver. In cancer cells, these processes are deregulated thus leading to cholesterol dyshomeostasis. In MCF-7 cells, *CETP* knock-down resulted in a downregulation of these cholesterol efflux genes in response to the reduced intracellular cholesterol

FIGURE 2 Cholesteryl ester transfer protein (CETP) knock-down inhibits cell proliferation and increases susceptibility to apoptosis in breast cancer. CETP knock-down decreased cell proliferation by (A) 68.2% in MCF-7 cells and (B) 51.3% in MDA-MB-231 cells. (C) Percentage change in apoptosis in CETP knock-down in MCF-7 cells, together with a combination treatment of tamoxifen (TAM) and acetyl plumbagin (AP) (5 μM and 10 μM) relative to the untreated CETP knock-down control. (D) Percentage change in apoptosis in CETP knock-down in MDA-MB-231 cells, together with a combination treatment of TAM and AP (5 μM and 10 μM) relative to the untreated CETP knock-down control. The increase in apoptosis was also observed in transfected MDA-MB-231 cells. 40 μM of PL was used as positive control. All treatments were incubated with the cells for 24 h. (E) In silico validation of CETP expression on cancer patient survival outcome breast cancer types: luminal A, luminal B, triple-negative breast cancer (TNBC) (basal-like) and HER2+ breast cancer subtype. (E [i]) The probability of survival for up to 150 months in luminal A (estrogen receptor positive [ER+]/PR+/HER2-/Ki67 low) breast cancer patients exhibiting high CETP levels, drops to 60%. On average, CETP overexpression reduces survival by 21 months in luminal A patients. (E [ii]) Luminal B tumours are characterised with elevated expression of Ki67 resulting in enhanced cancer proliferation and poorer patient prognosis.[53] Luminal B cancer patients with high CETP expression have reduced chance of survival from 80% to 60% over 100 months. (E [iii]) Similarly, basal-like (TNBC) patients with elevated levels of CETP expression have a poorer probability of survival and reduced predicted patient survival by 11 months. This finding is particularly noteworthy as TNBC is considered to be the most challenging subtype to treat, rendering the reduction of CETP levels a potential approach to suppress TNBC resistance, thereby increasing the efficacy of drug treatments (as demonstrated in Figure 1B and Figure SI4). (E [iv]) Finally, low CETP expression is favourable in HER2+ breast cancer patients, whereby survival probability is increased by 15 months compared with patients with high CETP expression. Survival plots were generated using the online Kaplan–Meier Plotter. Data represents mean ± standard deviation (SD) of raw data ($n = 3$), where ns = not significant, *$p \leq .05$, **$p \leq .01$ and ***$p \leq .001$ significant difference to non-transfected cells calculated using Student's *t*-test.

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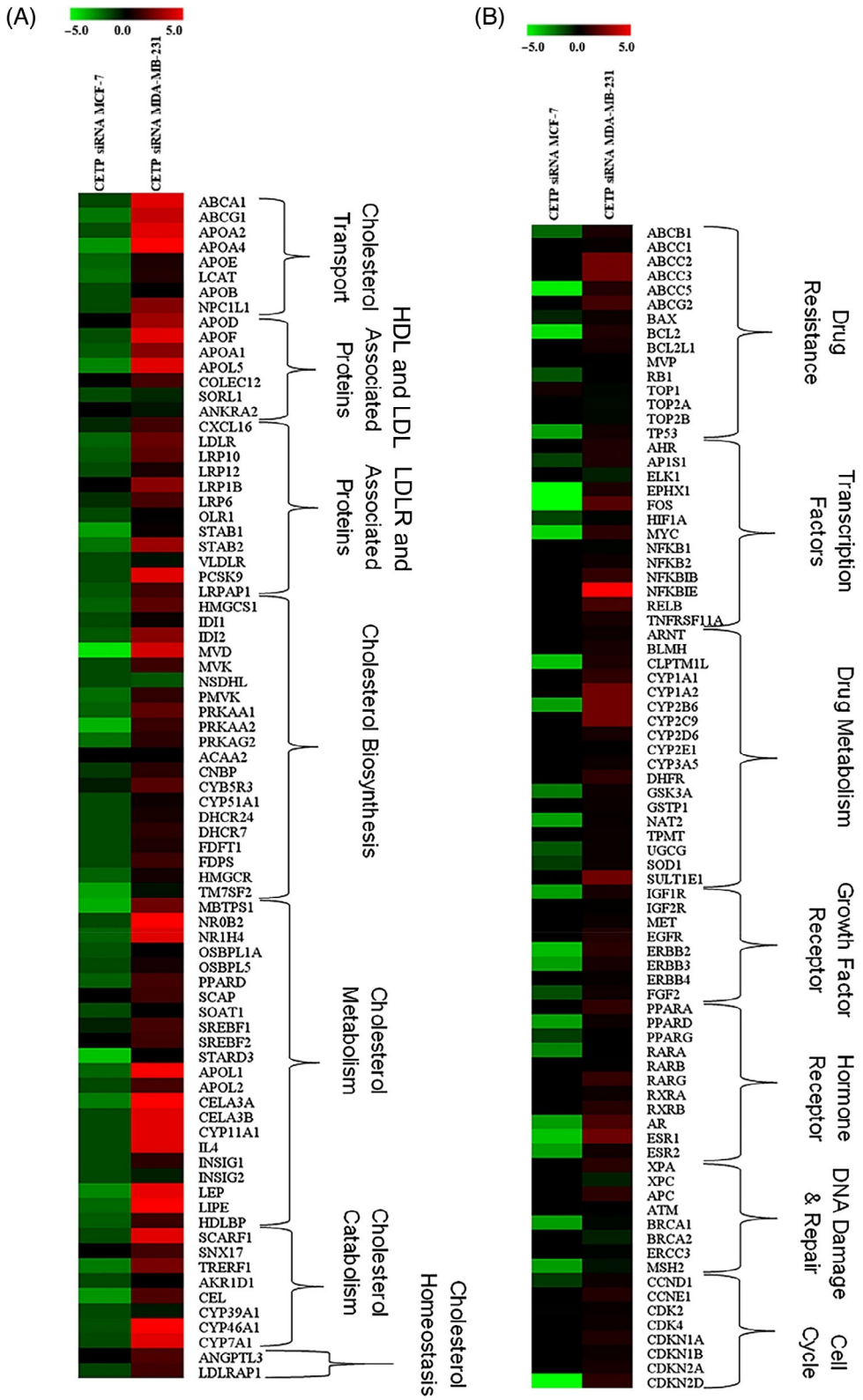


FIGURE 3 The effects of cholesteryl ester transfer protein (CETP) knock-down modifies human breast cancer resistance and lipoprotein signalling pathways. The heat map above is a comparison between transfected (72 h) MCF-7 and MDA-MB-231 cells to the non-transfected cells. These heat maps are representations of a total of 80–84 genes that are associated in breast cancer drug resistance pathway (A) and lipoprotein signalling and cholesterol metabolism pathway (B). Red colour shows increase in expression, green shows reduction in expression and black shows no change in expression of genes.

(Figure 4A). However, these genes were upregulated in MDA-MB-231 cells (Figure 4C), suggesting that cholesterol efflux may be one of the pathways attributed to resistance of MDA-MB-231 cells. Nonetheless, protein expression profiles need to be performed for accurate assessment. In line with the increased cholesterol efflux, there would be increased serum cholesterol and possibly increased RCT. Therefore, cholesterol in the medium would be of interest in future studies.

The lethality of breast cancer is associated with difficulties in treatment and associated drug resistance. Knock-down of *CETP* downregulated expression of

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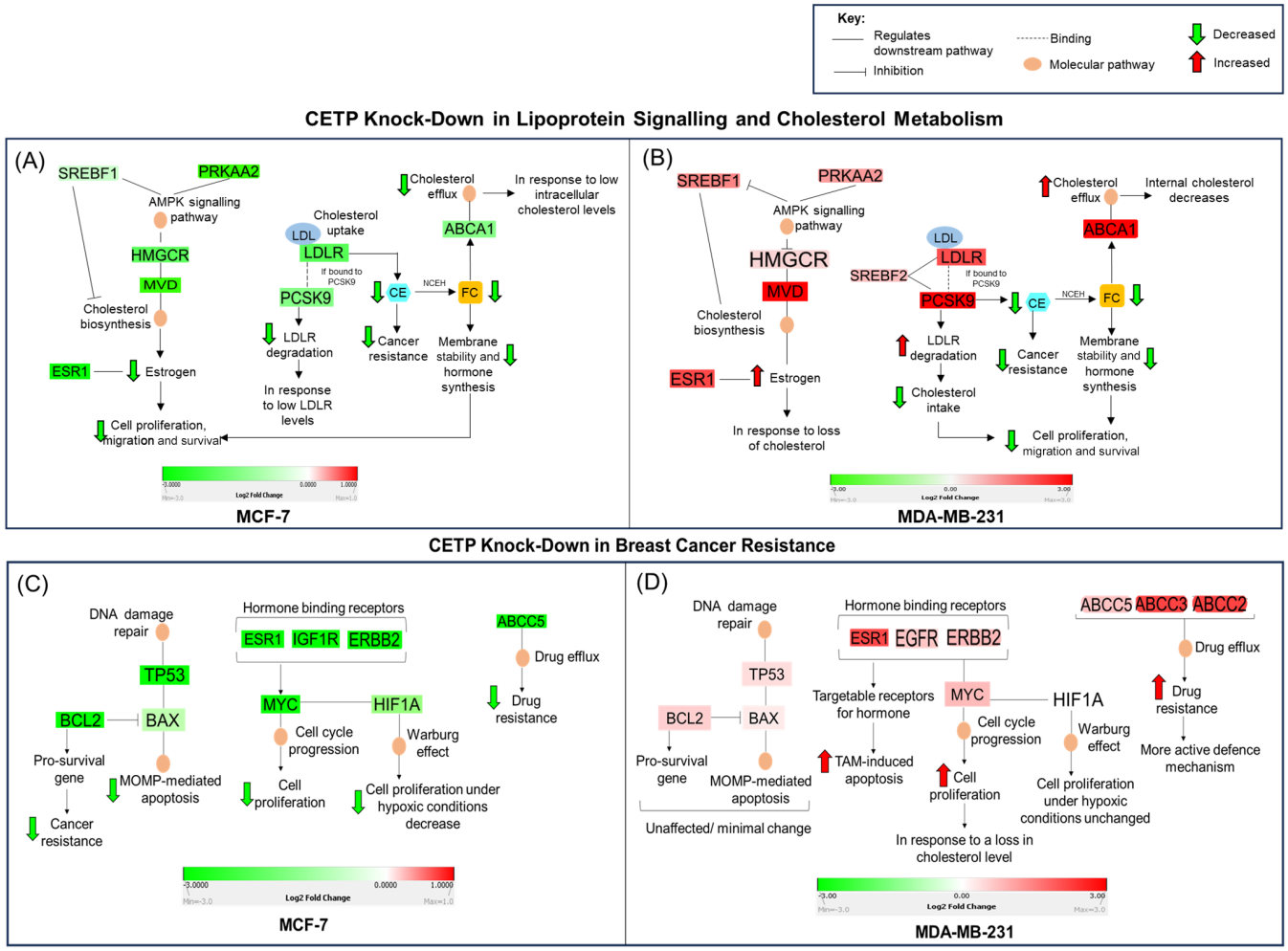


FIGURE 4 Diagram illustrating several genes and pathways affected post cholesteryl ester transfer protein (CETP) knock-down. Visual representation of CETP effects on lipoprotein signalling and cholesterol metabolism in (A) MCF-7 (estrogen receptor-positive [ER+]) and (C) MDA-MB-231 (triple-negative breast cancer [TNBC]) cells. The effects of CETP knock-down on genes involved in human breast cancer resistance in (B) MCF-7 (ER+) and (D) MDA-MB-231 (TNBC) cells. Green and red scale bar indicates the level of expression where green represents a reduced expression, whereas red indicates increased expression.

growth factor receptors (*IGF1R, ERBB2/ERBB3, FGFR*) and proliferative transcription factors (*HIF1A*, *MYC*, *AP1S1*) in MCF-7 cells, halting anaerobic glycolysis thereby inhibiting cancer cell growth and self-sustained proliferation (Figures 2A and 3C). Estrogen signalling is intimately associated with hormone-responsive breast cancer; the observed downregulation of hormone receptors (*ESR1*, *ESR2*) in ER+ MCF-7 cells is likely to mitigate oncogenic signalling between ER and growth factor receptors, consequently modulating drug resistance.[62] In addition, drug metabolising enzymes (*CYP1A1, CYP1A2, CYP2B6, CYP2C9*—minor contribution to TAM metabolism, *CYP3A5 and CYP2D6*—main metabolites[63]) were unaffected by the change in *CETP* expression in MCF-7 cells, whereas they were slightly increased in MDA-MB-231 cells (*CYP1A1, CYP1A2, CYP2B6, CYP2C9* and *CYP2D6*; Figure SI5B). As mentioned previously, TNBC cells lack hormone receptors, and thus endocrine therapies are ineffective in causing apoptosis in MDA-MB-231 cells. However, upon *CETP* knock-down, we observed an increase in *ER* in MDA-MB-231 cells (Figures 3D and 4D and Figure SI5), which in conjunction with an increase in *CYP* genes could explain the observed improvement of TAM efficacy in TNBC cells (Figure SI4).

The most pronounced gene expression changes were observed in cancer drug resistance-associated genes namely *ABCC5*, *ABCB1, BCL2, BAX* and *TP53* (Figure 3B). These genes are, however, slightly increased in MDA-MB-231 cells after *CETP* knock-down (Figure 3D). Multi-drug resistance (MDR) is a key barrier that prevents the effectiveness and efficiency of chemotherapies. The decreased expression of *ABCC5* and

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ABCB1 could possibly suggest a decline in extracellular transport of drugs and therefore providing additional insight into the increased cell death when treated with TAM and AP (Figures SI3 and SI4). However, in *CETP* knocked-down MDA-MB-231 cells, there was a slight increase in *ABCC5* and *ABCB1*, and an upregulation of *ABCC2* and *ABCC3* (Figure 3D and Table SI1). Hence, TAM and AP treatments were less effective in MDA-MB-231 cells compared with MCF-7 cells (Figures SI3 and SI4). Evasion of apoptosis is a key hallmark of drug resistance. BCL2 (anti-apoptotic) is often upregulated in resistant TNBC cells, to counteract the activity of BAX (pro-apoptotic, in response to cell stress or DNA damage).[64] For this reason, *BCL2* inhibitors or *BAX* overexpression/mimics have become a popular topic in research as a possible therapeutic strategy in treating cancer.[65–67] *CETP* knock-down lowered the expression of *BCL2* and *BAX* remained unchanged, mitigating MCF-7 resistance and thus correlating to the increase in growth inhibition and apoptosis that was observed post chemotherapeutic treatments (Figure 3C and Figure SI3).

In summary, the distinct mechanisms operative in MCF-7 and MDA-MB-231 cells shed light on disparate cholesterol homeostasis pathways inherent to these cell lines. Within MCF-7 cells, the depletion of CETP leads to a reduction in cancer resistance, manifested by decreased cholesterol accumulation as depicted in Figure 1 and Figures SI3 and SI4. This effect is compounded by diminished expression of hormone receptors and drug efflux pumps (Figure 4C). Conversely, the influence of CETP on cholesterol-mediated processes within MDA-MB-231 cells appears limited. Interestingly, *CETP* knock-down within MDA-MB-231 cells prompts a noteworthy increase in gene expressions governing cholesterol efflux (Figure 4B) and in the upregulation of *ER* (Figure 4D), possibly rendering these cells more receptive to therapeutic interventions, which was observed in both single treatments (Figures SI4) and enhanced significantly when used in combination with a cholesterol-depleting agent; AP (Figure 2C,D).

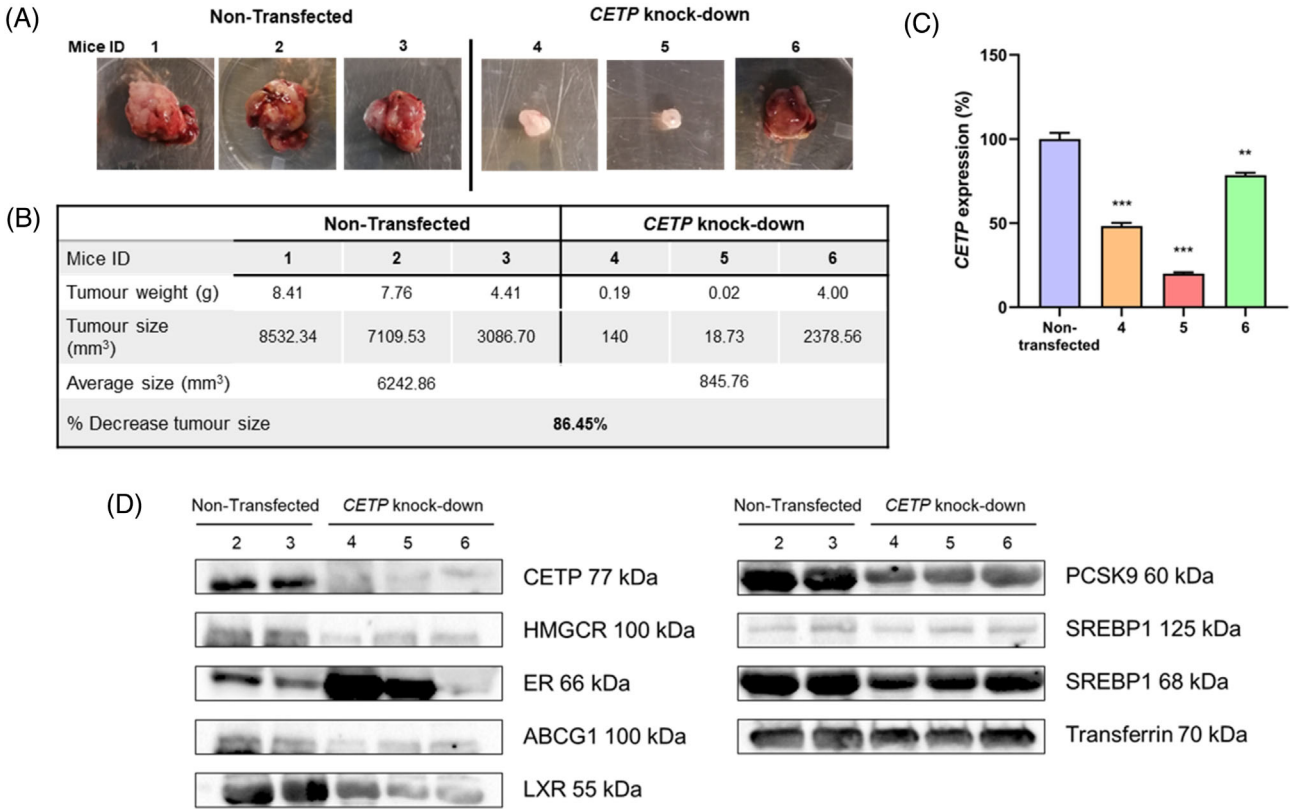
3.4 | *CETP* knock-down reduces tumour growth in vivo

After establishing the effect of CETP in vitro, a pilot xenograft study was performed to confirm the role of *CETP* in cancer cell survival. Mice injected with *CETP* knocked-down MDA-MB-231 cells had substantially reduced tumour sizes compared with non-transfected cells (an average of 845.76 mm^3 as opposed to 6242.68 mm^3, respectively) (Figure 5A,B). Overall, there was a notable 86.45% decrease in tumour growth rate. Reduced tumour growth was corroborated with decreased *CETP* expression in tumours (Figure 5C) and protein expression in serum (Figure 5D). The large tumour size of mice 6 (2378.56 mm^3) is presumably due to poor transfection supported by the higher *CETP* mRNA expression as compared with mice 4 and 5 (Figure 5A–C). Due to the small tumour size, we explored the effect of *CETP* knock-down on the cholesterol pathway in mice serum. Western blotting confirmed an overall decrease in cholesterol-related protein expression: HMGCR, ABCG1, liver X receptor (LXR), PCSK9, 125 kDa SREBP and 68 kDa SREBP. It has been found that PCSK9 expression is directly proportional to SREBP,[68] and *CETP* inhibitors have been found to downregulate PCSK9 protein and gene expression levels through a decreased SREBP levels.[69] Therefore, coinciding with the in vivo study on SREBP and PCSK9 serum protein expression (Figure 6D). In contrast, *CETP* knock-down increased ER expression levels in mice serum. This corroborated with the in vitro results, where a knock-down of *CETP* increased *ER* expression in MDA-MB-231 cells. It was interesting to note that in mice 6 that had higher levels of *CETP* compared with the other two mice with lower levels of *CETP* (Figure 5C) exhibited protein expression profiles that closely resembled that of the non-transfected phenotype (Figure 5D).

4 | DISCUSSION

Breast cancer, especially ER+ breast cancer cells, display elevated levels of cholesterol compared with the non-cancerous cells, as it is an essential component in hormone synthesis for cell proliferation.[70] Hence, cell proliferation is escalated in cancer cells. In line with Esau et al.,[30] *CETP* knock-down curbed cancer cell proliferation, as well as increased cell death in both ER+ breast cancer and TNBC cells treated with TAM. Cancer cell death was further enhanced when TAM and AP were used in combination, thus, confirming the role of *CETP* as a cell survival gene and its association with breast cancer resistance. Furthermore, the absence of *CETP* resulted in a decrease in intracellular CE levels, lipid rafts, as well as cholesterol droplets in both cell lines. Therefore, proving that depleting intracellular cholesterol levels reduces breast cancer resistance and as a result increases TAM efficacy. This result is especially significant for TNBC cells as these cells lack ER, which is necessary for the binding of TAM to impede growth promoting signals. Several studies have shown that there is a small subpopulation of TNBC patients that do respond to TAM through estrogen receptor alpha (ERα) independent mechanisms (i.e., estroge-related receptor

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	Non-Transfected			CETP knock-down		
Mice ID	1	2	3	4	5	6
Tumour weight (g)	8.41	7.76	4.41	0.19	0.02	4.00
Tumour size (mm^3)	8532.34	7109.53	3086.70	140	18.73	2378.56
Average size (mm^3)		6242.86			845.76	
% Decrease tumour size			**86.45%**			

FIGURE 5 Cholesteryl ester transfer protein (CETP) knock-down reduces tumour size through perturbation of cholesterol pathway. (A) Visual representation of tumour size in non-transfected vs CETP knock-down xenografts 18 days post-tumour identification. (B) Comparison of tumour weight and size in non-transfected versus CETP knock-down xenografts. (C) Reverse transcription - quantitative polymerase chain reaction (RT-qPCR) results showing CETP expression level in CETP knock-down xenografts relative to non-transfected tumours. CETP expression was normalised against actin beta (ACTB). (D) The effect of CETP knock-down on cholesterol-related proteins in mice serum of non-transfected and CETP knock-down mice. Transferrin was utilised as a loading control. The serum from mouse 1 could not be collected as the blood coagulated in the body during dissection. A one-way analysis of variance (ANOVA) test was performed for statistical analysis, followed by a Bonferroni post hoc test comparing the average of non-transfected groups to the CETP knock-down groups. Data represents mean ± standard deviation (SD) of raw data ($n = 3$), where $^{**}p < .01$ and $^{***}p < .001$ indicates significant difference.

alpha (ERRα) and estrogen receptor beta (ERβ)).[71–75] Furthermore, a study by Payandeh et al. showed that tamoxifen with radiotherapy did increase overall survival in TNBC patients.[76] In addition, ERβ have been observed to regulate cholesterol synthesis in TNBC patients.[77,78] Therefore, targeting cholesterol appears to be a promising strategy in reducing drug resistance in both ER+ and TNBC cells. Especially in TNBC as these cells are resistant to TAM and this reduction in resistance post CETP knock-down could possibly be due to the affiliation with ERβ; however, more research would be required to confirm these speculations.

Considering the effects of cholesterol lowering in reducing cancer survival and resistance, there have been increased interest in the use of statins as a possible anti-cancer therapy.[53] However, the outcomes of statin treatment have been contradictory and therefore warrants further attention.[79,80] In addition, 25% individuals do not respond to statins,[81] thus highlighting a population group where statins will not be effective as anti-cancer therapy. On the contrary to blocking the cholesterol synthesis pathway, our study focused more on depleting excess cholesterol, as it still plays a vital role in maintaining cell membrane for stability and shape, in addition to hormone synthesis in non-cancerous cells.[82] Furthermore, we looked at the molecular mechanisms underlying CETP's role in breast cancer.

CETP is an important protein in regulating cholesterol homeostasis both intracellularly and extracellularly.[27,30] Interestingly, *CETP* knock-down resulted in an overall decrease in a number of genes involved in various cholesterol-related pathways: cholesterol synthesis, transport, metabolism and catabolism in MCF-7 cells all leading to a decrease in cholesterol accumulation, whereas little to no change was observed in MDA-MB-231 cells (Figure 6). This is in alignment with various treatment

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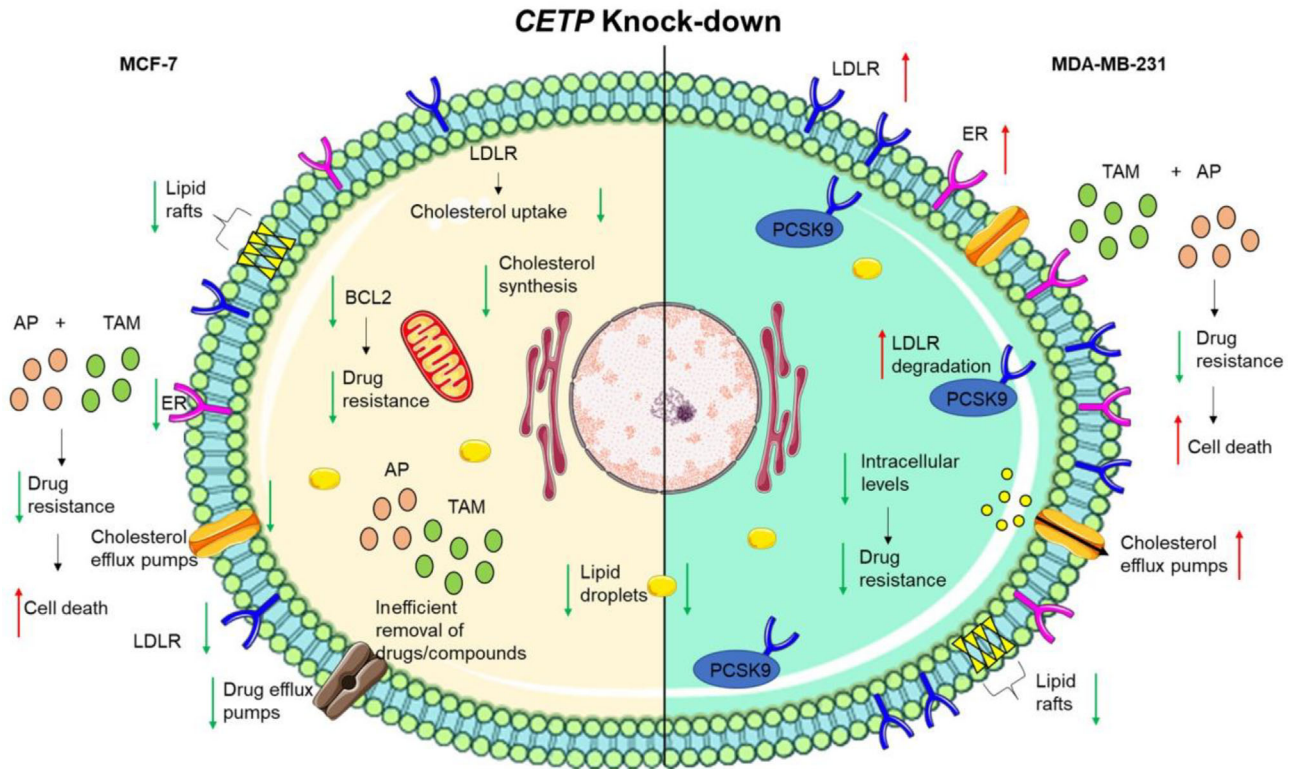


FIGURE 6 Elucidation of molecular mechanisms involved in cholesteryl ester transfer protein (CETP)-mediated drug resistance in estrogen receptor-positive (ER+) and triple-negative breast cancer (TNBC) cells. The figure demonstrates the predicted mechanisms that are involved post-CETP knock-down in both MCF-7 and MDA-MB-231 cells. Green arrow represents a decreased expression, whereas red arrow represents an increased expression or activity. The figure shows that CETP knock-down affected different pathways in hormone receptor-positive breast cancer subtype and TNBC subtype. In MCF-7 cells, cholesterol synthesis and drug efflux pathways are reduced, thus decreasing resistance. In MDA-MB-231 cells, hormone receptors are increased, and cholesterol efflux pumps are possibly increased to reduce resistance.

outcomes based on the type of breast cancer and therefore understanding the underlying molecular mechanisms provides important clinical relevance. MDA-MB-231 cells are inherently more resistant and there are currently no targeted therapies. However, excess cholesterol has been evident in various cancer malignancies including but not limited to: liver, gallbladder, colon, kidney and pancreatic cancer.[83–85] Furthermore, our other work has shown that a depletion in cholesterol levels has yielded in promising results in reducing breast cancer tumours in a mice xenograft study,[21] enhanced drug efficacy,[35] reduced breast cancer metastasis (patent: PCT/IN2020/050651) and reduced colorectal cancer tumours in mice (Pillay et al., unpublished data). This suggest that reducing surplus cholesterol could be advantageous in the management of various cancer types. In addition, measuring cholesterol levels in cancer patients could possibly provide useful insights into chemotherapeutic efficacy and outcomes.

We further looked at how *CETP* knock-down affected 84 genes involved in cancer resistance pathways as 40% of patients experience acquired drug resistance with initial positive response to drug treatments. In addition, *CETP* is a key gene that can affect/predict patient survival outcome.[30] It was observed that *CETP* knock-down resulted in an overall decrease in gene expressions involved in breast cancer resistance in MCF-7 cells while not affecting as many in MDA-MB-231 cells. Notably, there was a downregulation in *ER* and *IGF1R* upon *CETP* knock-down in MCF-7 cells, thus correlating to the decrease in cell proliferation. However, this is interesting as ER is an essential target for TAM. Furthermore, it was found that altered expression of ER and coactivators/corepressors leads to acquired TAM resistance.[86] Perhaps, the decreased cholesterol levels in MCF-7 cells suggest that small amount of the drug is sufficient to have a cytotoxic effect. In the case of TNBC cells, lacking ER expression, exhibit de novo TAM resistance. However, upon *CETP* knock-down, the ER expression was slightly increased in MDA-MB-231 cells and thus expressing ER, in addition to a reduction in cholesterol, resulted in an increased efficacy of TAM-mediated apoptosis in MDA-

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MB-231 cells. Another key determinant in cancer resistance is the increased drug export proteins such as the ABC proteins,[87,88] which were downregulated upon *CETP* knock-down in MCF-7 cells. On the contrary, this was not observed in MDA-MB-231 cells, where upon *CETP* knock-down increased expression of drug efflux pumps and thus suggest that TNBC cells have a more active defence mechanism and thus contributing to the resistant nature of these cells. Therefore, the resistance of these cells is possibly compromised through alternative pathways post-*CETP* knock-down, currently unknown. Drug metabolism or detoxification is another decisive factor for poor clinical outcome that works closely with drug efflux pumps. This means that with the increased drug efflux, there would be a comparable amount of drug elimination through the liver.[89]

Intracellular cholesterol levels were reduced upon *CETP* knock-down. However, in MCF-7 cells, the loss of cholesterol is mainly due to the decrease in genes involved in the signalling of cholesterol synthesis and uptake by LDLR. Although in MDA-MB-231 cells, it is due to an increase in the transcription of cholesterol efflux pumps that transports the cholesterol out of the cells and an increase in LDLR degradation in response to the increased LDLR levels. In addition, resistance was reduced in MCF-7 cells, upon CETP knock-down, by reducing drug efflux pumps and pro-survival gene, *BCL2*. In MDA-MB-231 cells, CETP knock-down increased ER transcription and possibly ER thus sensitising TNBC cells to TAM/ endocrine therapy. Furthermore, *CETP* knock-down resulted in a decrease in lipid rafts and lipid droplets in both MCF-7 and MDA-MB-231 cells. Therefore, the treatment of these cells with AP further decreases intracellular cholesterol levels thus sensitising the cells to TAM. The mice xenograft study supported the findings from,[30] where CETP was proven to be a cell survival gene and protein by regulating levels of cholesterol. Despite the benefits of breast cancer tumour size reduction in mice from *CETP* knock-down, the strategy cannot be employed as a chemotherapeutic strategy. Despite controversial results obtained in Esau et al.,[30] where the inhibition of CETP through the use of torcetrapib only reduced growth but did not cause apoptosis, other previous pre-clinical studies with torcetrapib resulted in severe cytotoxicity and increased mortality.[90,91] Hence, the unpredictable treatment outcomes resulting in varying effects of torcetrapib rendered it unsafe and ultimately leading to its discontinuation. This is possibly due to CETPs dual function in maintaining cholesterol homeostasis both intracellularly and extracellularly in normal and diseased cells.[30,31] Therefore, the untargeted complete inhibition of *CETP* would result in expected undesired treatment outcomes. However, a potential targeted strategy of using various nanoparticles,[92] lipid-based nanoparticles,[93] mesoporous silica nanoparticles,[94] polymeric nanoparticles[95] and magnetic nanoparticles[96] to encapsulate drugs and siRNAs, such as *CETP* siRNA in the aid to targeted reduction of CETP could be an interesting avenue to be explored in the future. The use of *CETP* inhibition as a plausible chemotherapeutic strategy would have to be thoroughly researched and validated. However, in this study, we showed that *CETP* could be a promising drug resistant marker, where a reduced CETP level in breast cancer patients is possibly linked to better treatment and survival outcomes; however, this need to be validated in breast cancer patient samples. Moreover, further protein expression profiles in the tumour samples would reveal more insights into the mechanisms affected post-*CETP* knock-down due to the small tumour size from the transfected mice, which was a limitation in this study and prevented a comprehensive comparison in protein levels in vivo.

5 | CONCLUSION

We demonstrated that *CETP* knock-down with the combination treatment of TAM and AP results in reduced intracellular cholesterol levels in both ER+ and TNBC cells and triggers molecular changes at both gene and protein expression levels, therefore, appears to be a promising strategy in reducing drug resistance. Moreover, *CETP* can be anticipated as a useful predictable marker for breast cancer resistance and thus patient survival outcome. In addition, the shift in expression profiles in the lipoprotein signalling and cholesterol metabolism pathways, as well as breast cancer resistance pathways post-*CETP* knock-down in MCF-7 cells provides novel insights into the mechanisms involved in reducing ER+ breast cancer resistance. However, a clear understanding of resistance mechanisms in TNBC cells is still lacking despite promising results and preliminary in vivo validation, where *CETP* knock-down not only affected tumour growth but also affected serum protein expression which could possibly affect normal RCT functions in cholesterol removal in mice. Therefore, this warrants further attention for a better understanding of the underlying molecular mechanisms involved. Furthermore, the effect of tumour microenvironment must also be investigated in the future studies. In addition, an overexpression study could strengthen and confirm the concept of using *CETP* as a drug resistance marker in both ER+ and TNBC subtypes.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Liang Gu https://orcid.org/0000-0002-4766-0650
Ruvesh Pascal Pillay https://orcid.org/0000-0002-5050-0754
Ruth Aronson https://orcid.org/0000-0002-3030-9573
Mandeep Kaur https://orcid.org/0000-0002-9757-4048

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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