

## ORIGINAL ARTICLE

# Comparative Study On Effects Of Lipoproteins On Growth And Migration Characteristics Of Tamoxifen Resistant Breast Cancer Cells

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## ABSTRACT

**Introduction:** Resistance towards treatment is one of the challenges in breast cancer therapy. Recent studies show the link between lipoprotein with cancer resistance and progression. Clinical data indicates that oxidized low-density lipoprotein (oxLDL) and very low-density lipoprotein (VLDL) play roles the progression of breast cancer. Therefore, purpose of this study was to determine the roles of lipoproteins on migration of breast cancer cell and compare the effects of oxLDL and VLDL. **Methods:** Parent MCF-7 cells were purchased from ATCC, while the Tamoxifen-resistant MCF-7 (Tam-R MCF-7) was developed by pulse treatment method. Tam-R cells were treated with gradual increase in tamoxifen concentration for 72 hours in Dulbecco's Modified Eagle's medium (DMEM) without phenol red. Cell viability test was done to measure the fold changes of Tam-R MCF-7 cells. Migration characteristics was studied using wound healing assay. Cells were treated with 10 µg/mL of oxLDL and VLDL up to 72 hours. **Results:** From the cell viability test, Tam-R MCF-7 cells had 4-fold increase of resistance than parental cells. Tam-R MCF-7 had acquired resistance to Tamoxifen and achieved a clinically relevant level of resistance. Lipoproteins were found to cause morphological changes, where cells exhibited elongation and dendritic-like growth compared to control cells. Both MCF-7 parental cells and Tam-R MCF-7 cells showed higher percentage of wound closure when treated with oxLDL. In contrast, VLDL treatment caused reduction in cell migration compared to oxLDL. **Conclusion:** Findings suggest that oxLDL may further promote resistant breast cancer cell migration compared to VLDL.

**Keywords:** Oxidized-low density lipoprotein, Very-low density lipoprotein, Tamoxifen-resistant breast cancer, Cell migration

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## INTRODUCTION

Chemotherapy resistance remains as one of the challenges in cancer treatment. About 30% early-stage breast cancer patients were found to have recurrent disease even with advances in early detection. In general, systemic treatment of breast cancer involves use of cytotoxic agents, hormonal therapies and immunotherapies. They are used in the adjuvant, neoadjuvant and metastatic settings. Systematic agents are effective at the beginning of therapy in 90% of primary breast cancers and only in 50% of patients with metastasis. After a certain period, relapse occurs and resistance towards therapy is expected (1). In patients with estrogen-receptor positive breast cancer, there is a sustained risk of disease recurrence and death after 5 years of tamoxifen therapy (2). Breast cancer is a heterogenous disease due to its morphologic appearances

and molecular genetic characteristics. Development of metastases represents the most important prognostic indicator among patients (3). Breast cancer is classified into distinct subsets which comprise of hormone receptor positive, human epidermal growth receptor 2 positive (HER2-positive), normal-like and basal-like (4). Endocrine therapy is the standard therapy for estrogen receptor positive metastatic breast cancer. However, its effectiveness is hindered by de novo resistance and resistance acquired during treatment. Reports claim that 30% of patients with metastatic disease show regression of tumour, while 20% patients had stable disease. This suggest there could be other survival pathways besides estrogen receptor for survival of tumours. Cancer cells may have other alternative pathways while being treated with estrogen receptor targeted therapy (5). Apart from estrogen receptor positive breast cancer, clinical studies on triple negative cohorts showed an inferior prognosis compared to other subtypes (6).

Cancer therapy failure occurs when drug-resistant cancer cells survive even after treatment with first-line standard therapy. This could initiate metastasis and recurrence.

Therefore, understanding of molecular mechanisms of drug-resistant and parental cells could provide insights on its molecular basis and possible methods to eradicate the resistant cells (7). Cancer cells proliferate rapidly compared healthy cells. This biological process demands for higher energy consumption and metabolism rate in cancer cells. Lipids are one of the sources of energy for cancer cell growth. Lipids are categorised into simple and complex lipids. Simple lipids are cholesterol and triglycerides. They are mainly transported in the blood circulation in oxLDL and VLDL, respectively.

So far, most of the in vitro studies are mainly focusing on low density lipoprotein, where breast cancer cells exposed to LDL may survive and become more aggressive breast cancer phenotype (8). There are emerging report on role of VLDL, where it VLDL promotes the mammo-sphere formation and resistance to radiation in inflammatory breast cancer (9). Clinical studies have shown increase in LDL and oxLDL among patients with cancer. Cancer cells survival would require continuous supply of energy either through glucose or lipid metabolism. A study found increased level of circulating cholesterol would promote the risk of aggressive prostate cancer (10). However, there is limited information with regards to roles of lipoproteins in breast cancer and resistance. Therefore, this study aims to assess roles of lipoprotein on parent and tamoxifen resistant (TamR) breast cancer cells on cell migration characteristics. This would provide knowledge on the effects of lipoproteins on breast cancer cells.

## MATERIALS AND METHODS

### Cancer cell culture

The human breast adenocarcinoma cell line MCF-7 was from American Type Culture Collection (ATCC, USA). It was grown in Dulbecco's Modified Eagle Medium (DMEM) without phenol red, provided with 10 % heat-inactivated fetal bovine serum, 1 % L-glutamine and 1 % antibiotics (10,000 U/ml of penicilin, 10,000 µg/ml of streptomycin). Tamoxifen-resistant (Tam-R) cells were derived from the MCF-7 parental cell line grown in gradually increased concentrations from 0.1 µM to 5.0 µM of tamoxifen over six months. Cells were exposed to Tamoxifen for 3 days, then culturing was continued with complete culture medium without tamoxifen and cells were left to recover before starting the next cycle of treatment. Cultures were incubated in 5 % CO<sub>2</sub> incubator at a temperature of 37 °C.

### Cell viability test

Cell viability assay was done by using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) following manufacturer's protocol. MCF-7 parental cells and Tam-R cells were seeded in a 96-well flat-bottomed plate. Each well contained 3 x 10<sup>3</sup> cells in 100 µL fresh culture medium. Cells were then left overnight to adhere to plate prior

treatment with varying concentration of tamoxifen (10 - 100 µM) for 3 days. After the treatment, medium was removed. Next fresh culture medium (100 µl) was mixed with 20 µl of CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) in each well. This was followed by incubation at 37 °C for 1.5 hours with 5% CO<sub>2</sub> atmosphere. Measurements were taken at 490 nm with a ELISA micro plate reader. The fold resistance of Tam-R MCF-7 was calculated as follows: Fold resistance = IC<sub>50</sub> of resistant cell line / IC<sub>50</sub> of parental cell line.

### Morphology of cells treated with lipoprotein

Phase contrast microscope images were taken of parent cells and resistant cells. Morphological changes were later captured after treatment with lipoproteins in both cells studied.

### Wound healing assay

Wound healing assay was performed to compare the effects of lipoproteins on both cells studied. Percentage of wound closure depicts the migration characteristics of the cells studied. MCF-7 parental cells and Tam-R MCF-7 were cultured in DMEM medium without phenol red as indicator. After trypsinisation, the total number of cells was determined using haemocytometer. Both parent and Tam-R MCF-7 cells were seeded in a 24-well plate. Each well contained 4 x 10<sup>5</sup> cells and the plate was left in a humidified CO<sub>2</sub> incubator for overnight. On the next day, culture medium was discarded then treated with colcemide at concentration of 0.1 mg/ml for 2 hours. After the treatment, two-hundred-microliter tip was used to create a denuded area ("wound") in middle of the well. This was followed by rinsing each well with PBS and treatment with 10 µg/ml of oxLDL and VLDL separately. Phase contrast images were captured at 0 h, 24 h, 48 h and 72 h. Cell migration distance was measured by subtraction of the values obtained at 0 h from 72 h. This done with 4 measurements per well. Distance of migration are expressed as percentage of the wound closure and analysis was done using ImageJ software.

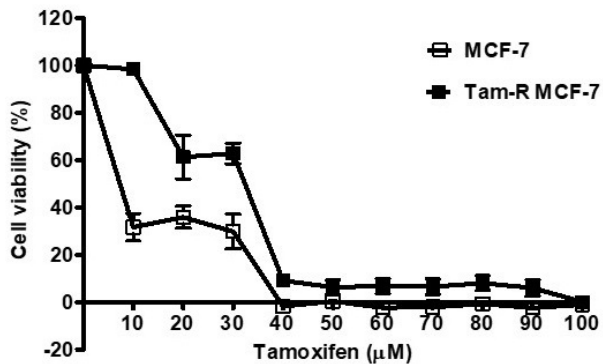
### Statistical analysis

Data obtained was analysed using SPSS software. Experiments were done in triplicates with the results are presented as means ± SEM. Differences are considered significant when p < 0.05.

## RESULTS

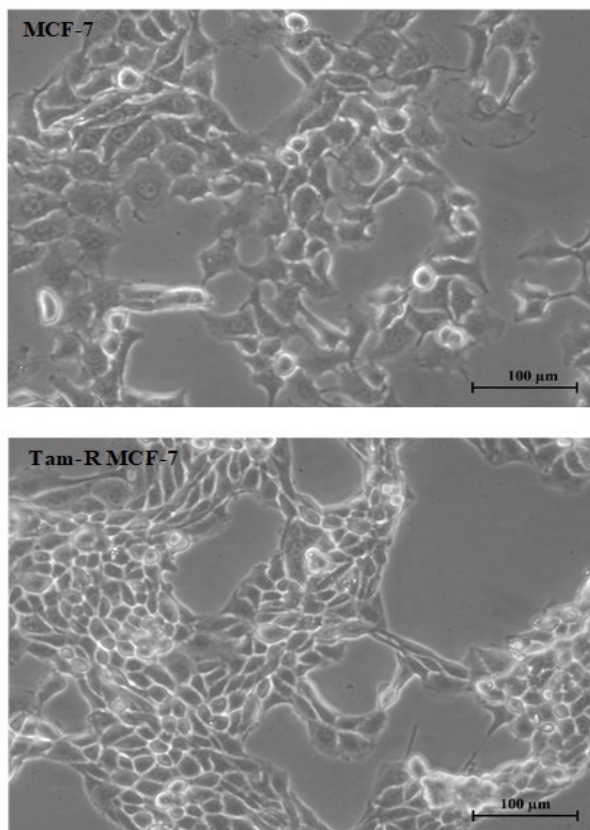
### MCF-7 cells acquired resistance to tamoxifen after pulse-stimulated treatment

MCF-7 was treated with a gradual increase in concentration of tamoxifen using pulse treatment method. After six months, the IC<sub>50</sub> value of tamoxifen-treated cells and IC<sub>50</sub> of MCF-7 parental cells were measured. Figure 1 shows that viability of Tam-R cells was higher compared to MCF-7 parental cells at all



**Figure 1: Cell viability assay shows the percentage of viable cells in parent and resistant cell lines.**

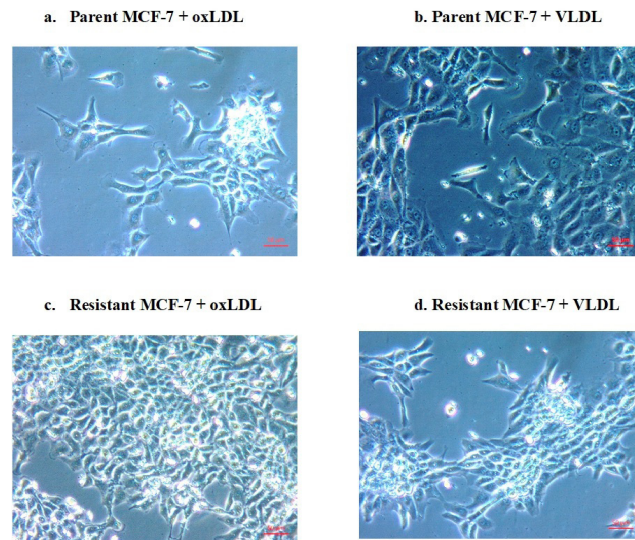
concentrations. Tam-R MCF-7 cells indicated IC50 value of 33 µM whereas MCF-7 parental cells had IC50 value of 8 µM. Hence, using the formula described above, the fold-resistance of Tam-R MCF-7 was 4-fold and confirmed that the cells had acquired a clinically relevant level of resistance for in vitro studies. Besides enhanced cell viability in Tam-R MCF-7, the cells also showed distinct changes in terms of their morphology. They indicated characteristics of epithelial to mesenchymal transition, with elongated cytoplasm and irregularity in shape. In contrast, MCF-7 parental cells showed regular epithelial-like morphology as depicted in Figure 2.



**Figure 2: Phase contrast images of parent and Tam-R MCF-7 cells**

### Morphology of cells treated with lipoproteins

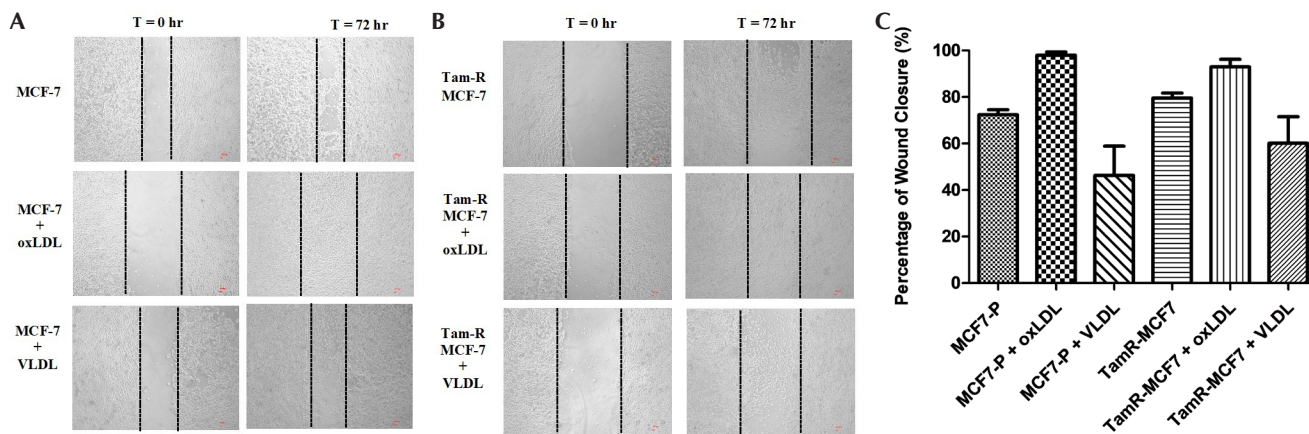
Morphology of MCF-7 parental cells and Tam-R cells were monitored following exposure to 10 µg/ml of oxLDL and VLDL separately. Generally, both cells showed changes in their morphology after the treatment as shown in Figure 3. Both parent and resistant cell shapes were elongated and exhibited more dendritic-like morphology in presence of lipoproteins.



**Figure 3: Morphological changes in parent and Tam-R MCF-7 cells with lipoprotein treatment.**

Comparison of wound closure with lipoprotein treatment Wound healing assay was performed to determine whether oxLDL and VLDL could induce the migration of breast cancer cells. Results showed that the oxLDL enhanced both MCF-7 parental and Tam-R MCF-7 cell migration, thus promoting in vitro wound closure. On the other hand, percentage of wound closure in both cells was lower after treatment with VLDL (Figure 4A and 4B). This suggests that VLDL had properties to reduce the migration of both the MCF-7 parental cells and Tam-R MCF-7 cells.

Mixed factorial two-way ANOVA was done to compare the effects of different lipoproteins on parent MCF-7 and Tam-R MCF-7 cells. Data obtained showed that lipoproteins had significant effect on wound closure ( $p=0.016$ ). In both parent and Tam-R MCF-7 cells, treatment with oxLDL increased the percentage of wound closure compared to control cells. In contrast, VLDL caused reduction in percentage of wound closure in both type of cells (Fig 4C). Group effect showed there was no significant difference between parent and Tam-R MCF-7 cells in wound closure ( $p=0.5$ ). Further analysis was done within each cell type using independent sample t-test. For parent MCF-7 cells, comparison with control showed significant difference in wound closure after oxLDL treatment ( $p$ -value = 0.003). However, VLDL did not cause significant difference in wound closure ( $p$ -value = 0.28). Similar finding was noted in resistant MCF-7 cells, where oxLDL caused significant



**Figure 4: Difference in wound closure (A) phase contrast image of wound healing assay of parent MCF-7 cells (B) phase contrast image of wound healing assay of Tam-R MCF-7 cells (C) Percentage of wound closure**

changes in percentage of wound closure (p-value = 0.026) compared to VLDL (p-value = 0.228).

## DISCUSSION

Resistance to chemotherapy remains as one of the challenges in treatment of cancer. Emerging reports link possible roles of lipoproteins with cancer resistance and progression. There are different types of lipoprotein with oxLDL and VLDL as major carriers of cholesterol and triglycerides in the blood circulation, respectively. They provide the energy source for cancer cell metabolism. However, there is limited information with regards to effects of lipoproteins particularly in breast cancer with endocrine therapy resistance. Tamoxifen is standard endocrine therapy which is used in treatment of estrogen receptor positive breast cancer. It is given along with aromatase inhibitor i.e. anastrozole as adjuvant therapy in post-menopausal women. Even with advancement in therapies, drug resistance is a hindrance in delivering effective cancer treatment. Estrogen receptor positive (ER+) cells for example, can evade anti-estrogen treatment through up-regulation of other signalling pathways related to cell proliferation and survival. Activation of Akt signalling was significantly correlated with a poor outcome in metastatic disease (11). Additionally, upregulation of signaling via EGF receptor (EGFR) (12) and HER receptor 2 (HER2) (13) were noticed with acquired resistance due to endocrine therapy.

To date, findings from cell culture studies of tamoxifen resistance have contributed informative data that can be further evaluated through in vivo and clinical study (14). According to previous reports, Tam-R breast cancer cell lines could be developed with long term exposure of MCF-7 cells to 10<sup>-6</sup> to 10<sup>-7</sup>M of tamoxifen. The durations ranged from over a period of six to twelve months (15) or more (16). In present study, tamoxifen-resistant MCF-7 cells were developed by gradual treatment with 5 μM tamoxifen for six months using pulse treatment method. This concentration was chosen

in order to simulate the pharmacological dosage that is prescribed to patients (17). The tamoxifen-resistant cells generated in this study had achieved a 4-fold increase in resistance, based on the cytotoxicity test. The level of resistance is clinically relevant when it is between two- to five-fold compared to the IC<sub>50</sub> value of parent cells (18). Thus, the resistant cells are relevant as a model to mimic clinical conditions. In this study, phenol-red free DMEM medium was used. This was to ensure that the background estrogen level would not induce adaptive changes due to estrogen deprivation and also to minimize the agonistic action of tamoxifen in ER(+) breast cancer cells.

Previously, LDL was shown to promote cell proliferation and migration of ER(-) breast cancer cell lines (19). Similarly, findings from present study on TamR MCF-7 cells indicated that oxLDL induced proliferative and migratory effects. The percentage of wound closure in MCF-7 parental cells was higher by 25% with oxLDL treatment. While in Tam-R MCF-7, it was 13% after treatment with the lipoprotein (Fig. 4). This finding was in parallel with clinical findings in which serum oxLDL level was higher among stage II and III breast cancer patients (20). This provides evidence on roles of oxLDL may play a role in metastasis. Additionally, oxLDL is stated as a potent independent mitogenic factor that causes either cell proliferation or cell death (21). It also may be involved in the development and progression of cancer through generation of cytokines and growth factors (22). Furthermore, LDL is prone to oxidation and this may result in high lipid peroxidation and initiate carcinogenesis.

On the other hand, VLDL is the major carrier of triglycerides compared to LDL. It was found to promote malignancy and enhanced cancer cell migration through PIK3/Akt/Slug pathway. In vivo model showed that VLDL induced metastasis to the lung in breast cancer (23). However, in this study, VLDL was found to lower cell migration. The percentage of wound closure in both MCF-7 parental cells and Tam-R cells decreased in

presence of VLDL.

## CONCLUSION

Lipoproteins may play a significant role in breast cancer cell growth. Findings obtained from this study showed that both lipoproteins had different effects on breast cancer cells. Both caused morphological changes in cancer cells. Interestingly, oxLDL was found to significantly promote cancer cell migration of parent and resistant breast cancer cells. This showed the possible role of oxLDL in inducing migration capability particularly in resistant cells. Further investigation is needed to identify lipid related target for managing recurrence of breast cancer in particular.

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## REFERENCES

1. Gonzalez-Angulo AM, Morales-Vasquez F, Hortobagyi GN. Overview of resistance to systemic therapy in patients with breast cancer. *Breast Cancer Chemosensitivity*: Springer; 2007. p. 1-22.
2. Group EBCTC. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *The Lancet*. 2005;365(9472):1687-717.
3. Luck AA, Evans AJ, Green AR, Rakha EA, Paish C, Ellis IO. The Influence of Basal Phenotype on the Metastatic Pattern of Breast Cancer. *Clinical Oncology*. 2008;20(1):40-5.
4. Surlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proceedings of the National Academy of Sciences*. 2003;100(14):8418-23.
5. Osborne CK, Schiff R. Mechanisms of Endocrine Resistance in Breast Cancer. *Annual Review of Medicine*. 2011;62(1):233-47.
6. Dent R, Hanna WM, Trudeau M, Rawlinson E, Sun P, Narod SA. Pattern of metastatic spread in triple-negative breast cancer. *Breast cancer research and treatment*. 2009;115(2):423-8.
7. Jeon JH, Kim DK, Shin Y, Kim HY, Song B, Lee EY, et al. Migration and invasion of drug-resistant lung adenocarcinoma cells are dependent on mitochondrial activity. *Experimental & molecular medicine*. 2016;48(12):e277-e.
8. Rodrigues dos Santos C, Domingues G, Matias I, Matos J, Fonseca I, de Almeida JM, et al. LDL-cholesterol signaling induces breast cancer proliferation and invasion. *Lipids in Health and Disease*. 2014;13(1):16.
9. Wolfe AR, Atkinson RL, Reddy JP, Debeb BG, Larson R, Li L, et al. High-density and very-low-density lipoprotein have opposing roles in regulating tumor-initiating cells and sensitivity to radiation in inflammatory breast cancer. *International Journal of Radiation Oncology\* Biology\* Physics*. 2015;91(5):1072-80.
10. Pelton K, Freeman MR, Solomon KR. Cholesterol and prostate cancer. *Current Opinion in Pharmacology*. 2012;12(6):751-9.
11. Tokunaga E, Kimura Y, Mashino K, Oki E, Kataoka A, Ohno S, et al. Activation of PI3K/Akt signaling and hormone resistance in breast cancer. *Breast cancer*. 2006;13(2):137-44.
12. Hutcheson IR, Knowlden JM, Madden T-A, Barrow D, Gee JM, Wakeling AE, et al. Oestrogen receptor-mediated modulation of the EGFR/MAPK pathway in tamoxifen-resistant MCF-7 cells. *Breast cancer research and treatment*. 2003;81(1):81-93.
13. Kurokawa H, Lenferink AE, Simpson JF, Pisacane PI, Sliwkowski MX, Forbes JT, et al. Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. *Cancer research*. 2000;60(20):5887-94.
14. Fan P, Yue W, Wang JP, Aiyar S, Li Y, Kim TH, et al. Mechanisms of resistance to structurally diverse antiestrogens differ under premenopausal and postmenopausal conditions: evidence from in vitro breast cancer cell models. *Endocrinology*. 2009;150(5):2036-45.
15. Loh YN, Hedditch EL, Baker LA, Jary E, Ward RL, Ford CE. The Wnt signalling pathway is upregulated in an in vitro model of acquired tamoxifen resistant breast cancer. *BMC cancer*. 2013;13(1):174.
16. Tegze B, Sz6116si Z, Haltrich I, P19nznv6116lty Z, Tyth Z, Lily I, et al. Parallel evolution under chemotherapy pressure in 29 breast cancer cell lines results in dissimilar mechanisms of resistance. *PLoS One*. 2012;7(2):e30804.
17. Shaw LE, Sadler AJ, Pugazhendhi D, Darbre PD. Changes in oestrogen receptor-alpha and -beta during progression to acquired resistance to tamoxifen and fulvestrant (Faslodex, ICI 182,780) in MCF7 human breast cancer cells. *The Journal of steroid biochemistry and molecular biology*. 2006;99(1):19-32.
18. McDermott M, Eustace A, Busschots S, Breen L, Clynes M, O'Donovan N, et al. In vitro development of chemotherapy and targeted therapy drug-resistant cancer cell lines: a practical guide with case studies. *Frontiers in oncology*. 2014;4:40.
19. Antalis CJ, Uchida A, Buhman KK, Siddiqui RA. Migration of MDA-MB-231 breast cancer cells depends on the availability of exogenous lipids and cholesterol esterification. *Clinical & experimental metastasis*. 2011;28(8):733-41.
20. Delimaris I, Faviou E, Antonakos G, Stathopoulou E, Zachari A, Dionyssiou-Asteriou A. Oxidized

- LDL, serum oxidizability and serum lipid levels in patients with breast or ovarian cancer. *Clinical biochemistry*. 2007;40(15):1129-34.
21. Hamburger AW. The human tumor clonogenic assay as a model system in cell biology. *International journal of cell cloning*. 1987;5(2):89-107.
  22. Shumay E, Tao J, Wang H-y, Malbon CC. Lysophosphatidic Acid Regulates Trafficking of  $\beta$ 2-Adrenergic Receptors THE  $G\alpha_{13}/p115$ RhoGEF/JNK PATHWAY STIMULATES RECEPTOR INTERNALIZATION. *Journal of Biological Chemistry*. 2007;282(29):21529-41.
  23. Lu C-W, Lo Y-H, Chen C-H, Lin C-Y, Tsai C-H, Chen P-J, et al. VLDL and LDL, but not HDL, promote breast cancer cell proliferation, metastasis and angiogenesis. *Cancer Letters*. 2017;388:130-8.