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Valentina Rossi[a], Marzia Govoni[a], Fulvia Farabegoli[b], Giuseppina Di Stefano[a#]

a) Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Via San Giacomo 14, 40126 Bologna, Italy.

b) Department of Pharmacy and Biotechnology, University of Bologna, Via San Giacomo 14, 40126 Bologna, Italy.

Corresponding Author

Prof. Giuseppina Di Stefano

Department of Experimental, Diagnostic and Specialty Medicine

University of Bologna, Via San Giacomo 14, 40126 Bologna, Italy.

Email: giuseppina.distefano@unibo.it

Abstract

Background - Tamoxifen is a widely used estrogen receptor inhibitor, whose clinical success is limited by the development of acquired resistance. This compound was also found to inhibit mitochondrial function, causing increased glycolysis and lactate production. Lactate has been widely recognized as a signaling molecule, showing the potential of modifying gene expression. These metabolic effects of tamoxifen can by hypothesized to contribute in driving drug resistance.

Methods - To test this hypothesis, we used MCF7 cells together with a tamoxifen resistant cell line (MCF7-TAM). Experiments were aimed at verifying whether enhanced lactate exposure can affect the phenotype of MCF7 cells, conferring them features mirroring those observed in the tamoxifen resistant culture.

Results - The obtained results suggested that enhanced lactate in MCF7 cells medium can increase the expression of tafazzin (TAZ) and telomerase complex (TERC, TERT) genes, reducing the cells' attitude to undergo senescence. In long term lactate-exposed cells, signs of EGFR activation, a pathway related to acquired tamoxifen resistance, was also observed.

Conclusions - The obtained results suggested lactate as a potential promoter of tamoxifen resistance. The off-target effects of this compound could play a role in hindering its therapeutic efficacy.

General Significance - The features of acquired tamoxifen resistance have been widely characterized at the molecular level; in spite of their heterogeneity, poorly responsive cells were often found to display upregulated glycolysis. Our results suggest that this metabolic asset is not simply a result of neoplastic progression, but can play an active part in driving this process.

Keywords: Breast cancer; Drug resistance; Glycolysis; Lactate; Tamoxifen.

1. Introduction

Tamoxifen (TAM) is a competitive inhibitor of the transcriptional activity of estrogen alpha-receptor (ERα); it was discovered about fifty years ago and was subsequently introduced in the clinical practice for the treatment of ER+ breast cancer, the most common form of this tumor [1,2]. During its long-lasting clinical use, this pioneering compound proved to reduce disease recurrence and mortality rate by 50 % and 30 %, respectively. Furthermore, it appeared to be devoid of relevant side effects in the majority of patients. For its efficacy and tolerability, after so many years TAM is still considered the first-choice medication in the adjuvant therapy of pre- and post-menopausal women and has also been evaluated in the chemoprevention of breast cancer.

Unfortunately, the success of this lifesaving compound can be undermined by the development of acquired resistance, which was found to occur in about 30% treated patients [3,4]. The possible mechanisms underlying this phenomenon have been extensively investigated and a number of complex pathways leading to a reduced response to TAM have been identified in resistant breast cancer cells cultured in vitro.

Altered expression of ERα and/or ERβ and change in co-regulatory proteins are frequent causes of TAM resistance [5]; furthermore, genetic polymorphisms involved in the compound metabolism have been identified [6]. Different miRNA expression profiles have also been observed in TAM resistant and sensitive breast cancer cell lines, by microarray analysis [7]. By using this technique, 97 miRNAs differentially expressed in MCF7 endocrine-sensitive versus resistant LY2 breast cancer cells have been identified.

A number of studies reported the upregulation of growth factor receptors (HER2, EGFR, FGFR, IGF1R) and the consequent activation of the PI3K-PTEN/AKT/mTOR pathway to be closely related to acquired TAM resistance [8]. Finally, in recent years, a large body of evidence has shown a relationship between TAM resistance and protective

autophagy and identified in resistant cells an increased level of factors promoting Cyclin D1 transcription and G1-S transition [9].

Interestingly, in spite of the varied mechanisms potentially involved in the onset of TAM resistance, several studies showed that breast cancer cells with acquired resistance to TAM seem to display a similarly up-regulated glycolytic metabolism and increased lactate production [10,11]. Furthermore, inhibition of glycolysis was found to hinder some of the pathways leading to TAM resistance and to restore the cell response to this compound [12].

Actively pursued studies aimed at characterizing additional properties of TAM molecular structure also evidenced for this compound ER-independent effects. In particular, one of these seems to be strictly related to the lipophilic nature of the molecule, which facilitates its partition into membrane lipid bilayers [13]. This feature could explain the inhibition of mitochondrial respiratory rate observed in cells exposed to TAM, probably exerted at the level of complex I. In particular, Daurio et al. showed for TAM a pronounced, ER-independent effect on cancer cell metabolism, consisting in increased glycolysis and lactate production [14]. Interestingly, this observation could have clinical relevance: breast neoplastic lesions of patients undergoing FDG-PET scans after TAM administration often show a “metabolic flare”, a picture that could be easily explained by the increased glucose consumption caused by the drug mitochondrial inhibition [15].

Lactate, the end-product of glycolysis, is now considered an “onco-metabolite” and evidences have been obtained suggesting for this molecule a role in the transcriptional regulation of cancer-related genes in breast cancer cells [16].

Based on all the above cited data, it can be hypothesized that the increased glycolysis and lactate production which characterize TAM resistant cells, but is also observed early after the drug administration, could not only be epiphenomena of TAM resistance, but might also play an active role in the onset of this detrimental condition. The experiments described in this manuscript were aimed at shedding light on this question.

2. Materials and Methods

2.1 Cell cultures and treatments

All the materials used for cell culture and all the reagents were obtained from Sigma-Aldrich, unless otherwise specified. MCF7, MDA-MB-231 and MCF10A cells were grown in low-glucose (1 g/l) DMEM medium, supplemented with 100 U/ml penicillin/streptomycin, 2 mM glutamine and 10% FBS. Medium of MCF10A cultures also contained 0.5 μg/ml hydrocortisone and 100 ng/ml cholera toxin. MCF7-TAM [17] were maintained in α-MEM without phenol red, supplemented with 10% charcoal-stripped FBS, 100 U/ml penicillin/streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 10^{-7} M 4-hydroxy-tamoxifen (TAM). For the experiment shown in Fig. 7C, MCF7-TAM were grown in L15 medium; this medium does not contain glucose and is supplemented with 10% dialyzed FBS and 4 mM glutamine. For of its formulation, L15 medium does not allow glycolysis and lactate production. In TAM including experiments, media were supplemented with 0,6% DMSO. Lactate (L-isomer) was dissolved in culture medium at a 20mM concentration; MCF7 cultures were exposed to 20 mM lactate for both conditional (72 h) and sustained (≥ 4 months) treatments. In these experiments, the 20 mM lactate supplement was directly added to the medium; medium was changed every 72 h since in preliminary testing we found that lactate concentration was not significantly affected within this time interval.

2.2 Assay of lactate levels

Cells (5×10^5 / well) were plated in triplicate in 6-wells plates and let to adhere overnight. Medium was then replaced with Krebs-Ringer buffer and released lactate was measured after 1-6 h incubation at 37°C using the method described in [18]. The same procedure was adopted to evaluate released lactate in the presence of 1 μM TAM.

2.3 Cell proliferation

These experiments were performed in MCF7 cultures to both identify the TAM lowest active concentration and study the proliferation dynamics in lactate-exposed cells. In both

experiments, cell proliferation was assessed through the detection of ATP levels, by using the CellTiter-Glo Assay (Promega). A Fluoroskan Ascent FL reader was used to evaluate plates' luminescence.

For the TAM experiments, 15-20 × 10^4 cells/well, plated in triplicate in clear bottom 96-well white plates were incubated with 1 μM TAM for 24-120 h. For studying the proliferation dynamics of lactate-exposed cells, 20 × 10^4 cells, were plated as described above; they were let to adhere for 16 h, after which the number of living cells was detected in three wells by applying the CellTiter-Glo Assay (Time = 0). Plated cells were then grown for 24-48 h in a medium with different FBS concentration (10, 2 and 1%).

2.4 Real-time PCR

MCF7 cells were seeded in T25 flasks and allowed to adhere overnight. Exponentially growing cultures were conditionally exposed to 20 mM lactate (72 h). RNA was extracted as described in [19] and was quantified spectrophotometrically (ONDA Nano Genius Photometer). Retro-transcription to cDNA was performed by using the Revert Aid First Strand cDNA Synthesis Kit (ThermoFisher), in different steps: 5 min denaturation at 65 °C, 5 min annealing at 25 °C, 1 h retro-transcription at 42 °C and 5 min at 70 °C. Real Time PCR (RT-PCR) analysis of cDNA (20 ng) was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and different primers mixtures. Table 1 shows the complete list of examined genes, grouped by their relevance in biological processes; the list of primers sequences used for both the examined genes and the internal controls of the reaction is shown in Table S1. For all genes, annealing temperature of primers was 60 °C and the thermal cycler (CFX96 TM Real Time System, Bio-Rad) was programmed as follows: 30 sec at 95 °C; 40 cycles of 15 sec at 95 °C; 30 sec at 60 °C.

For comparison, the same experiment was also performed on lactate-exposed MCF10A cells and on MCF7-TAM cultures, maintained in their routinely growth medium.

The data of RT-PCR experiments were analyzed by applying the $2^{-\Delta\Delta CT}$ method [20].

Table 1

Complete list of examined genes in lactate-exposed MCF7 cells

Gene	Extended name	Gene	Extended name
Proliferative Potential		CYCs	Cytochrome C, Somatic [36,37]
ERBB1	Epidermal Growth Factor Receptor [21]	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase [38]
ERBB2	HER2 Receptor [22]	GLUT1	Glucose Transporter Type 1 [39]
MKI67	Marker Of Proliferation Ki-67 [23]	GLUT4	Glucose Transporter Type 4 [40]
NANOG	Homeobox Protein NANOG [24]	GPER1	G Protein-Coupled Estrogen Receptor 1 [41]
NOTCH1	Notch Receptor 1 [25]	LDHA	Lactate Dehydrogenase A [42]
OCT4	Octamer-binding Transcription Factor 4 [26]	LDHB	Lactate Dehydrogenase B [43]
PCNA	Proliferating Cell Nuclear Antigen [27]	MCT1	Monocarboxylic Acid Transporter 1 [44]
SRC	Proto-Oncogene c-Src [28]	MCT4	Monocarboxylic Acid Transporter 4 [44]
TAZ	Gene for Tafazzin [29]	NDUFA5	NADH:Ubiquinone Oxidoreductase Subunit A5 [36,37]
TERC	Telomerase RNA Component [30]	NDUFS3	NADH:Ubiquinone Oxidoreductase Core Subunit S3 [36,37]
TERT	Telomerase Reverse Transcriptase [30]	***Prognostic Markers***	
YAP	Yes-Associated Protein 1 [31]	ALDH1A3	Aldehyde Dehydrogenase 1 Family Member A3 [45]
Infiltrative Growth		BCL2	BCL2 Apoptosis Regulator [46]
MMP2	Matrix Metallopeptidase 2 [32]	CD24	CD24 Antigen [47]
PLAU	Urokinase-type plasminogen activator [33]	CD44	CD44 Antigen [48]
SERPINB2	Serpin Family B Member 2 [34]	CDKN1A	Gene for p21/WAF1 Protein [46]
VIM	Gene for Vimentin [35]	CDKN2A	Gene for p16 Protein [49]
Metabolism		CENPF	Centromere Protein F [50]
ATP5A1	ATP Synthase F1 Subunit Alpha [36,37]	E-CAD	Epithelial Cadherin [51]
ATP5B	ATP Synthase F1 Subunit Beta [36,37]	N-CAD	Neuronal Cadherin [51]
CYC1	Cytochrome C1 [36,37]	PTEN	Phosphatase and Tensin Homolog [52]

2.5 Immunoblotting

These experiments were performed on MCF7 cells after the conditional (72 h) exposure to lactate, to assess histone acetylation and expression level of some proteins identified following the RT-PCR experiments. A similar experiment was performed in cells with sustained (≥ 4 months) lactate exposure to obtain evidence on the activation of the EGFR pathway. For both experiments, control and treated MCF7 cultures were harvested and lysed in 50 µl RIPA buffer containing protease and phosphatase inhibitors. To evidence the activation of EGFR pathway, a 3-h pretreatment with 10 μg/ml Insulin was applied to cells with sustained lactate exposure and their untreated controls before culture harvesting.

80 µg of protein (measured according to Bradford) was loaded into 4–12% polyacrylamide gel for electrophoresis and run at 170 V. The separated proteins were blotted on a low fluorescent PVDF membrane (GE Lifescience) using a standard apparatus for wet transfer with an electrical field of 60 mA for 16 h. The blotted membrane was blocked with 5% BSA in TBS-TWEEN and probed with the primary antibody. The antibodies used were: rabbit anti-H3 (Cell Signaling); rabbit anti-Panacetyl-H3 (Active Motif); rabbit anti-TAZ (Cohesion Biosciences); rabbit anti-LDH-A (Cell Signaling); rabbit anti-(Tyr10)-phospho-LDH-A (Cell Signaling); rabbit anti-c-Myc (Abcam); rabbit Ab-21 polyclonal anti-EGFR (Neomarkers/Labvision Inc.); rabbit anti-phospho-(Tyr1068)-EGFR (Novex); rabbit anti-AKT (Cell Signaling); rabbit anti-phospho-(Ser473)-AKT (Cell Signaling). Binding was revealed by a Cy5-labelled secondary antibody (goat anti rabbit-IgG, Cytiva Life Sciences). Fluorescence of the blots was assayed with the Pharos FX Scanner (Bio-Rad) at a resolution of 100 µm, using the Quantity One software (Bio-Rad).

2.6 Telomerase assay

Telomerase activity in control and conditionally (72 h) lactate-exposed MCF7 cells was measured using a quantitative real-time telomeric repeat amplification protocol (RTQ-TRAP), widely described in literature [53-55].

For this experiment, cultures were scraped-off and washed with cold PBS. Subsequently, 1-2 × 10^6 cells were lysed in 50 μl CHAPS buffer, left on ice for 30 min and sonicated for 15 sec in an ice-submerged tube. A Heat System Model XL2020 sonicator was used, applying a power of 50-60 W for 5 sec, with 15 sec intervals.

Lysates were centrifuged at 14000g for 20 min at 4°C; surnatant was recovered, aliquoted and stored at -80°C until used. Proteins of the lysates were quantified using the Bradford method.

The RTQ-TRAP assay mixture contained 0.25 μM TS primer (5'-AATCCGTCGAGCAGAGTT-3'), 0.25 μM ACX primer (5'-GCGCGG(CTTACC)$_3$CTAACC-3'), 1x SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 2-5 μl of cell lysate, in a final volume of 25 μl. Mixture was incubated 20 min at 25°C to allow TS primer elongation with TTAGGG repeats by the telomerase enzyme in cell lysates. Quantification of the added telomeric sequences was assessed by RT-PCR using the following conditions: 10 min denaturation at 95°C and 40 amplification cycles (20 sec at 95°C, 30 sec at 50°C, 90 sec at 72°C). PCR reactions were performed in a CXF96 Real Time System (Bio-Rad). Negative controls (2-5 μl lysis buffer) and telomerase-negative controls (2-5 μl heat-inactivated cellular lysates) were used in each experiment.

2.7 Wound healing assay

Control and conditionally (72 h) lactate-exposed MCF7 cells were seeded in triplicate in 6-well plates (1.5 × 10^6 /well) and cultured until they had reached 100% confluence. Artificial wounds were then created using a 10 μl pipette tip. The detached cells were washed away with PBS and cultures were exposed to a medium supplemented with a lowered serum content (1 and 2%). 20 mM lactate was also added to the medium of lactate-exposed cultures. The wound areas were captured with an inverted microscope at 0, 24 and 36 h and their repopulation was analyzed by using the ImageJ software.

2.8 Senescence associated β-galactosidase staining

This experiment was performed in MCF7 cultures exposed to a sustained (≥ 4 months) 20 mM lactate treatment. Control and lactate-exposed cells (1×10^5 / well) were seeded in triplicate in 6-well plates and treated with 1 μM TAM for 7 days. After treatment, cells were washed twice with PBS and fixed with a 2% formalin / 0.2% glutaraldehyde solution, for 5 min at room temperature. After fixation, they were washed again with PBS and incubated overnight at 37°C with X-gal (1 mg/ml), dissolved in a staining solution containing 40 mM citric acid pH 6, 5 mM potassium ferrocyanide II, 5 mM potassium ferrocyanide III, 150 mM NaCl and 2 mM $MgCl_2$. After a 16-h incubation, cultures' images were captured using an inverted microscope. The development of a perinuclear blue color was indicative of senescent cells.

2.9 Statistical analyses

All data were analyzed by using the GraphPad Prism software. All results were obtained from at least two independent experiments, performed with triplicate samples. They are expressed as mean values ± SE and have been calculated using all the data obtained from the independent experiments; the significance level was set at $p < 0.05$.

3. Results

3.1 Short-term exposure to TAM causes enhanced lactate release by MCF7 cells

To establish a correlation between lactate-induced changes and reduced TAM response in MCF7 cells, we took advantage of a TAM-resistant clone of this line (MCF7-TAM), obtained by one of the co-authors (FF) [17]. MCF7-TAM cells are routinely cultured in the presence of TAM and differ from their parental line in a markedly higher level of released lactate (> 1.5-fold in 4 h, Fig. 1A); furthermore, in agreement with previous studies showing upregulated glycolysis as a hallmark of poor pharmacological response, they show

a pattern of metabolite production superimposable to that of MDA-MB-231 cells, a well-studied model of triple-negative, drug resistant breast cancer.

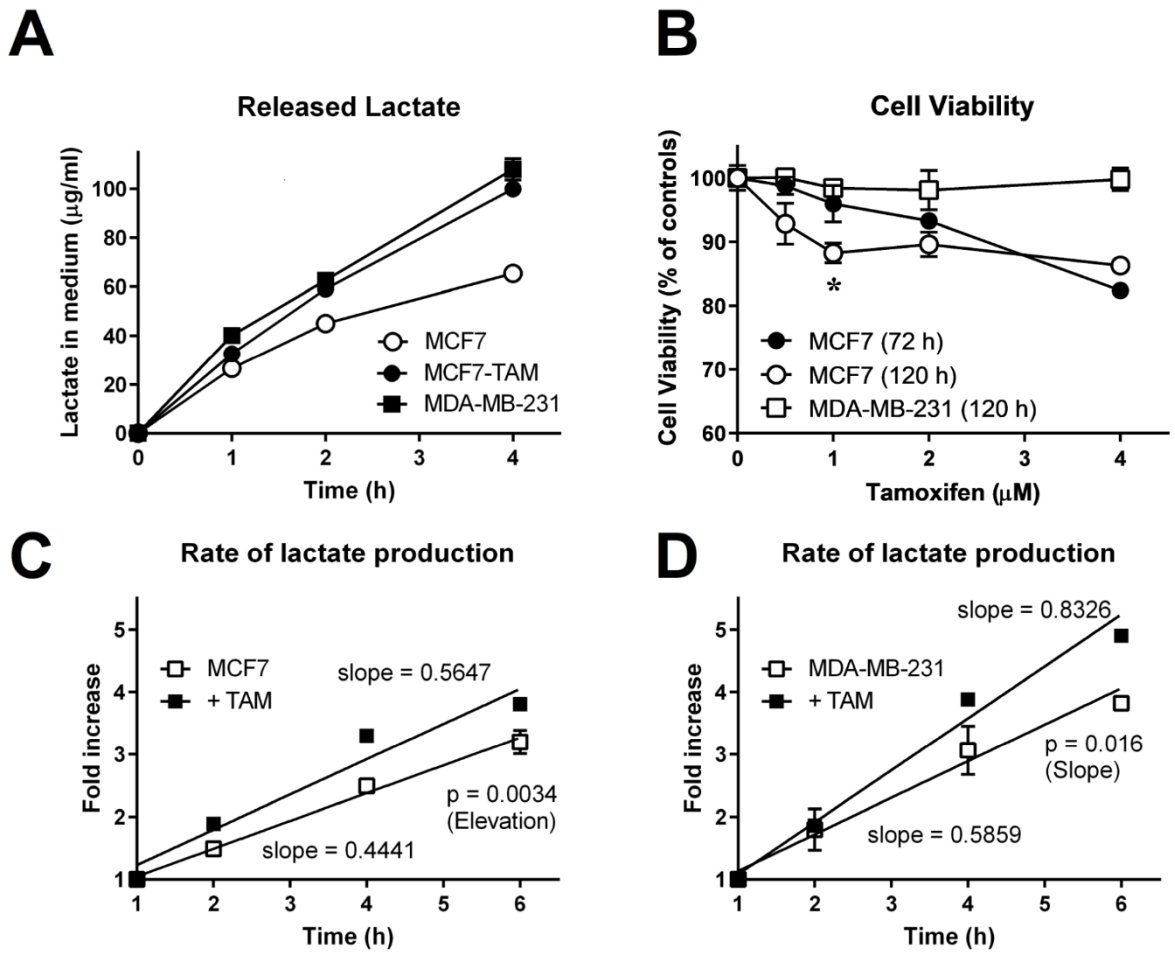

Figure 1 – Assay of lactate levels. (A): Lactate released in medium was assessed as described in Materials and Methods. Data were analyzed by applying the linear regression; the curve slopes measured in MCF7-TAM and MDA-MB-231 cells were significantly higher ($p < 0.05$) than that shown by MCF7 cells. (B): Effect of TAM on the viability of MCF7 and MDA-MB-231 cells. Data were analyzed by multiple *t*-test; no statistically significant effect was evidenced in MDA-MB-231 cells, which do not express ER. In MCF7 cells, a statistically significant reduction of cell viability was observed at 120 h with 1 μM TAM, the dose used for the experiments shown in (C) and (D). (C),(D): Rate of lactate production assessed in MCF7 (C) and MDA-MB-231 (D) cells exposed to 1 μM TAM. Data were analyzed by linear regression; curves' slopes and statistically significant parameters are shown in the graphs.

In a following experiment, parental MCF7 cells were exposed to scalar doses of TAM (0-4 μM) for 72 – 120 h, in order to identify the lowest drug concentration able to reduce cell viability. As shown in Fig. 1B, a statistically significant effect was observed with 1 μM TAM, only after the 120-h treatment. Interestingly, when this dose was applied to both MCF7 and MDA-MB-231 cultures, a rapid increase (2-6 h) in the lactate production rate was detected (Fig. 1C,D); in TAM-exposed MCF7 cells, the curve elevation of lactate production rate resulted significantly higher ($p = 0.0034$) with a 27%-increased slope, compared to the untreated cultures. Even more marked effects were measured in MDA-MB-231 cells, which do not express ER. These results suggest that the metabolic changes caused by TAM definitely forestall the emergence of its antiproliferative effects, paving the way to phenotypic adaptations which could potentially interfere with the drug antineoplastic action.

3.2 Conditional exposure of MCF7 cells to lactate leads to gene expression changes similar to those constitutively observed in MCF7-TAM cells

To explore whether lactate is involved in the transcriptional regulation of genes potentially leading to TAM resistance, we conditionally exposed MCF7 cells to increased level of this metabolite (72 h). In planning this experiment, we referred to the linear regression curve obtained from the data of lactate levels released in medium by MCF7-TAM cells (Fig. 1A). The equation obtained from the data regression analysis (μg/ml Lact = (26.20 ± 0.86) × h) indicated that the lactate level released in medium from these cultures could reach a concentration of about 7 mM in 24 h, theoretically growing up to 20 mM in 72 h. 20 mM lactate fits well with the level of metabolite usually detected in the microenvironment of different tumor tissues [56]; the experiments aimed at evaluating upregulated gene expression were then performed by exposing the parental MCF7 cultures to this lactate concentration for 72 h.

RT-PCR assays examined a number of genes selected among those with a documented relationship with TAM resistance or having prognostic significance in breast cancer; a total of 40 genes were considered (Table 1).

Genes with a statistically significant up-regulation induced by lactate are shown in Fig. 2A, grouped by their relevance in biological processes. Interestingly, the gene cluster shown in the “Proliferative Potential” graph was found to be involved in the control of cancer cells’ stem properties. In particular, TAZ, a transducer of the Hippo pathway, was shown to sustain self-renewal and tumor-initiation capacities in breast cells [57]. Together with its partner protein YAP, it was also found to be involved in metabolism regulation and glycolysis promotion, suggesting a role in coordinating nutrient availability with cell proliferation [58]. The marked up-regulation of LDHA observed in lactate-exposed MCF7 cells (Fig. 2A, “Metabolism” graph) is in line with this idea.

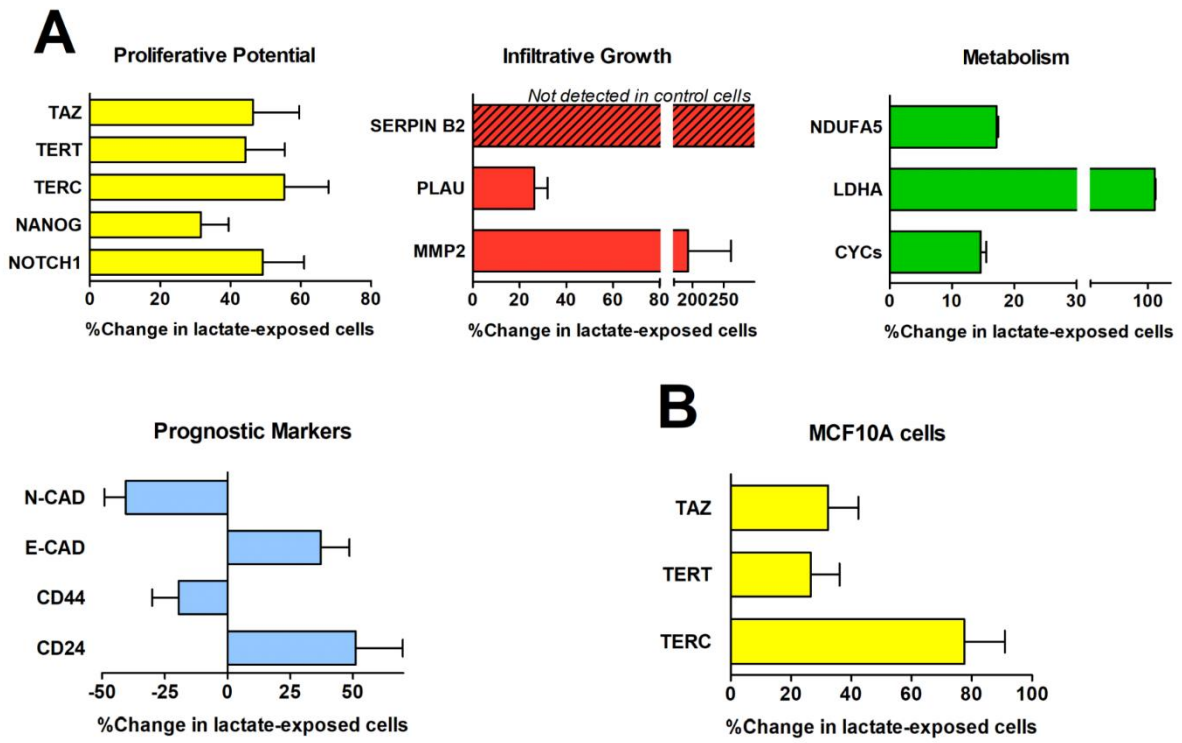

Figure 2 – Real-time PCR experiments performed in MCF7 and MCF10A cells after a conditional (72 h) lactate exposure. The expression levels of the 40 genes reported in Table 1 was assessed in lactate-exposed MCF7 cultures. Results were evaluated using the column statistics' analysis of the GraphPad software, which applies the one sample *t*-test and calculates whether the mean of each data set is different from a given hypothetical value (0, i.e. no change, compared to untreated cultures). The graphs show only the statistically significant changes; p values ranged from 0.04 to < 0.0001.(A): Experiments performed in MCF7 cells; genes have been grouped in graphs according to their biological function. (B): Experiments performed in MCF10A cells; in these cultures, only three genes were found to be upregulated.

Fig. 2A also shows that some statistically significant up-regulations were also found in genes related to infiltrative growth; however, these findings were not completely confirmed by the analysis of some prognostic markers with consolidated value in breast cancer: E- and N-CAD [51]; CD24 and CD44 [47,48]. In lactate-exposed MCF7 cells, E-CAD was found to be increased, CD44 decreased. These inconsistent results did not allow to relate the enhanced self-renewal potential induced by lactate with features suggesting cancer progression, at least in the short time. This finding is in line with the results of a previous study which examined the effects on lactate in different tumor contexts [59].

Following these results, we wondered whether lactate exposure could in the same way affect the gene expression of non-cancerous cells. As a model of non-neoplastic breast cells, we adopted the MCF10A line [60]; in this culture, the 72-h treatment with 20 mM lactate resulted in fewer gene expression changes. When the lactate responsive genes identified in the MCF7 culture were evaluated in MCF10A cells, only TAZ and the telomerase complex genes (TERC, TERT) were found to be significantly up-regulated by lactate (Fig. 2B). This finding poses a clinically relevant question on the effects potentially induced by the metabolites released by highly glycolyzing cancer cells on the surrounding normal tissue.

Fig. 3 shows a comparison between MCF7-TAM and its parental culture, concerning the expression of lactate-upregulated genes identified with the RT-PCR experiments shown in Fig. 2A.

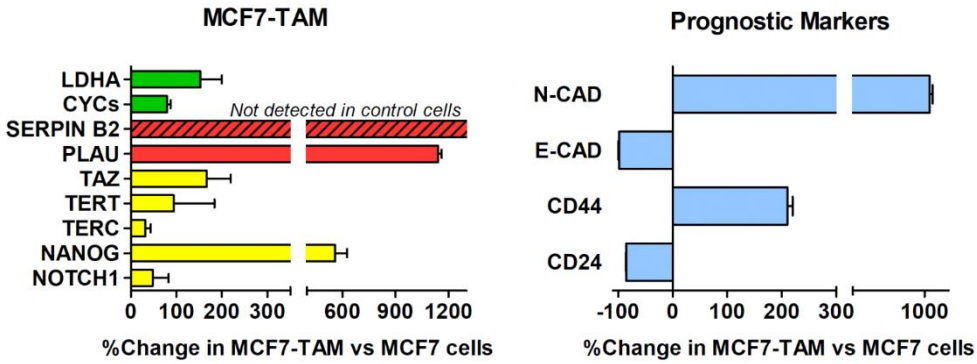

Figure 3 - Real-time PCR experiments performed in MCF7-TAM. In these experiments the expression of the lactate-upregulated genes (Fig. 2A) was evaluated in MCF7-TAM and compared to their parental culture. The increased expression shown by lactate-exposed cells was found further enhanced in MCF7-TAM culture and prognostic markers appeared worsened. Results were statistically evaluated as described for Fig. 2; the level of statistical significance was reached for all genes, with the exception of NOTCH1 and TERT; p values ranged from 0.03 to < 0.0001.

The upregulated gene expression pattern observed in lactate-exposed MCF7 cells was confirmed in the TAM resistant line. In these actively glycolyzing cells, which produce elevated lactate levels (Fig.1A), genes' upregulation was markedly higher than that observed in the parental culture exposed to 20 mM lactate. Interestingly, in MCF7-TAM cells the considered prognostic parameters appeared to be markedly worsened. Taken together, the results of Fig. 2A and 3 warranted further experiments to highlight the role of lactate in the onset of neoplastic progression and TAM resistance.

3.4 Conditional exposure to lactate increases the proliferative potential of MCF7 cells

The next step of our study was aimed at exploring whether the observed gene expression changes could also be evidenced at protein and/or functional level.

Because of the perceived inconsistent results, the genes related to prognosis and infiltrative growth were not furtherly considered. Moreover, we hypothesized that some changes in the metabolism-related genes could be linked to the upregulation of TAZ function

[58], instead of a direct effect of lactate. For these reasons, among the results obtained with the RT-PCR experiments, we mainly focused our attention on the lactate-dependence shown by genes correlated with proliferative potential.

As a first step, we verified whether the observed genes' upregulation could be related to enhanced histone 3 (H3) acetylation and whether it also resulted in increased protein levels, as assessed by immunoblotting experiments or by functional assays. Unfortunately, among the "Proliferative Potential" proteins, NANOG and NOTCH1 were not evidenced in the immunoblotting experiments, both in control and in lactate-exposed cells; the low-level expression of these genes in the RT-PCR assay (Ct ≥ 28) could account for this result.

Fig. 4 showed that the adopted conditional exposure to lactate caused a ≈30% increased level of H3 pan-acetylation and that this effect resulted in a similarly increased level of TAZ protein. HDAC inhibition and increased H3 acetylation are recognized mechanisms underlying the epigenetic effects of lactate [61].

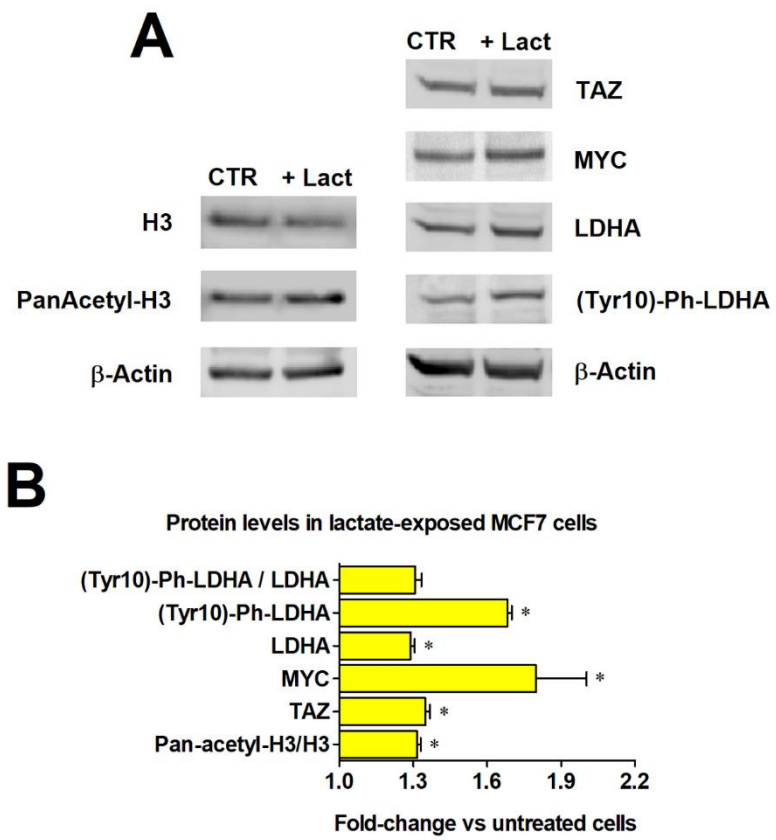

Figure 4 – Immunoblotting evaluation of the lactate-upregulated proteins. (A) Immunoblotting images. (B) Protein level changes, assessed through bands' densitometric reading. Results were statistically evaluated by multiple *t*-test; *, $p < 0.05$, compared to control cultures.

The increased level/function of the transcriptional co-activator TAZ was also confirmed by the enhanced level of MYC and LDHA proteins. MYC is one of the targets of the activated Hippo pathway [62] and is known to directly activate the transcription of the LDHA gene [63]. Concerning LDHA, we also measured the levels of its phosphorylation on Tyr10, a post-translational modification enhancing the enzymatic activity of the protein and promoting cancer cell invasion and anoikis resistance [64]. According to the data of Fig. 4A,B, the increased level of (Tyr10)-phosphorylation fits well with the increased level of the total protein, which suggested that the lactate-triggered effects did not affect post-translational LDHA changes.

For the two identified telomerase complex gene components TERC and TERT (Fig. 2A), only a functional assay was adopted, since only one of them (the reverse transcriptase TERT) is translated into a protein, while the other (TERC) encodes for the RNA component of the enzymatic complex. The assay of telomerase activity was performed using scalar amount of cells proteins (1-20 μg); it measured, in control and lactate-exposed MCF7 cells, the elongation with TTAGGG repeats operated by the cell TERT enzyme on a primer sequence added in the reaction mix (TS primer). Results (Fig. 5A-D) showed no difference between the two cultures when the experiment was performed with 1 μg proteins. However, when the telomerase reaction assay was repeated with higher amounts of cell lysates, a progressively increasing difference in the reaction yield was observed between the two cultures, suggesting a higher-level activation of the telomerase complex in lactate-exposed cells.

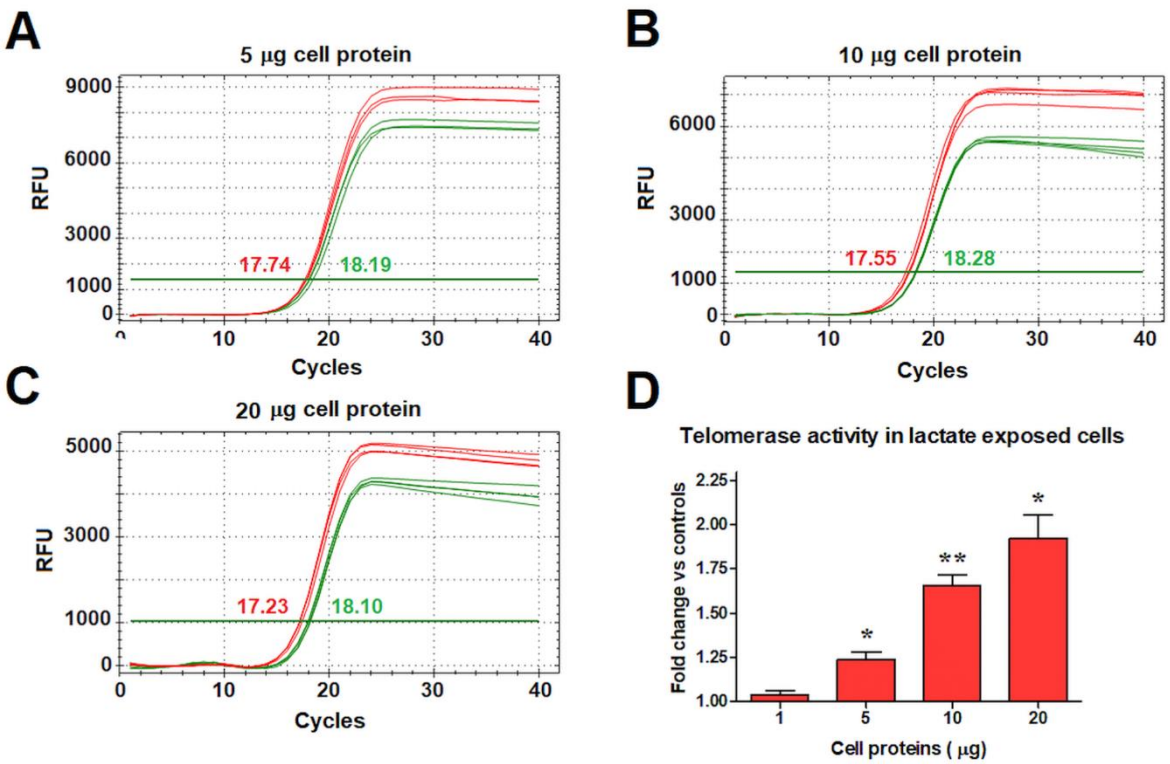

Figure 5 – Assay of telomerase activity. Telomerase complex activity was assessed using scalar amounts of protein extracts from control and lactate-exposed MCF7 cells. (A-C) Exemplificative plots showing the PCR amplification curves obtained from control (green) and lactate-exposed (red) cells. The numbers reported in plot images show the Ct mean values. Differences were evaluated by applying the $2^{-\Delta Ct}$ method and are shown in (D); * and **, $p < 0.05$ and 0.01 compared to control cultures, as evaluated by multiple *t*-test.

As shown in Fig. 5C,D, the telomerase activity of lactate-exposed cells appeared to be 2-fold higher when the assay was performed with 20 µg cell proteins. Previous studies showed TAM to be involved in the control of TERT expression with opposite functions, both involving ERs: in endometrial cells it acts as a receptor agonist, stimulating proliferation and activating TERT expression [65], whereas in breast cancer cells it may suppress TERT by functioning as a receptor antagonist [66]. The results of Fig. 2A and 5 suggest that the off-target metabolic effects of this drug can contribute to hinder one of the effects of its primary mechanism of action.

To gain further confirmation about the lactate-induced enhancement of replicative potential, which was suggested by the results of Fig. 2, 4 and 5, other assays were performed (Fig. 6). The proliferation of lactate-exposed MCF7 cells was compared to that of the parental culture by applying a wound healing assay (Fig. 6A) and by measuring the increase in cell number through the detection of ATP levels (Fig. 6B). Both experiments were performed by maintaining the cultures in a medium with low serum levels (1-2%), to reduce stimulation by growth factors. Fig. 6A shows that in lactate-exposed cells maintained at 2% FBS the percentage of repopulated wound area was significantly higher at 24 h; in this condition, the advantage of lactate treatment appeared to disappear at 36 h, when, however, the proliferative advantage of observed in lactate-exposed cells became more evident and reached statical significance in cultures maintained at 1% FBS. The experiments of Fig. 6B showed that after 24 h culture in the conventional medium supplemented with 10% FBS, cell number was 30% higher in lactate-exposed cells; furthermore, when both cultures were maintained in serum-deprived conditions, the proliferation of lactate-exposed cells showed a significantly lower level of inhibition.

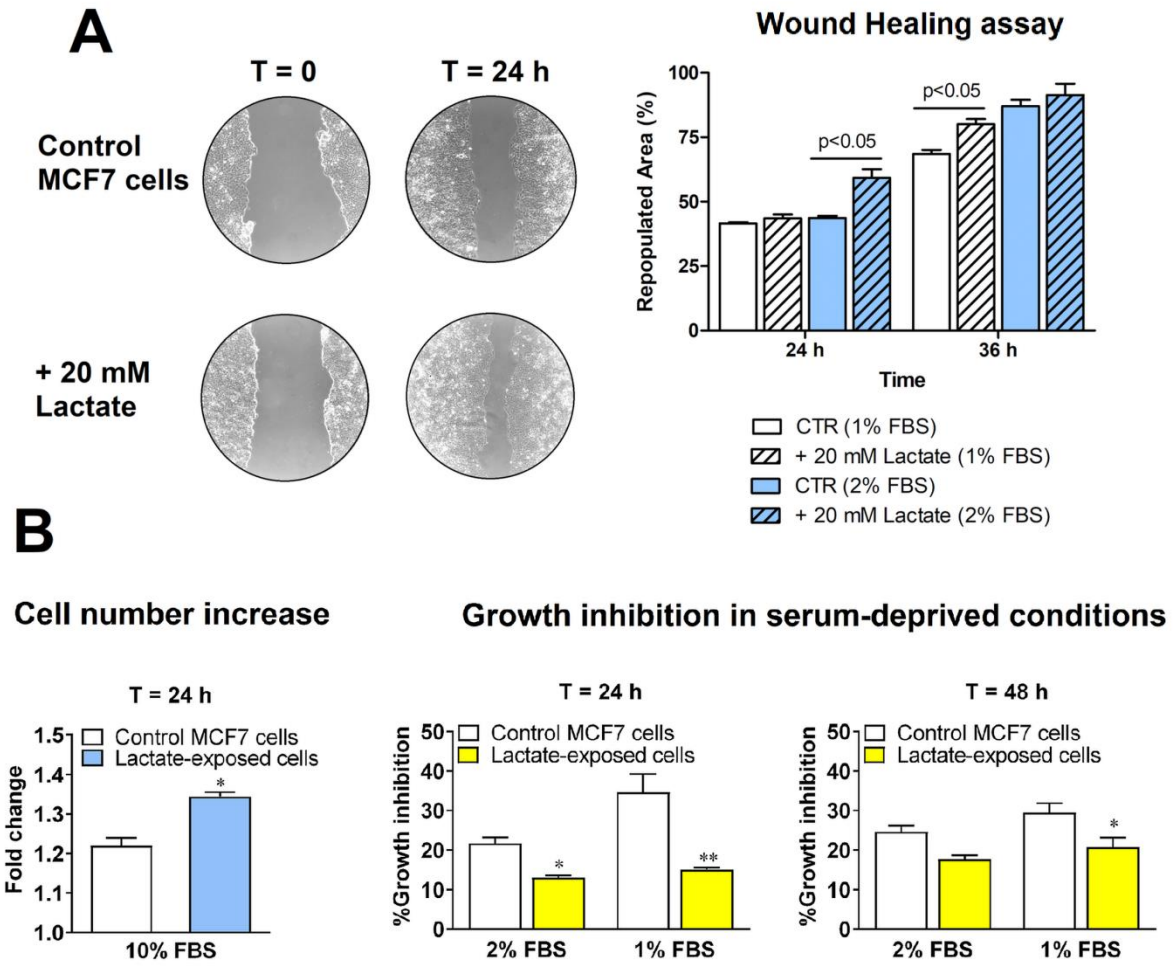

Figure 6 – Evaluation of proliferative potential in lactate-exposed MCF7 cells. (A) Wound healing assay; the percentage of wound repopulated area was evaluated using the ImageJ software. Data were statistically analyzed using two-way ANOVA followed by Bonferroni post-test. (B) When maintained in the conventional culture medium, lactate-exposed MCF7 cells showed a 30% increased proliferation at 24 h. The growth inhibition caused by serum deprivation was significantly reduced by lactate. Data were evaluated by multiple t-test; * and **, $p < 0.05$ and 0.01, respectively.

3.5 Sustained lactate-exposure reduces the senescence of MCF7 cells treated with TAM.

The conclusive section of our study was aimed at evidencing signs of a subsiding response to TAM in lactate-grown MCF7 cells. For these experiments, MCF7 cultures were adapted to grow in a medium containing 20 mM lactate for ≥ 4 months. No evident

morphological changes were observed in cells after sustained lactate exposure. To compare the antineoplastic effect of 1 μM TAM in control and lactate-exposed cultures, we tried to evidence a p53-mediated response, by an immunoblotting evaluation of p53 levels, followed by a RT-PCR detection of p21 mRNA. No significant difference was observed between the two cultures, up to a 7-days TAM treatment (data not shown). We hypothesized that this missing result could be a linked to the ER-mediated p53 regulation [67]; as a consequence of the antagonism between anti-estrogens and p53, these compounds were previously found to reduce both breast cancer cell proliferation and their p53 levels [68]. In agreement with this hypothesis, a marked difference between the two treated cultures was observed when the effect of TAM was evaluated by assessing β-galactosidase (β-GAL) activity, a widely used biomarker of replicative senescence. Fig. 7A shows an overview of parental and lactate-exposed MCF7 cultures, tested for β-GAL activity. Untreated cultures did not show appreciable differences; however, after the 7-days exposure to 1 μM TAM, marked and diffuse β-GAL staining was clearly more evident in control MCF7 cells. This result suggested that the lactate-awarded proliferative advantage, together with the activated telomerase function can impact on the cellular response to TAM and foster drug resistance.

Several studies highlighted progressive activation of the EGFR pathway as a salvage mechanism adopted by TAM-exposed breast cancer cells in which the ER functions are repressed [69]. Expression of EGFR was found to slightly increase shortly after the beginning of TAM treatment and to become markedly increased in resistant tumors [70].

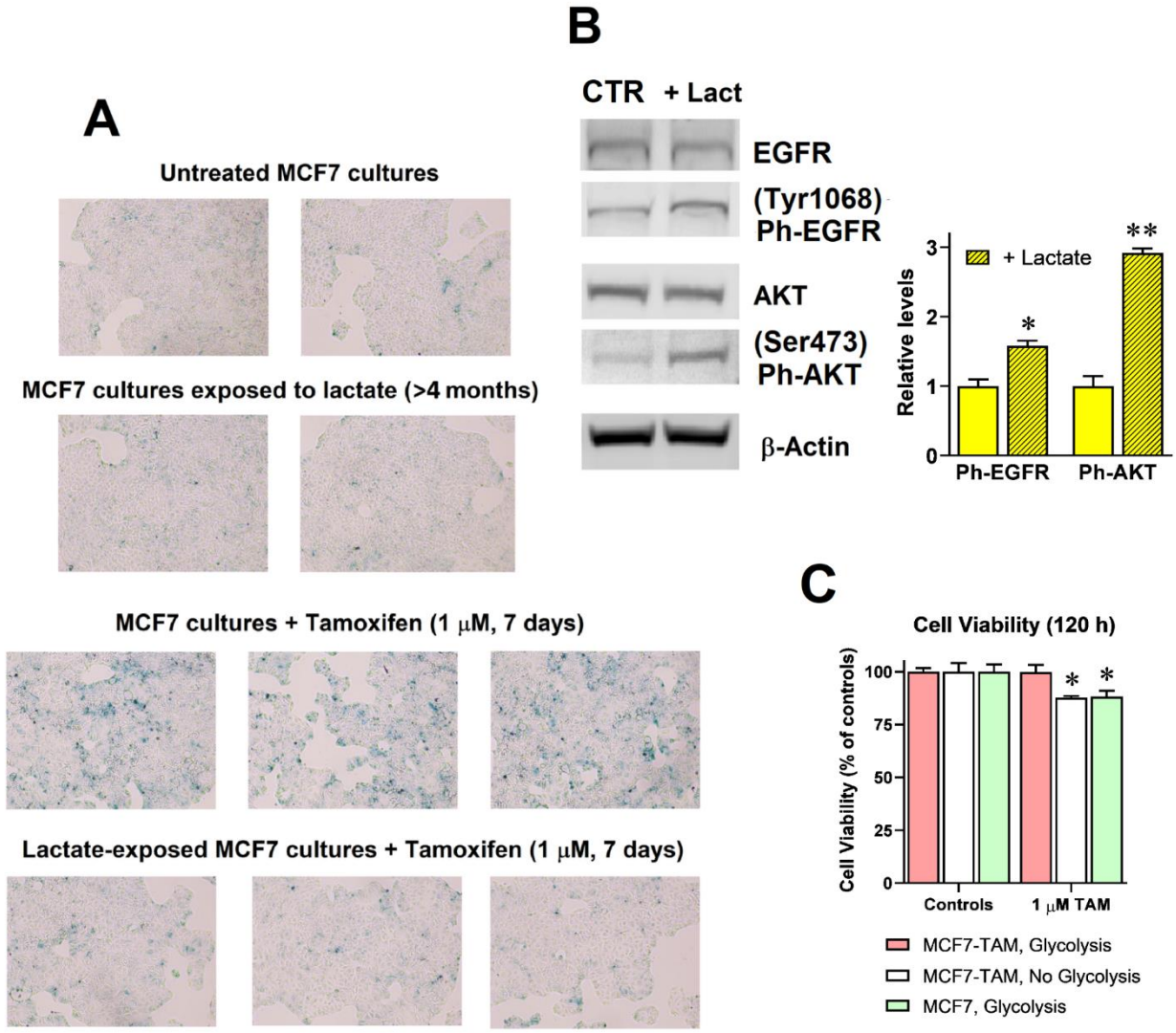

Figure 7 – Experiments performed in MCF7 cells after a sustained exposure (≥ 4 months) to lactate and restoration of TAM response after lactate deprivation. (A) Pictures showing parental and lactate-exposed MCF7 cells treated for 7 days with 1 μM TAM and stained for β-galactosidase activity. Lactate-exposed cells showed a markedly lower development of the β-galactosidase reaction product. (B) Immunoblotting evaluation of activated EGFR pathway. The level of (Tyr1068)-phospho-EGFR and (Ser473)-phospho-AKT appeared to be significantly enhanced in lactate-exposed cells. Differences were assessed by multiple *t*-test; * and **, $p < 0.05$ and 0.01 compared to control cultures. (C) Lactate deprivation in MCF7-TAM cells leads to a restored TAM response. Lactate deprivation was obtained by culturing the cells in L15 medium, which does not allow glycolysis. TAM was administered for 120 h at 1 μM, which are the same conditions used in the experiment of Fig. 1B. Differences were assessed by multiple *t*-test; *, $p < 0.05$ compared to the untreated cultures.

Fig. 7B shows an immunoblotting evaluation of EGFR pathway activation, performed in MCF7 cells after the sustained exposure to lactate and in their parental culture. Phosphorylation on Tyr1068 was shown to positively regulate EGFR signaling [71] and promote AKT activation, evidenced by phosphorylation on Ser473. As shown in Fig. 7B, both these phosphorylation events appeared to be significantly increased by lactate exposure. Activation of EGFR pathway has been shown to promote glycolytic metabolism [72-74]. Interestingly, it can be concluded that in our experiments lactate exposure appeared to trigger a cell response that, by promoting glycolysis, should lead to further increased lactate levels. Finally, the experiment shown in Fig. 7C attempted to verify whether lactate deprivation in MCF7-TAM cells could recover, at least in part, their response to TAM. To impede lactate production, MCF7-TAM cells were cultured in L15 medium which, because of its formulation, does not allow glycolysis and lactate production. In this medium the proliferation rate of MCF7-TAM appeared to be compromised, but cells maintained their viability. The bar graph shown in Fig. 7C compares the effect caused by 1 μM TAM administered for 120 h (dose and time used for the experiment of Fig. 1B) to MCF7-TAM and MCF7 cells maintained in their conventional medium and to MCF7-TAM maintained in L15 medium. This dose of TAM did not compromise the viability of MCF7-TAM cells when they were grown in their conventional medium, allowing glycolysis and lactate production. On the contrary, when this culture was maintained in L-15 medium, TAM was found to significantly affect cell proliferation, causing an inhibitory effect very similar to that observed in the parental MCF7 cell culture. This result can be considered a further confirmation of the role of lactate in maintaining the TAM-resistant cell phenotype.

4. Discussion

Our results showed that a lactate level potentially achievable in cancer cell microenvironment could affect gene expression in a way that might lead to reduced TAM response. In the microenvironment of breast cancer tissues, enhanced lactate levels can be linked to the increased glycolytic metabolism which characterize neoplastic tissues, but, in treated patients, it could also derive from the documented inhibitory effects caused by TAM on mitochondrial respiratory chain [13]. Interestingly, evidence of these effects was also obtained in TAM-treated patients undergoing diagnostic procedures [15]. According to our data, the contribution of lactate in reducing TAM efficacy could derive from its capacity to enhance the proliferative potential of cells and, as a consequence of the activation of the telomerase complex, to reduce their attitude to undergo senescence. The same features can be expected to impact also on the response of cancer cells to different antineoplastic agents. Our data are in complete agreement with the findings of Hamadneh et al. [43], who showed that the development of TAM resistance in MCF7 cells correlates with upregulated LDHA/B expression and increased lactate concentration in cell culture medium. Our data support this study by suggesting that the product of LDH reaction (either deriving from the basal cancer cell metabolism, or from the TAM side-effects) can by itself play a direct role in promoting a reduced drug response. Furthermore, as also proposed by Das et al. [42], our results suggest that targeting LDHA could open a novel strategy to interrupt TAM resistance in breast cancer.

Interestingly, experiment shown in Fig. 2B suggested that tumor released lactate could also exert phenotypic modifications on normal bystander breast cells; this is an interesting and unexplored issue, worth of further study which, however, is out of the scope of the present manuscript. The observed phenotypic modifications caused by lactate in both cancer and normal cells are completely coherent with the role of glycolytic metabolism in

embryonal development and in the maintenance of stem compartment in normal tissues [75].

Cancer promoters are defined as agents that, without changing DNA sequence, influence cell proliferation, also inhibiting programmed cell death; this epigenetic process ultimately results in the generation of neoplastic cell foci [76]. In line with this concept, our results suggest that lactate could be viewed as a promoter of TAM resistance in the MCF7 breast cancer model. In MCF7 cultures exposed to lactate for ≥ 4 months, we obtained evidence of EGFR activation, which has been previously documented as a crucial pathway controlling the proliferation of TAM-resistant cells [8]. A direct correlation between the epigenetic effects caused by lactate and EGFR activation, is suggested by previously published data showing that in breast cancer, but also in different neoplastic cells, TAZ overexpression promotes EGFR signaling, leading to AKT/ERK activation and increased cell proliferation [77-79]. The increased AKT phosphorylation also shown in Fig. 7B is a further evidence of activated EGFR pathway and is in line with the results reported in refs. [77-79]. Because of the promoting effect on aerobic glycolysis of EGFR-mediated signaling [72-74], on the basis of our results we can speculate that the increased lactate levels in the microenvironment of TAM-exposed breast cancer cells can fire up a self-feeding loop where a metabolic product (lactate) promotes epigenetic changes ultimately resulting in the amplification of its generation (Fig.8). In the long term, the cell phenotypic changes induced by the activation of this self-supporting cycle could lead to TAM resistance.

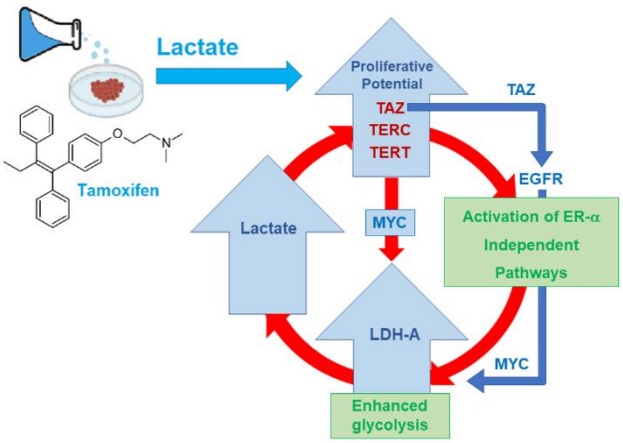

Figure 8 – Self-supporting cycle potentially induced by lactate in MCF7 cells. Increased lactate exposure increases the proliferative potential of MCF7 cells by upregulating the expression of TAZ, TERC and TERT. TAZ overexpression was found to activate EGFR pathway and to increase MYC level, leading to increased LDHA expression/activity. On the basis of our results, we propose that, by causing increased lactate production in cells, the TAM associated metabolic changes could activate ER-α independent pathways, paving the way to a progressive reduction of therapeutic effects.

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