Warburg Effect Inversion: Adiposity shifts central primary metabolism in MCF-7 breast cancer cells

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A B S T R A C T

Aims: Obesity is a complex health disorder and a trigger to many diseases like Diabetes *mellitus* (DM) and breast cancer (BrCa), both leading causes of morbidity and mortality worldwide. Also evidence demonstrates that abnormal glucose metabolism termed 'the Warburg effect' in cancer cell is closely associated with malignant phenotypes and promote the aggressiveness of several types of cancer, including BrCa.

In this study, we evaluated the breast cancer cell metabolism in normoglycemia, hyperglycemia and in an obesity condition in order to clarify the potential underlined mechanisms that link these disorders.

Materials and methods: MCF-7 cells were exposed to low and high glucose levels, the latter either in the presence of 3T3-L1 adipocyte conditioned medium (CM), thus mimicking the adiposity observed in obese patients. Cell viability, migration, proliferation, cytotoxicity and cell death assays were performed under the different culture conditions. Hormonal and lipid profile were also characterized by biochemical assays and primary metabolism was determined by Nuclear Magnetic Resonance (NMR)-based metabolomics.

Results: Our results show an increased aggressiveness in the condition mimicking diabetogenic obesity with an altered energy/lipid metabolism. Interestingly in the experimental obesity-mimicking status, lipids and amino acids were expended while glucose was produced by tumor cells from lactate. These findings reveal a shift on tumor cells metabolism that is opposite to 'the Warburg effect'.

Conclusions: Overall, this experimentally obesity-mimicking condition not only revealed an increased tumor proliferation and aggressiveness but also disclosed a new mechanism of cancer metabolism, the 'Warburg Effect Inversion'.

Keywords: Type 2 diabetes Obesity Breast Cancer MCF-7 Metabolism Warburg effect

1. Introduction

According to the International Diabetes Federation, Diabetes *mellitus* (DM) affected > 425 million people in 2017 worldwide. Diabetes is not only a disease responsible for > 4 million deaths over the world but also a trigger to other non-communicable diseases (like cancer) and it is directly associated with its progression, severity and therapeutic strategy. Type 2 diabetes (T2D) is characterized by a state of hyperglycemia with underlined mechanisms such as insulin resistance, inflammation and steroid hormones dysregulation that have been reported to be also implicated in tumorigenesis [1].

One major risk linking both diseases is obesity. Accordingly, adipose tissue is considered an endocrine organ that produces a large variety of products and metabolites [2]. Adipose tissue is composed by adipocytes, connective tissue matrix, nerve tissue, stromal-derived vascular and immune cells, and is nowadays considered a metabolic, neuroendocrine and immune tissue. Adipose tissue is spread throughout the body. However, its location can determine its contribution to an overall

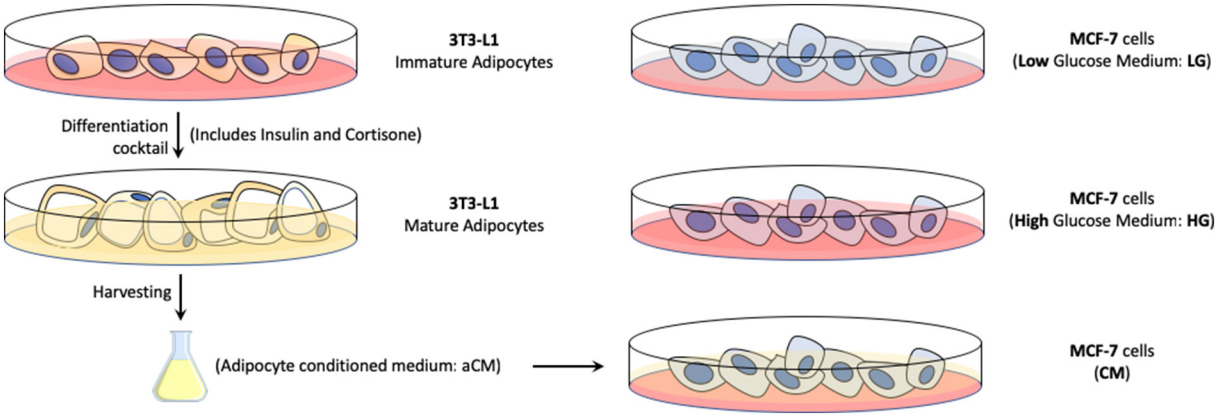

Fig. 1. MCF-7 breast cancer cells were exposed to different medium environments regarding its nutritional and hormone status. LG: Low glucose medium; HG: high glucose medium; CM –adipocyte conditioned medium.

disease state. Visceral adipose tissue reflects an increased risk for multiple states of disease including metabolic syndrome and T2D [3]. Adipocytes, representing approximately 50% of adipose tissue cells [4], are known to express several endocrine factors directly involved in metabolic functions, as well as enzymes implicated in sex steroids and glucocorticoids metabolism. The majority of these endocrine factors are also associated with disease, namely by interfering with immune modulation, angiogenesis and tumorigenesis.

Recently, it has been reported that obesity increases the risk of cancer in 13 anatomic sites [5], including breast, prostate, endometrium, colon and gallbladder cancer, which have a significant hormonal basis. In agreement, adipocytes are able to synthesize high levels of estrogens by expressing aromatase [6]. Estrogens, in turn, promote cell proliferation, invasiveness and decrease cell apoptosis, inducing tumor aggressiveness. Studies also suggest that obesity can affect the therapeutic efficacy [1], leading to high resistant tumors. Therefore, understanding the metabolic cues of tumor cells becomes mandatory in order to identify accurate treatment approaches of metabolic disease-associated cancers.

Finally, approaching tumor metabolism is to address the Warburg's effect [7]. Considered the father of cancer cell metabolism, Otto Warburg, discovered in the 1920s that tumor cells had a number of common metabolic features, namely (1) high glucose consumption, (2) with low oxygen consumption (3) but increase of lactate production. For > 90 years this metabolic switch has passed discreetly to the scientific community, but the increase of knowledge in this field has grown enormously in the last 10 years [8]. It was shown that aerobic glycolysis occurs in the adjacent stroma, rather than in cancer cells [9]. Some discussion has been generated around this topic, whether nutrients provided by stromal cells (lactate or ketones) or by the products of autophagy [10,11] which seems to provide fatty acids, and free amino acids that are ready-for-use by cancer cells. This mechanism is known as the reverse Warburg effect. Nonetheless, the metabolic interrelationships underlying the cancer cell in an obesity microenvironment are not yet understood [12].

The aim of the present study is to analyze the metabolic behavior of MCF-7 breast cancer cell line when exposed to normal and increased glucose and obesity conditions, simulating the overall metabolism observed in an experimental setup mimicking normoglycemic, hyperglycemic and obesity microenvironment of cancer cell but in the absence of the stromal cells.

2. Material and methods

2.1. Adipocyte differentiation and adipocyte conditioned medium production

3T3-L1 (ATCC CL-173) is a cell line of pre-adipocytes obtained from murine strain 3T3 embryo (ATCC CL-173, purchased to American Tissue and Cell Culture, Manassas, USA). 3T3-L1 were maintained in Dulbecco's modified eagle's medium (DMEM; Sigma–Aldrich, St. Louis, USA), with 10% Newborn Calf Serum (Sigma–Aldrich, St. Louis, USA), 2% Glutamine (Sigma–Aldrich, St. Louis, USA), 1% penicillin/streptomycin (Invitrogen Life technologies, Waltham, USA), 1.5 g/L sodium bicarbonate and allowed to reach confluence. After 2 days (day 0), the differentiation was initiated by addition of an induction medium. This medium was composed by complete medium which is a mixture of high glucose (4.5 g/L) DMEM (Sigma–Aldrich, St. Louis, USA), supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen Life technologies, Waltham, USA), 1% penicillin/streptomycin (Invitrogen Life technologies, Waltham, USA), and 1.5 g/L sodium bicarbonate with a hormonal mixture composed of 2 mM insulin (Sigma–Aldrich, St. Louis, USA), 1 mM dexamethasone (Sigma–Aldrich, St. Louis, USA) and 0.25 mM isobutylmethylxanthine (Fluka, Sigma–Aldrich, St. Louis, USA). Three days later (day 3), the induction medium was replaced by complete medium supplemented with insulin (10 mg/L). At day 7, cultures with a differentiation yield higher than 80% were washed with phosphate buffered saline (PBS) and incubated in serum-free DMEM. Working solution of PBS (1xPBS) was prepared with 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and adjusted to 7.4 pH. After 24 h (day 8), the adipocyte conditioned medium was harvested from the adipocyte's cultures (Fig. 1), stored at −20 °C for the subsequent treatments [13]. This supernatant is also referred as the adipocyte secretome, since is composed by all the hormones, metabolites and cytokines and adipokines secreted by the mature adipose cells.

2.2. Cell cultures and in vitro experimental design

Human breast carcinoma cells MCF-7 (ATCC HTB22, purchased to American Tissue and Cell Culture, Manassas, USA) were cultured in DMEM (Sigma–Aldrich, St. Louis, USA), supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen Life technologies, Waltham, USA), 1% penicillin/streptomycin (Invitrogen Life technologies, Waltham, USA) and 3.7 g/L sodium bicarbonate and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were used between passages 17 to 29. Treatments were performed using DMEM, without supplementation, using different concentrations of glucose (1 g/L for low glucose and 4.5 g/L for high glucose). Conditioned medium was obtained of mature adipocyte growth from differentiation of fibroblasts

3T3-L1. Therefore, three experimental groups were used according to MCF-7 exposure to nutrients and adiposity conditions (Fig. 1). MCF-7 breast cancer cells were exposed to (1) low glucose medium (LG), (2) high glucose medium (HG) and to (3) adipocyte conditioned medium (CM) as defined previously [13].

2.3. Metabolic activity assay (MTT)

MCF-7 cells (1×10^5 cells/mL) were cultured following the described treatment procedures for 24 h. Cells were then washed twice with 1xPBS and subjected to MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, an index of cell viability and cell growth. Cells were incubated with MTT solution (Abcam, Cambridge, UK) at a final concentration of 0.5 mg/mL for 3 h and then lysed in DMSO. Optical density was measured at 550/560 nm in a UV–Vis spectrophotometer (MultiSkan Ascent, Thermo Fisher, Massachusetts, USA). The background absorbance of the medium in the absence of cells was subtracted. All samples were assayed in triplicate, and the mean value for each experiment was calculated. The results are given as mean ± SD and are expressed as percentage of control (LG), which was considered to be 100%.

2.4. Cell death assay

Cell death was determined by lactate dehydrogenase (LDH) activity of such enzymes released from dying cells. This reaction assumes stoichiometric relation of lactate to NADH (1:1) since NADH is formed for each lactate consumed. MCF-7 cells (1×10^5 cells/mL) were cultured 24 h with standard treatments and the analysis was performed spectrophotometrically by examination of the rate of NADH depletion at 340 nm using the Multiskan Ascent spectrophotometer (Thermo Fisher, Massachusetts, USA) with a Liquick Cor-LDH 30 kit (Cormay, Łomianki, Poland), according to the manufacturer's instructions.

2.5. Oxidative stress assay

Total antioxidant status (TAS) is a simple colorimetric method intended to analyze the global antioxidant capacity of sample, *i.e.*, measures everything with antioxidant effect. It was used TAS Randox® assay (Cat No. NX2332, Randox Laboratories, Ltd, Crumlin, UK) on the Multiskan Ascent spectrophotometer (Thermo Fisher, Massachusetts, USA), according to the manufacturer's instructions.

2.6. BrdU proliferation assay

This method is based on the cell ability to incorporate 5-bromo-2′-deoxyuridine (BrdU) into cellular DNA during cell proliferation. Then incorporated BrdU is detected using an anti-BrdU antibody. MCF-7 cells (1×10^5 cells/mL) were cultured with standard treatments for 24 h. Bromodeoxyuridine (BrdU) was added to each well and sequentially incubated for 24 h. The detection was performed using the colorimetric Cell Proliferation ELISA, BrdU (ab126556, Abcam, Cambridge, U.K.), according to the manufacturer's instructions.

2.7. Injury assay

The injury assay (or scratch-wound assay) is a simple, reliable, and reproducible assay commonly used to measure fundamental cell migration parameters such as speed, persistence, and polarity.

Confluent MCF-7 monolayers on 24-plate well, were wounded with a 10-µl pipette tip. 24 h after treatment, the migrated distance was photographed under an inverted microscope (Nikon Instruments Inc., Melville, USA) at a 100× magnification and the scratch closure was determined by measuring the injury width with Image J software (U. S. National Institutes of Health, Bethesda, USA).

2.8. Hormone and biochemical profile

MCF-7 cells (1×10^5 cells/mL) were cultured with standard treatments for 24 h. Analysis to estradiol (E2), prolactin, and luteinizing hormone (LH) were determined by electrochemiluminescence immunoassay (ECLIA) by modular analytics E170 COBAS e8000. Triglycerides and cholesterol were determined by enzymatic colorimetric method in modular analytics COBAS c111.

2.9. NMR (nuclear magnetic resonance)-based metabolomics

1H nuclear magnetic resonance (NMR) spectroscopy was used to quantify metabolites in MCF-7 cell culture media. NMR spectra were acquired using VNMRS 600 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with a 3 mm 1H(X)-PFG inverse configuration probe. Solvent-suppressed ^{1}H NMR spectra were acquired using following acquisition parameters: spectral width of 12 kHz, 4 s relaxation delay, 3 s acquisition time, 300 pulse angle, and 32 scans at 298 K. Sodium fumarate was used as internal reference (6.50 ppm) to quantify the following metabolites (multiplicity, ppm): lactate (doublet, 1.33), alanine (doublet, 1.45), acetate (singlet, 1.9), H1-α glucose (doublet, 5.22), glutamine (multiplet, 2.44), and pyruvate (singlet, 2.38) as previously described [14]. Spectra were processed using Topspin 3.2 software (Bruker, Ettlingen, Germany). The relative areas of 1H NMR resonances were quantified using integration routine provided by Amix 3.9.15 (Bruker Biospin, Germany) and the results were normalized by the number of cells present in the cell medium at the time of sample collection.

2.10. Statistical analysis

Results were expressed as mean ± SEM. Data were analyzed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA). Differences between samples and parameters were evaluated by Bonferroni. Statistical analysis was conducted through one-way ANOVA. Significance was set at $p < 0.05$.

3. Results

3.1. Adipocyte conditioned medium significantly increases viability and proliferation of MCF-7 cells

The results reveal a stable profile regarding proliferation and viability. A slight increase in viability was observed upon HG medium in comparison to cells exposed to LG, representing normoglycemia (Fig. 2A). Moreover, HG led to a significant augment in MCF-7 proliferation (Fig. 2B), being further enhanced after exposure to adipocyte CM.

No significant differences were found regarding cell death analyzed by LDH activity nor in the oxidative status analyzed by TAS (Fig. 2C and D).

3.2. Adipocyte conditioned medium modulates migration and cell spreading

We then performed the injury assay to examine cell motility. A significant increase in migration rate was observed when MCF-7 cells were exposed to adipocyte secretome, revealing a significantly higher aggressiveness ($p < 0.001$) under such conditions (Fig. 3).

3.3. MCF-7 cells hormone profile is altered in adipose conditioned medium

Hormone profile was determined in the medium surrounding MCF-7 cells at beginning of the treatment and after 24 h of exposure. Thus, levels of hormones are expressed as a differential in order to time. Interestingly, when exposed to adipocyte secretome, estradiol significantly diminished, whereas LH has significantly increased in

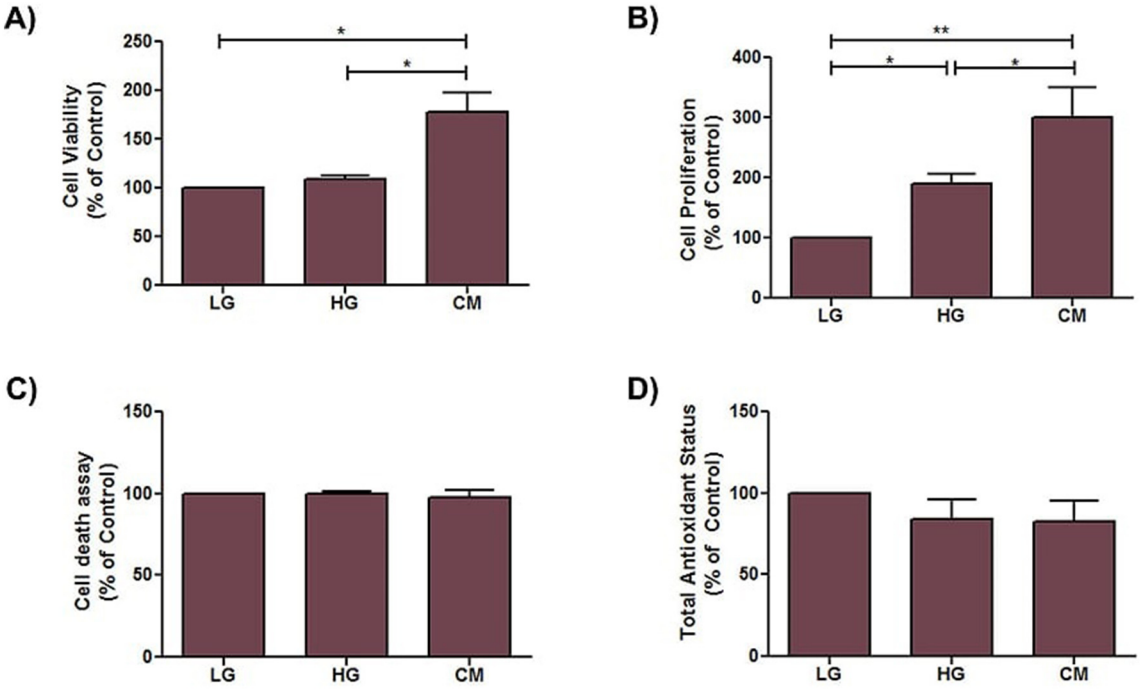

Fig. 2. Cell assays. (A) Cell Viability assay by MTT (B) cell proliferation assay by BrdU (C) cell death assay by LDH (D) cytotoxicity assay by TAS. LG: Low glucose; HG: high glucose; CM: conditioned medium. *$p < 0.05$; and **$p < 0.01$.

comparison to both LG and HG conditions (Fig. 4 and Table 1). Prolactin pattern did not change within the experimental conditions analyzed (data not shown).

3.4. Adipocyte conditioned medium changes MCF-7 cell lipid profile

Although HG incubation did not significantly affect triglyceride and cholesterol content as compared to LG conditions, MCF-7 cells exposed to the adipocyte conditioned medium significantly decreased both triglycerides ($p < 0.001$) and cholesterol ($p < 0.01$) as illustrated in Fig. 5 and Table 1.

3.5. NMR-metabolomics revealed altered glycolytic metabolism in MCF-7 cells cultured in adipose conditioned medium

No significant differences were observed between incubation with LG and HG for any of the glycolytic metabolites analyzed. However, in comparison to HG treatment, cells exposed to CM (adipocyte secretome) present an inverted tendency in glucose, lactate, alanine and pyruvate levels. CM led to a significant release of glucose, pyruvate and acetate, which were consumed upon hyperglycemia status (HG). In contrast, cells consumed a significant amount of lactate and glutamine when cultured in the presence of CM (Fig. 6 and Table 1).

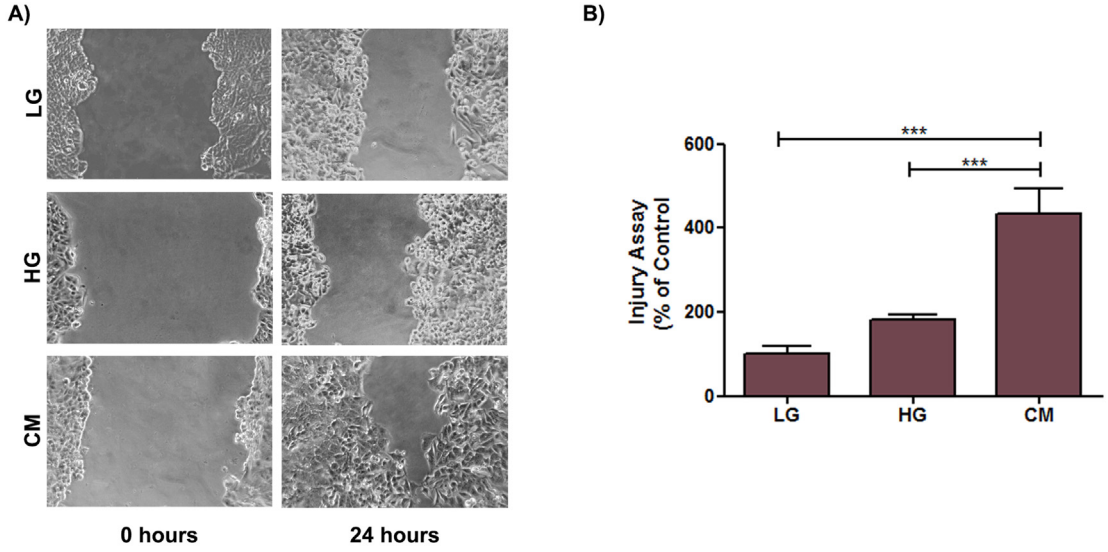

Fig. 3. *In vitro* motility, migration and spreading analysis of MCF-7 breast tumor cells. (A) Migration of MCF-7 cell after 24 h incubation period with the different treatments. Cell cultures were visualized under an inverted microscope at a 200× magnification. (B) Quantification of MCF-7 migration: Mean values (± SEM) of the three conditions in evaluation. LG: Low glucose; HG: high glucose; CM: conditioned medium. ***$p < 0.001$.

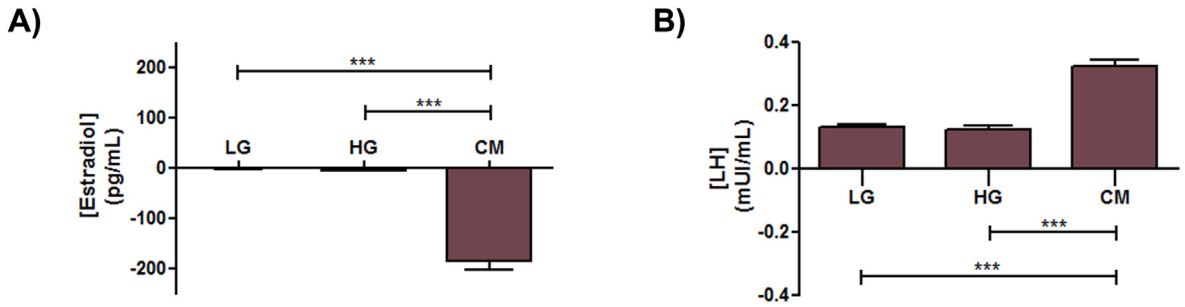

Fig. 4. Hormone profile of MCF-7 breast tumor cells. (A) Estradiol; (B) LH (luteinizing hormone). Plotted hormone levels were obtained by the difference between the hormone levels measured at 24 h and at the initial time point. LG: Low glucose; HG: high glucose; CM: conditioned medium. ***$p < 0.001$.

Table 1
Metabolites and hormones composition in the different mediums before and after cell treatments.

Analytes		Low glucose medium (LG)		High glucose medium (HG)		Adipocyte conditioned medium (CM)	
		Mean	SEM	Mean	SEM	Mean	SEM
Acetate	Time 0	1.84	0.11	1.71	0.07	2.03	0.04
(μmol $\times 10^{-9}/10^{6}$ cells)	End point	2.16	0.10	1.92	0.02	7.03	0.45
Alanine	Time 0	0.99	0.07	1.13	0.05	8.07	0.09
(μmol $\times 10^{-9}/10^{6}$ cells)	End point	1.58	0.10	1.73	0.04	8.04	1.35
Glucose	Time 0	3.91	0.07	21.8	0.62	6.71	0.08
(μmol $\times 10^{-8}/10^{6}$ cells)	End point	3.58	0.12	20.1	0.10	15.6	0.98
Glutamine	Time 0	2.33	0.05	3.71	0.12	3.08	0.04
(μmol $\times 10^{-8}/10^{6}$ cells)	End point	2.11	0.30	2.93	0.04	0.95	0.11
Lactate	Time 0	2.62	0.55	1.72	0.33	61.8	1.32
(μmol $\times 10^{-9}/10^{6}$ cells)	End point	5.83	0.07	7.41	0.05	41.6	3.46
Pyruvate	Time 0	5.24	0.02	5.43	0.22	4.34	0.14
(μmol $\times 10^{-9}/10^{6}$ cells)	End point	4.39	0.17	4.84	0.28	4.97	0.40
Cholesterol (mg/mL)	Time 0	0.67	0.58	1.00	0.00	3.33	1.15
	End point	1.00	0.00	1.33	0.58	1.33	0.58
Triglycerides (mg/mL)	Time 0	2.00	1.00	2.33	0.58	99.33	2.52
	End point	2.33	1.15	3.67	0.58	36.33	11.59
Estradiol (E2) (pg/mL)	Time 0	9.20	3.64	10.73	5.00	238.87	9.76
	End point	5.00	0.00	5.00	0.00	52.60	17.50
Luteinizing hormone (LH) (mU/mL)	Time 0	0.459	0.15	0.45	0.10	0.21	0.04
	End point	0.59	0.01	0.57	0.02	0.53	0.04

SEM - standard error of the mean.

4. Discussion and conclusions

Our *in vitro* experimental setup revealed that the presence of HG resulted in a significant increase in MCF-7 cell proliferation but not cell viability, apoptosis, antioxidant status or migration capacity. Nevertheless, obesity mimicking conditions led to increased aggressiveness of breast tumor cells, by significantly exacerbating cell viability, proliferation, and migration. Several studies have mentioned that adiposity is associated with a worse prognosis due to a more effectiveness survival profile which is in agreement with our results that show an increase of proliferation and tumor cell viability. Also, such observational studies have already demonstrated that a high Body Mass Index (BMI) is significantly associated with an increased risk of breast cancer [15,16].

Moreover, our findings that cancer cells become more aggressive in obese conditions, may result in enhanced recurrence of BrCa. Corroborating our findings, several epidemiological studies demonstrated worse survival rates among obese women with breast cancer in comparison to non-obese women. In addition, obese women are at higher risk of developing a second primary breast cancer [17].

One of the reasons for this worse prognosis might be explained by the estrogen source of adipose tissue. Most epidemiological evidence

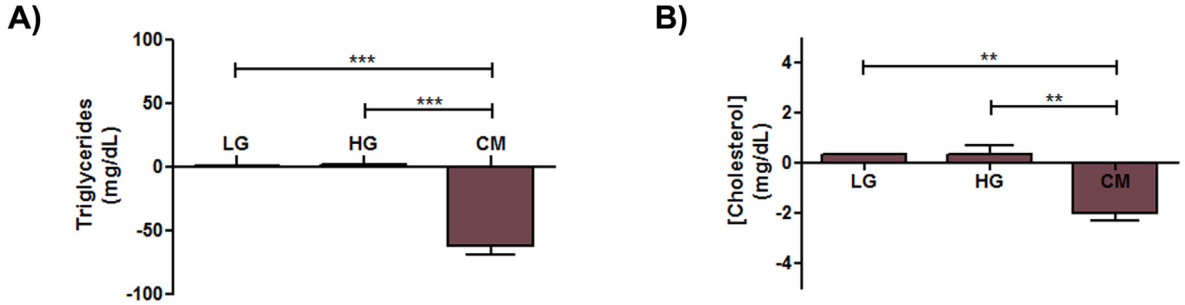

Fig. 5. Lipid profile of MCF-7 breast tumor cells. (A) Triglycerides; (B) cholesterol. Exhibited values represent the difference between the 24 h treatment period value and the initial value. LG: Low glucose; HG: high glucose; CM: conditioned medium. **$p < 0.01$; and ***$p < 0.001$.

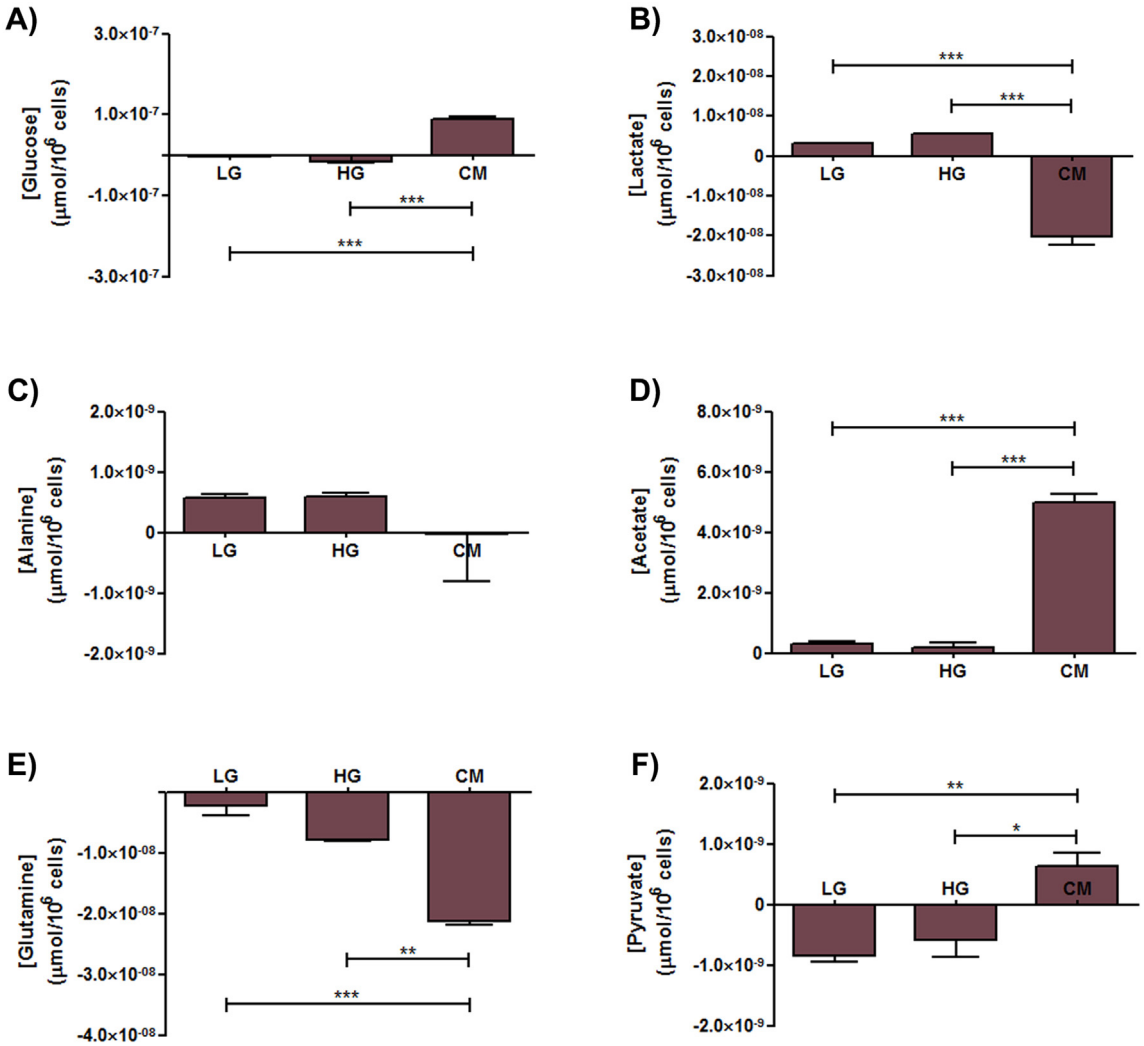

Fig. 6. Glycolytic profile MCF-7 breast tumor cells. (A) Glucose; (B) lactate; (C) alanine; (D) acetate; (E) glutamine; (F) pyruvate. Exhibited values represent the difference between the 24 h treatment period value and the initial value. LG: Low glucose; HG: high glucose; CM: conditioned medium. Significance levels: (*) $p < 0.05$; (**) $p < 0.01$; and (***) $p < 0.001$.

suggest that increase adiposity is associated to increase steroid hormone levels and that obesity may encourage carcinogenesis by increasing estrogen levels [38]. Adipose tissues up-regulate the conversion of androstenedione to estrone and testosterone to estradiol by aromatase activity, which is increased in obesity [18,19] while at the same time reducing sex hormone-binding globulin capacity which leads to increased levels of free, biologically active estrogens [20]. In postmenopausal women, aromatization of androgens in the adipose tissue by aromatase elevates local and circulating levels of estrogen [21]. It has been known for long, that estradiol has proliferative effects on estrogen-dependent cancer cells like MCF-7 cell line [22]. Moreover, MCF-7 cells may produce endogenous levels of estradiol from estrone by aldo-keto reductases (AKRs), which act in a paracrine fashion in tissues such as the breast [23,24].

According to our study, estradiol was not detected neither in normoglycemia nor in hyperglycemia medium (LG and HG). Conversely, estradiol levels were detected in adipocyte conditioned medium (CM). However, after 24 h, its levels significantly decreased in cell medium, suggesting that Estradiol has entered MCF-7 cells, activating estrogen receptors-depending genes involved in proliferation. These findings led us to hypothesize that Estradiol triggers proliferation of MCF-7 tumor cells in adipocyte conditions.

Although estrogens are involved in the association between obesity and breast cancer, we cannot exclude other mechanisms. Accordingly, increased levels of insulin and IGF-I have been reported in elevated adiposity conditions [25].

Another important pathway involves LH. Chronic administration of agonistic analogues of luteinizing hormone-releasing hormone (LHRH) causes regression of mammary tumors in experimental animals [26], thus indicating that LH may have an anti-proliferative effect on breast cancer cells. Recently LH and FSH were described to act directly on breast cancer cells, increasing motility and invasion [27]. Accordingly, the levels of LH were also highly increased in our CM which also promote a more aggressive profile under adiposity conditions.

Interestingly enough, the overall energy metabolism is altered when cells are cultured with adipocyte CM. Consumption of triglycerides and cholesterol was induced in MCF-7 cells upon adipose conditions, probably due to the availability of these compounds in CM. It has been established that higher levels of triglycerides and cholesterol in human serum are associated with a worst prognosis in breast cancer [28,29]. An increase of cholesterol uptake renders cells the building blocks for membrane synthesis, conferring cells to undergo cell division [30], thus promoting proliferation observed in adipose secretome medium. Previous studies reveal that the cholesterol metabolite, 27-hydroxycholesterol (27HC) is associated with tumorigenesis and tumor growth both in mouse models and in human breast cancers by being a partial agonist for the estrogen receptor and the liver X receptor [31].

Triglyceride metabolism in breast tumor cells provides cells with a

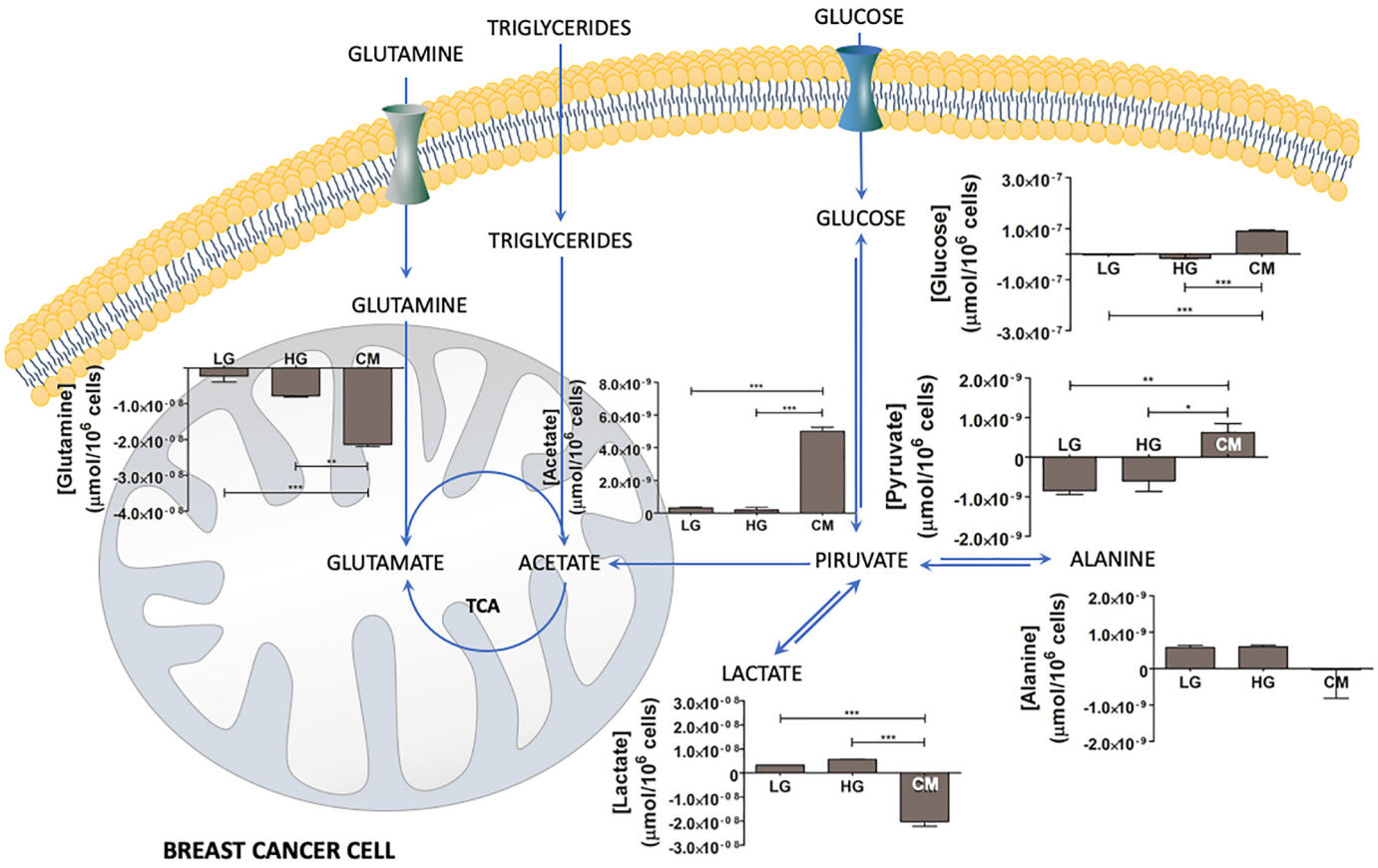

Fig. 7. Representative model of the central energetic metabolism of MCF-7 breast cancer cell under different nutritive status in which under adiposity generated by adipose conditioned medium Warburg effects inverts with lowering lactate and *de novo* synthesis of glucose. Increased pyruvate in adiposity benefits from the contribution not only from lactate but also from amino acid catabolism. Cancer Krebs cycle under adiposity is feed from lipid and amino acid catabolism. In particular lipolysis and beta-oxidation but also from deamination of glutamine (glutaminolysis). TCA: Tricarboxylic acid cycle. LG: Low glucose environment; HG: high glucose environment; CM: adipose conditioned environment.

source of fatty acids for energy metabolism. Fatty acids can be catabolized into acetyl-CoA, thus justifying the production of acetate in adipose CM conditions. We, therefore, hypothesize that the increase of acetate and the triglycerides consumption are associated with the catabolism of fatty acid, providing fuel for breast cancer cells.

Inversely, glycolytic metabolism was downregulated as observed by the increase in glucose and pyruvate release concomitant with the reduction in lactate in media of MCF-7 cells incubated with CM for 24 h. An abnormal glucose metabolism has been observed in several disorders like Non-Alcoholic Fatty Liver Disease (NAFLD), pancreatic cancer, cystic fibrosis, and in Diabetes [32–34]. Abnormal glucose metabolism has been transversally associated with obesity and estrogen receptors activity.

In addition, cancer cells are high glutamine consumers. The use of glutamine by tumor cells is associated with increased cancer aggressiveness and progression. This amino acid plays a central role in protein metabolism, being used for amino acid derived molecules or incorporated within proteins. Our study showed that glutamine is consumed in the three conditions, with a higher consumption in obesity, confirming the high metabolic status of MCF-7 in obesity mimicking conditions.

Altogether, the findings obtained in the current study confirm that obesity secretome plays a crucial role in aggressiveness of MCF-7 breast cancer cells, by conferring these cells the capacity to grow and migrate. These effects are accompanied by increased consumption of estradiol, as well as energy lipid fuels, common released by adipocytes such as triglycerides and cholesterol. The use of lipids as an energy source is corroborated by the increased release of acetate, an acetyl-coA-derived molecule, but also of glucose and pyruvate, accompanied by a decrease in lactate. Amino acids, such as alanine and glutamine are also significantly consumed by MCF-7 cells when exposed to adipocyte CM.

Fig. 6 illustrates the array of metabolites changed, underlying the metabolism of tumor cells when exposed to an adipose environment. Tumor cells undergo aerobic glycolysis even in the presence of effective levels of oxygen. This outcome, known as the Warburg effect, is not the most efficient mechanism to generate ATP, but is thought as an adaptation tool to facilitate the uptake and incorporation of nutrients [35]. In normoglycemia and hyperglycemia conditions, there was a direct consumption of glucose and pyruvate to the production of glucose end products like lactate, alanine and acetate, as expected by the Warburg effect. However, it is interesting to observe a metabolic shift pattern on breast cancer under adiposity in the substrates involved in Warburg effect. In such adiposity environment, our results suggest that breast cancer cells may produce glucose *de novo* from pyruvate. Also, our results indicate that pyruvate is increasing essentially by the contribution of lactate and alanine. This inversion to Warburg effect in cancer cells under an adiposity environment may be also driven in part to the high beta-oxidation that increases enormously acetate levels and the increased glutaminolysis which feed tricarboxylic acid cycle (TCA).

In such adiposity environment, breast cancer cells undergo towards a catabolic pattern of lipids and amino acids as observed in the reverse Warburg effect [10,11]. Amino acid catabolism (oxidative deamination and transamination), in particular from alanine and glutamine which provide for the increase of pyruvate and anaplerotic contributes to TCA respectively, although computational studies revealed that contribution of glutamine alone was not significant for the tumor feeding [36].

Regarding to lipid metabolism the amount of triglycerides in the tumor cell vicinity favors lipolysis and further beta-oxidation with the generation of considerable amounts of acetate. Therefore, the increase of energy production by aerobic respiration of cancer cells under an adiposity environment as well as the increase of gluconeogenesis substrates drives tumors cell to the "Warburg Effect Inversion" (Fig. 7). This is an important finding in the sense that, conversely to reverse Warburg effect which depends strictly from the stromal microenvironment, cancer cell will shift it metabolic pattern in high adiposity context inverting its routes without stromal contribution. This was also observed in other tumors [37].

The mechanisms underlying tumor pathogenesis are complex and multifactorial. Therefore, further studies are necessary to clarify these events in breast tumor cells subjected to an adipose environment. Nevertheless, the present results show that changes in metabolic pathways are probably an important feature to address for developing innovative therapeutic approaches in breast cancers under an obesity phenotype.

Conflicts of interest

Author declaration

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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