MitoFit Preprints 2022.24 https://doi.org/10.26124/mitofit:2022-0024

Open Access

Article type

Cite
Moreno-Sánchez R, Robledo-Cadena DX, Pacheco-Velázquez SC, Rodríguez-Enríquez S (2022) Estimation of energy pathway fluxes in cancer cells- beyond the Warburg effect. https://doi.org/10.26124/mitofit:2022-0024

Author contributions
Data collection and evaluation was performed by DXRC, SCPV, SRE. RMS and SRE wrote the manuscript. SRE and RMS designed the framework of the review.

Conflicts of interest
The authors declare no conflict of interest.

Received 2022-06-08
Accepted 2022-06-20
Online 2022-06-21

Data availability
Raw data used for this manuscript is available upon reasonable request to the corresponding author.

Keywords – Cell ATP supply; Cancer cell migration; Glycolysis; Oxidative Phosphorylation; Oxygen uptake.

Estimation of energy pathway fluxes in cancer cells - beyond the Warburg effect

Rafael Moreno-Sánchez[1*], Diana Xochiquetzal Robledo-Cadena[2], Silvia Cecilia Pacheco-Velázquez[3], Sara Rodríguez-Enríquez[3*]

[1] Fac Estudios Superiores Iztacala, Univ Nacional Autónoma de México. Los Reyes Ixtacala, Hab. Los Reyes Ixtacala Barrio de los Árboles/Barrio de los Héroes, Tlalnepantla 54090. México
[2] Departamento de Bioquímica, Instituto Nacional de Cardiología, Ciudad de México 14080. México.
[3] Carrera de Medicina. Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México. Los Reyes Ixtacala, Hab. Los Reyes Ixtacala Barrio de los Árboles/Barrio de los Héroes, Tlalnepantla 54090. México

* Corresponding authors:
rafael.moreno@iztacala.unam.mx;
saren960104@hotmail.com

Abstract

Glycolytic and respiratory fluxes were analyzed in cancer and non-cancer cells. The steady-state fluxes in energy metabolism were used to estimate the aerobic glycolytic and mitochondrial (oxidative phosphorylation, OxPhos) contributions to the cellular ATP supply. The rate of lactate production — corrected for the fraction generated by glutaminolysis — is proposed as the appropriate way to estimate glycolytic flux. In general, the glycolytic rates estimated for cancer cells are higher than those found in non-cancer cells, as originally observed by Otto Warburg. The rate of ROUTINE *R* cellular O_2 consumption corrected for LEAK respiration *L* measured after inhibition by oligomycin (a specific, potent and permeable ATP synthase inhibitor) becomes the respiratory *R-L* net ROUTINE capacity, which is proposed as the appropriate way to estimate mitochondrial ATP synthesis-linked O_2 flux or net OxPhos flux in living cells. Detecting non-negligible O_2

consumption rates in cancer cells has revealed that the mitochondrial function is not impaired, as claimed by the Warburg effect. Furthermore, when calculating the relative contributions to cellular ATP supply, under a variety of environmental conditions and for several different types of cancer cells, it was found that OxPhos was the main ATP provider over glycolysis. Hence, OxPhos targeting can be successfully used to block in cancer cells ATP-dependent processes such as migration. These observations may guide the re-design of novel targeted therapies.

1. Introduction

Nowadays it seems a common practice to make predictions and reach conclusions on biological behaviors such as metabolic traits, acquisition of specific phenotypes, drug effects and targeting based on DNA sequencing (and presence of certain genes or genomics), gene expression (mRNA levels or transcriptomics) or even protein contents (proteomics). These "omics" approaches are used as monitors or markers assessing cell behavior to follow-up drug treatments and phenotype variations. However, it should be noted that (*1*) the existence of genes coding for relevant proteins does not warrant their efficient and timing expression; (*2*) the transcript levels do not always correlate with the corresponding protein contents; (*3*) the protein levels do not always correlate with the enzyme/transporter activities either; and (4) enzyme/transporter activities do not often correlate with variations in the corresponding metabolite concentrations or pathway fluxes.

Regrettably, these "omics" approaches do not include the analysis of the *function* under study and the multitude of *regulation mechanisms* involved at the different levels of biological complexity [1]. Thus, trying to understand biological behaviors based solely on mRNA or protein level variations may clearly lead to partial, biased overall pictures and even misinterpretations. Therefore, to overcome these severe limitations of the typical "omics" approaches, the cellular or biological function must be directly assessed [2, 3]. This can be done (*1*) locally, by determining the enzyme/transporter activities contained within the cell and their kinetic properties (i.e. kinetomics) under physiological temperature, pH, and high K^+ media; and (*2*) systemically, by measuring the fluxes of the metabolic pathway (i.e, fluxomics) or the biological function (cell growth, migration, motility) under study [1]. Then, by applying a more "omics" integral analysis, including function determination, it may be achievable to fully understand how a biological function or behavior works, is regulated, and could be manipulated.

It is worth noting that the assessment of functional variations carries the advantage of intrinsically incorporating the changes made in gene expression and translation, in the correct protein folding to generate an active protein (and not an inactive protein), and in the synthesis/degradation of each protein component of the function evaluated, as well as the regulatory mechanisms involved in each of the processes.

It is well established that energy metabolism of cancer cells is altered and this feature has become one of the emerging cancer hallmarks [4]. Such cancer hallmarks are

currently studied at the genomics, transcriptomics and proteomics levels, leading to conclusions and proposing clinical treatments. However, the altered or reprogrammed cancer energy metabolism has also been analyzed at the functional level allowing to reach conclusions that are not always in consonance with those derived from the canonical omics approaches.

For instance, in the early and pioneering studies of cancer energy metabolism by Warburg (1956) [5], it was described that tumor cells have an increased glycolytic capacity in the presence of oxygen. Indeed, the glycolytic flux is several-fold higher in hepatoma than in normal liver under normoxia ($[O_2]$>25 μM); glycolytic flux is even much greater in cancer cells than in non-cancer cells under hypoxia ($[O_2]$ <20 μM). However, Warburg originally proposed that mitochondria (and hence mitochondrial function) were impaired in cancer and the ensuing energy deficiency induced the observed increased glycolysis. The Warburg hypothesis has been profusely confirmed throughout the years regarding the glycolytic statement, i.e. that the majority of cancer cell types show an enhanced glycolytic capacity in comparison to their healthy tissues of origin [3, 6]. However, the statement on mitochondrial impairment in the Warburg hypothesis has not been experimentally examined in a systematic fashion.

As cancer glycolysis is enhanced, it has been generally assumed that the ATP required for the cell functions is mainly or only provided by this cytosolic energy metabolism pathway, without an appropriate experimental assessment of both glycolysis and oxidative phosphorylation (OxPhos) pathways in each cancer cell type [2]. The glycolytic flux has been determined by measuring the rate of lactate production in cells incubated with added external glucose. Cancer cells actively consume glutamine and glutaminolysis as well as glycogen degradation may generate lactate. Then, to correct for any overestimation of the glycolytic flux from external glucose in cancer cells, lactate production should be also determined in the absence of external glucose (to assess lactate derived from glycogen degradation) and in the presence of a glycolytic inhibitor such as 2-deoxyglucose (2-DG). The remaining lactate production in the presence of 2-DG may be assumed to derive from glutaminolysis, which should be completely inhibited by a respiratory electron transfer inhibitor.

Often, the rate of external glucose uptake is used to assess glycolysis. However, one has to be aware that not all metabolized glucose through the glycolytic pathway generates lactate (and cytosolic ATP), because a significant glucose fraction may be drained to the branching pathways. The lactate/glucose ratio for glycolysis under physiological conditions is usually lower than 2 (1.3-1.9) [3]. Then, it is more accurate to determine the rate of lactate production, instead of glucose uptake or extracellular acidification, as a more adequate parameter of glycolytic ATP synthesis (1 lactate/ 1 ATP) and correcting for lactate formed from other sources.

Lately, the advent and availability of high-resolution respirometry has allowed to assess respiratory rates of smaller samples of living cells, revealing functional and very active mitochondria in many cancer cell types [3]. Likewise, OxPhos, as determined by the rate of cellular oxygen consumption in studies with cancer cells, is also usually over-estimated since the activity of several non-mitochondrial oxygen-consuming enzymes (heme oxygenase and cytochromes P450) are significantly over-expressed in cancer cells [7, 8], and the inner membrane mitochondrial H^+ passive diffusion may also consume oxygen at significant rates.

To correct for any over-estimation from total O_2 uptake and specifically assess OxPhos flux, the rate of oligomycin sensitive O_2 consumption has to be evaluated. To transform this measurement into the actual rate of mitochondrial ATP synthesis, a P/O ratio of approximately 2.5 for NADH-linked oxidizable substrates and 1.5 for $FADH_2$-linked substrates is applied. For intact cell determinations, the P/O ratio for NADH-linked substrates is commonly used because most of the carbon sources oxidized by *in situ* mitochondria generate NADH [9]. These P/O ratios were experimentally determined in hepatoma isolated mitochondria [10]. Oligomycin is a highly specific inhibitor of the mitochondrial ATP synthase; it is also membrane permeable and exhibits high potency (tightly-bound inhibitor with a dissociation constant K_d in the low nanomolar range). The only apparent drawback of this molecule is that it is a slow inhibitor *i.e.*, it may take several minutes to fully interact with its specific target when mixed with cell suspensions (approximately up to 5 min at 37 °C and longer at lower temperatures).

Therefore, the generalized belief in the cancer biology field that glycolysis predominates over OxPhos for ATP supply in cancer cells and tumors should be re-evaluated and experimentally determined for each particular type of cancer cells under each particular environmental condition. In the present work, we have experimentally assessed the contributions of glycolysis and OxPhos to the cell ATP supply in different metastatic and non-metastatic cancer cells and these values were compared to non-cancer cells.

2. Material and methods

2.1. Cancer cells in bi-dimensional cultures

Several human cancer cells from different origins and metastatic stages were used in this study as well as non-cancer cell lines. Human triple negative breast cancer (metastatic MDA-MB-231 and MDA-MB-468), estrogen receptor positive breast cancer (low metastatic MCF-7), cervix cancer (metastatic HeLa), HEK-293 (human embryo kidney) cells and non-cancer rodent 3T3 fibroblasts (American Type Culture Collection; Rockville, MD, USA) were grown in Petri culture dishes in 20 mL DMEM medium supplemented with 10 % fetal bovine serum and 10,000 U penicillin/streptomycin. The cells were incubated under 5 % CO_2/95 % air at 37 °C [11]. After reaching 80–90 % confluence, cells were carefully removed with trypsin/EDTA and washed with fresh Krebs-Ringer (KR, containing 125 mM NaCl, 5 mM KCl, 25 mM HEPES, 1.4 mM $CaCl_2$, 1 mM KH_2PO_4, 1 mM $MgCl_2$, pH 7.4) buffer at 25 °C. Cell viability (> 90 %) was determined by using the trypan blue assay [11]. For authentication, cancer cell genotyping analyses were performed by the National Institute of Genomic Medicine (INMEGEN, México); the results revealed that the cancer cell lines used shared more than 90 % out of 100 % alleles, reported by the ATCC.

2.2. Multi-cellular tumor spheroids (MCTS)

For MCTS, HeLa and HEK-293 cells were used. MCTS were formed from monolayer cultures by using the liquid overlay modified technique [6]. Briefly, 1×10^5 cells were seeded in 2 % (w/v) agarose-coated Petri dishes. After five days of culture spheroids reached a diameter of ≈100 μm. Then, old medium was replaced with fresh DMEM medium and MCTS were placed under slow (20–50 rpm) orbital shaking for further 20 days at 37°C under 95 % air/5 % CO_2. Old medium was replaced with fresh medium every

2 to 3 days to remove cellular debris. MCTS spheroid size was measured daily with a graduated reticule (1/10 mm; Zeiss, NY, USA) in an inverted phase contrast microscope (Zeiss, NY, USA) [6].

2.3. Glycolysis determination

For glycolytic flux, intact cancer cells (2-3 mg protein/mL) were incubated in 1.5 mL KR buffer. Aerobic glycolysis was carried out by using cells incubated in plastic flasks in an orbital shaking water bath at 37°C. The reaction was started by the addition of exogenous glucose (5 mM). At time zero and 10 minutes, the reaction was stopped by mixing a cell aliquot with 3 % (w/v) cold perchloric acid and centrifuged. In parallel sets of experiments, cells were also incubated for zero and 10 min with 2-deoxyglucose (2-DG, 10 mM) to correct for lactate production by glutaminolysis. Residual lactate production (2-DG insensitive lactate formation, *i.e.* glutaminolysis) was totally blocked by 5 µM rotenone (inhibitor of respiratory chain site 1). For cellular extracts, each supernatant was neutralized with 1 N KOH/100 mM Tris. Lactate was enzymatically determined by using lactate dehydrogenase (LDH) Roche, Mannheim, Germany) following the NADH formation at 340 nm [12].

2.4. Oxidative phosphorylation (OxPhos) determination

For OxPhos flux, cancer cells (1 mg protein/mL) were incubated at 37°C in 1.9 mL of air-saturated KR buffer *plus* 5 mM glucose and the ROUTINE *R* cellular O_2 consumption and LEAK *L* respiration rates (which was apparent after adding 5 µM oligomycin) were determined by using a high resolution Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria). To estimate mitochondrial ATP synthesis-linked O_2 flux or net OXPHOS flux in living cells, the respiratory *R-L* (ROUTINE O_2 consumption *minus* LEAK respiration) net ROUTINE capacity was calculated [13].

2.5. ATP determination from energy metabolism pathways

The contribution of OxPhos and glycolysis to the cellular ATP supply was determined, respectively, from the respiratory net ROUTINE capacity (after acquiring the oligomycin sensitive respiration rate, as explained above) multiplied by the ATP/O ratio of 2.5 [10] and from the rate of lactate production, assuming a stoichiometry of 1 mol of ATP produced per 1 mol of lactate produced.

2.6. Invasiveness assay in metastatic cancer cells in bidimensional cultures

MDA-MB-231 and MDA-MB-468 cells were incubated in free-serum DMEM for 24 h under normoxia (21 % O_2). Afterwards, the cells were washed, resuspended in free-serum DMEM medium, and placed in the upper compartment of 96-multiwell Boyden chambers (Merck Millipore, MA, USA) at a final concentration of $5 \cdot 10^4$ cells per well and 37 °C. The Boyden chamber lower compartment was filled with free-serum DMEM. After 24 h, the number of cells collected in the lower chamber compartment was determined with 60 nM calcein-AM after 60 min incubation. Fluorescence was detected at 485 nm excitation and 520 nm emission in a microplate reader (Nunclon, Roskilde, Denmark). By titrating with oligomycin (0, 0.1, 1 and 10 µM) the dependence of invasiveness on OxPhos was assessed [14].

2.7. Migration assay in metastatic cancer cells in bidimensional cultures

MDA-MB-231 and MDA-MB-468 cells were grown in complete DMEM medium in multiwell-plates (5 × 10^5 cells/well) under normoxia (21 % O_2). After 80–90 % confluence was reached, the cell culture was wounded by using a plastic tip (wound healing assay), washed twice with 37°C PBS (155 mM NaCl, 1.5 mM KH2PO4, and 2.7 mM NaH2PO4, pH 7.2), and further incubated with fresh non-serum DMEM with oligomycin (0, 0.1, 1 and 10 μM). The cellular migration was taken at 0 and 24 h with an inverted microscope (Zeiss, Thornwood, NY, USA). For each experiment, the cellular migration distance from the border to the center of the Petri dish was measured with a graduated reticule (Zeiss, Thornwood, NY, USA) [14]. Titrating with oligomycin the dependence of migration on OxPhos was assessed.

3. Results

3.1. Glycolytic flux determination in cancer cells

Glycolytic flux was determined by measuring the total lactate production by each cell type (cytosolic + external), after adding external glucose. Lactate was enzymatically determined by using commercial lactate dehydrogenase (LDH) following the NADH formation at λ_{exc}= 340 nm [11] or following the changes in the NADH fluorescent signal at λ_{em}= 450-460 nm and λ_{exc}= 340 nm (Figure 1A). Total lactate content in all cancer bi-dimensional (1.4-3 times, except for MDA-MB-231 cells) and MCTS (3-6.7 times) cultures was higher than that found in non-cancer 3T3 fibroblasts (Table 1), supporting the well-known glycolytic phenotype of cancer cells. It is widely known that lactate can also derived from other sources such as glycogen breakdown and glutamine oxidation in cancer cells [15]. Thus, to reveal the actual glycolytic flux, cancer cells must be incubated in the presence of the glycolytic inhibitor 2-deoxyglucose (2-DG) and in the presence of any respiratory chain inhibitor such as rotenone (Table 1).

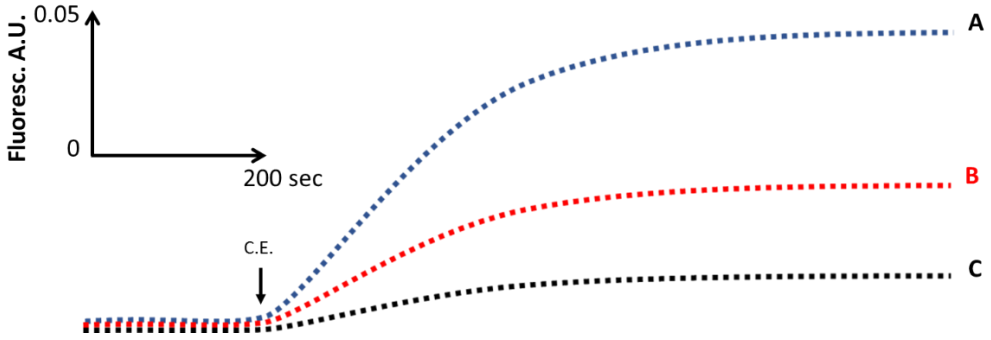

Figure 1. Representative image showing glycolysis determination in HeLa cancer cells by measuring the content of lactate. HeLa cells were exposed to 5 mM glucose for 10 min incubation (time=10 min) in the absence **(A)** or in the presence of 2-DG **(B)**. In **(C)** HeLa cells were exposed to 5 mM glucose for 15 s incubation. (time= zero). Lactate was enzymatically determined by using commercial LDH following the NADH formation, after stopping the reaction by mixing cells with PCA. Cellular extracts were added at 2-3 mg/mL protein. Abbreviations: C.E., cellular extracts; Fluoresc. A.U., Fluorescence arbitrary units.

Glycolytic net flux (2-DG sensitive lactate production flux) was close (HeLa and HeLa mutated p53 R248Q) or similar (young and mature HeLa and Hek-293 MCTS) to the

measured total lactate production (Table 1), indicating that glutaminolysis for lactate contribution was zero in MCTS or negligible (<10 %) in bi-dimensional cultures.

For the other cancer cell lines, glutaminolysis was an important carbon source for lactate production providing >20 % of total lactate. Similar to total lactate production, glycolysis was elevated (1.6-3.4 times) in cancer cells compared to 3T3 fibroblasts, except for MDA-MB-231 and HEK-298 cells. Regarding glutamine oxidation, analysis was solely performed in bi-dimensional cancer cultures. Glutaminolysis (rotenone-sensitive lactate formation) was negligible (<10 %) in HeLa and HeLa mutated p53 R248Q cells; however, in Hela cells with overexpressed wild type p53, MCF-7, and MDA-MB-231 cells the contribution of glutaminolysis to the total lactate production was from 25 to 50 % (Table 1).

Table 1. Total lactate production and glycolytic fluxes in cancer and non-cancer cells in bi-dimensional cultures and in multicellular tumor spheroids (MCTS). For young MCTS, diameters were <500 μm; for mature MCTS, diameters were >500 μm. Total lactate production and glycolytic ratesare expressed in nmol/min/mg cellular protein. (*n*=2). N.D., not determined.

	Total lactate	Glycolysis	Glutaminolysis
Cancer cells			
human cervix HeLa cells	17	16	1
human cervix HeLa cells with overexpressed wild type-p53	34	17	17
human cervix HeLa cells with overexpressed mutated p53 (R248Q)	35	34	1
ER+ human breast low metastatic MCF7 cells	23	17	6
human breast triple negative MDA-MB 231 cells	14	10	4
human breast triple negative MDA-MB-468 cells	19	16	3
transformed human embryo kidney HEK-293 cells		9	N.D.
Non-cancer cells			
rodent 3T3 fibroblasts	12	10	N.D.
Multicellular tumor spheroids (MCTS)			
human cervix HeLa young MCTS	39	39	N.D.
human cervix HeLa mature MCTS	60	60	N.D.
transformed human embryo kidney HEK-293 young MCTS	38	38	N.D.
transformed human embryo kidney HEK-293 mature MCTS	81	81	N.D.

3.2. OxPhos flux determination in cancer cells

In several studies in cancer cells, the total cellular oxygen consumption has been measured as indicative of OxPhos flux [16]. This assumption is incorrect because there are several over-expressed non-mitochondrial oxygen-consuming enzymes (heme oxygenase and cytochromes P450) in cancer cells, as well as a high inner mitochondrial membrane H^+ passive diffusion that contribute to a high oxygen consumption at significant rates, masking the actual OxPhos flux value. Therefore, total (ROUTINE) oxygen consumption must be corrected by using oligomycin, a canonical ATP synthase inhibitor (Figure 2).

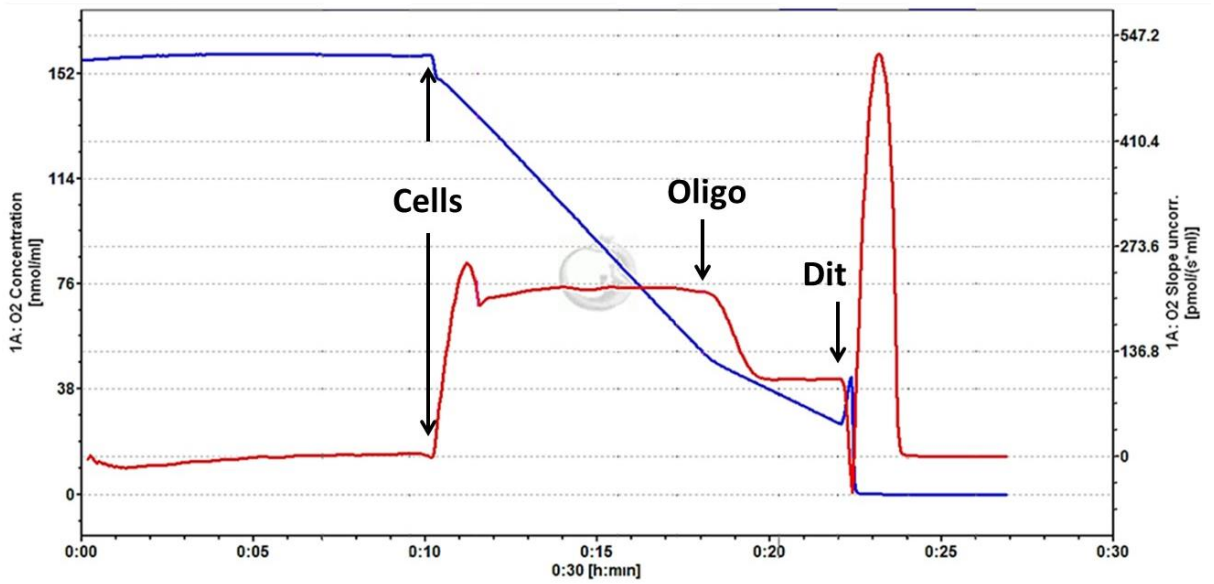

Figure 2. Representative image showing ROUTINE oxygen consumption in MDA-MB-231 (1 mg/mL) cells by using an Oroboros high-resolution respirometer. To reveal net OxPhos flux, the ATP synthase inhibitor oligomycin (Oligo, 5 µM) was added. Sodium dithionite (Dit) was added to the end of each experiment to reach oxygen chemical zero. Blue line represents the oxygen concentration; red line represents the oxygen flux (pmol/s*ml) . Cellular viability was higher than 80 % in the presence of oligomycin. Slope uncorrected

Indeed, total cellular ROUTINE oxygen consumption in all assayed bi-dimensional cultured cells was higher (35-64 %) than net OxPhos (oligomycin-sensitive oxygen consumption, Table 2) flux, including non-cancer cells, indicating the presence of active oxygen-consuming enzymes not associated to mitochondrial ATP synthesis. Net OxPhos flux was very similar in HeLa, HeLa mutated p53, MDA-MB-231 and MDA-MB-468 cancer cells *vs.* fibroblasts. Moreover, the net OxPhos flux in HeLa wild type p53, MCF-7 and HEK-293 cells was higher (2.7-3.8 times) *vs.* non-cancer fibroblasts (Table 2). Similarly, small and young cancer spheroids with diameters less than 500 µm (HeLa, HEK293) maintained an OxPhos flux greater than that of large mature cancer spheroids with diameters upper than 500 µm (Table 2).

3.3. ATP contribution from glycolysis and OxPhos

The contribution of glycolysis to the cellular ATP supply ($J_{ATPglyc}$) was determined from lactate production, assuming a stoichiometry of 1 mol of ATP produced *per* 1 mol of

lactate produced. The contribution of OxPhos to the cellular ATP supply ($J_{ATPOxPhos}$) was determined from the net respiratory ROUTINE (*R-L*) capacity (*i.e.*, using the oligomycin-sensitive respiration rate) and assuming an ATP/O ratio of 2.5 [10].

Table 2. Total oxygen consumption (ROUTINE respiration) and net OxPhos fluxes (*R-L* net ROUTINE capacity) in cancer and non-cancer cells in bi-dimensional cultures and in multicellular tumor spheroids (MCTS). For young MCTS, diameters were <500 µm; for mature MCTS, diameters were >500 µm. Total O_2 consumption and OxPhos are expressed in ng atoms oxygen/min/mg cellular protein (*n*=2).

	Total O_2 consumption	OxPhos
Cancer cells		
human cervix HeLa cells	16	10
human cervix HeLa cells with overexpressed p53	25	16
human cervix HeLa cells with overexpressed mutated p53 (R248Q)	9	5
ER+ human breast low metastatic MCF7 cells	38	18
human breast triple negative MDA-MB 231 cells	17	8
human breast triple negative MDA-MB-468 cells	28	10
transformed human embryo kidney HEK-293 cells		23
Non-cancer cells		
rodent 3T3 fibroblasts	10	6
Multicellular tumor spheroids (MCTS)		
human cervix HeLa young MCTS		27
human cervix HeLa mature MCTS		5
transformed human embryo kidney HEK-293 young MCTS		21
transformed human embryo kidney HEK-293 mature MCTS		8.5

Therefore, the total rate of ATP production from both energy pathways was calculated as follows: $J_{ATP\ cellular\ production} = J_{ATPglyc} + J_{ATPOxPhos}$. In the steady state, $J_{ATP\ production}$ is equal to the total rate of ATP consumption, $J_{ATP\ consumption}$. For comparison, $J_{ATP\ cellular\ production}$ was normalized to 100 % and the contribution to ATP supply from each pathway is given as a percentage (Table 3).

Most of the evaluated cancer cells and non-cancer cells in bidimensional cultures, as well as young MCTS, mainly dependeded on OxPhos for ATP supply (Table 3). In contrast, HeLa mutated p53 cells and mature MCTS depended mainly on glycolytic ATP.

3.4. Dependence of cellular migration and tumor invasiveness on OxPhos ATP supply

Our results (Table 3) clearly indicated that the principal ATP supplier in cancer cells was OxPhos. Because metastasis has been proposed as a highly ATP-dependent pathway in cancer cells [14], two processes involved in metastasis such as cellular migration and invasiveness were determined in the high metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-468; and the sensitivity to the ATP synthase inhibitor, oligomycin (Figures 3A and 3B) was determined. Cancer cell invasiveness was not significantly affected by oligomycin at doses (0.1 and 1 μM) in which OxPhos was severely suppressed (40-80 %). In this condition and applying metabolic control analysis (MCA) [17, 18], OxPhos exerted low control on cancer cell invasiveness; the flux control coefficient (calculated from the initial slope starting at the 100 %, 100 % coordinates, which is the reference, control point) was zero (MDA-MB-468 cells) or <0.01 (MDA-MB-231 cells) (Figure 3A). These observations suggested that cancer cell invasiveness was not controlled by ATP supply from OxPhos, and that other processes perhaps like specific protein synthesis (exoproteases; epithelial-mesenchymal transition proteins) mainly govern this cell function. To test this hypothesis, experimentation with specific glycolytic inhibitors (2-DG, gossypol or iodoacetate) or antibodies or gene transcription downregulation against glycolytic pathway should be carried out in future work.

Table 3. Contribution of glycolysis and OxPhos to total ATP supply in cancer and non-cancer cells in bi-dimensional cultures and in multicellular tumor spheroids (MCTS). For young MCTS, diameters were <500 μm; for mature MCTS, diameters were >500 μm (*n*=2).

	ATP supply	
	% Glycolysis	% OxPhos
Cancer cells		
human cervix HeLa cells	39	61
human cervix HeLa cells with overexpressed p53	30	70
human cervix HeLa cells with overexpressed mutated p53 (R248Q)	73	27
ER+ human breast low metastatic MCF7 cells	27	73
human breast triple negative MDA-MB 231 cells	33	64
human breast triple negative MDA-MB-468 cells	39	61
transformed human embryo kidney HEK-293	13.5	86
Non-cancer cells		
rodent 3T3 fibroblasts	40	60
Multicellular tumor spheroids (MCTS)		
human cervix HeLa young MCTS	37	63
human cervix HeLa mature MCTS	83	17
transformed human embryo kidney HEK-293 young MCTS	42	58
transformed human embryo kidney HEK-293 mature MCTS	79	21

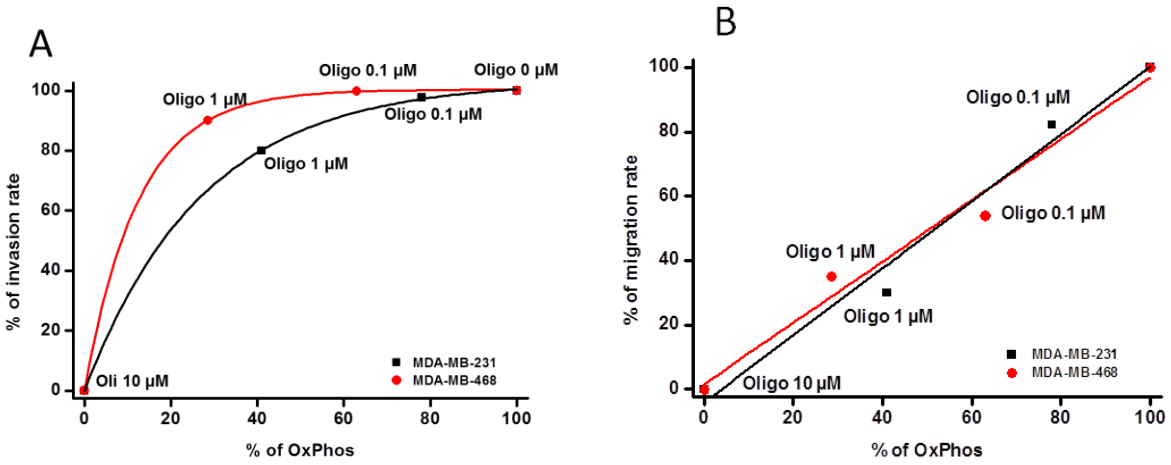

Figure 3. Dependence on OxPhos ATP supply of cancer cell invasion (A) and migration (B) of highly metastatic human breast triple negative cancer cells. OxPhos and metastasis processes were titrated with oligomycin (Oligo) as it is described in the Material and Methods section. Cellular viability was higher 90 % in non-treated cells; higher than 70-80 % in cells exposed to 0.1, and 1 µM oligomycin and less than 5 % in cells exposed to 10 µM oligomycin. For cell invasion, the 100 % value for net OxPhos flux was 17 ngAt O/min/mg cell protein for MDA-MB-231 and 28 ngAt O/min/mg cell protein for MDA-MB-468. For cell migration, the 100 % value was 200-250 nm for MDA-MB-231 cells and 150-200 nm for MDA-MB-468 cells; whereas for OxPhos, the 100 % value for net OxPhos flux was 17 ngAt O/min/mg cell protein for MDA-MB-231 cells and 28 ngAt O/min/mg cell protein for MDA-MB-468 cells.

In contrast to what was observed for cancer cell invasiveness, MDA-MB-231 and MDA-MB-468 cell migration was strongly inhibited by oligomycin, i.e. cellular migration was highly dependent on mitochondrial ATP (Figure 3B). MCA revealed a flux control coefficient near unity in both cancer cells, indicating that OxPhos exerted high control on cancer cell migration.

4. Discussion

4.1. High glycolytic rate in cancer cells

The rate of glycolysis in cultured cells has been determined in a variety of ways. For example by (*1*) measuring the increment in [H^+] due to the release of lactate and H^+ into the extracellular milieu [19,20]; (*2*) measuring external glucose consumption indicative of glycolysis onset; or (*3*) directly measuring the content of lactate, the end-product of the pathway.

There are several disadvantages related with the determination of extracellular acidification rate or glucose consumption to measure glycolytic rate. Unfortunately, the relationship between glycolytic rate and extracellular acidification is spoiled by CO_2 generated in the Krebs cycle, which is hydrated to H_2CO_3 and dissociated to HCO_3^- + H^+ at physiological pH, catalyzed by highly active carbonic anhydrase. Thus, it has been estimated that extracellular acidification is three times higher when glucose is transformed to CO_2 than lactate production from glucose [19]. Regarding glucose

consumption, it is well established that glucose transformation may provide intermediaries connected to glycolytic branches (pentose phosphate pathway, glycogen biosynthesis, amino acids and lipids syntheses, Krebs cycle); thus, glycolysis estimated from glucose consumption could be overestimated.

In the present study, lactate was enzymatically determined in cells incubated with external glucose by using an excess of exogenous LDH and NAD^+, following the formation of NADH. Although absorption spectrophotometry can be used for NADH/NAD^+ detection, fluorometry is more sensitive [21, 22]. When extracellular glucose is added to cells, it is transformed into pyruvate. Pyruvate has two possible major fates. (*1*) It may complete anaerobic glycolysis and generate lactate through lactate dehydrogenase (using NADH generated by glyceraldehyde 3-phosphate dehydrogenase) followed by export with H^+ to the medium in a reaction catalyzed by the monocarboxylate transporter isoform 4 (MCT-4). (*2*) Pyruvate may also be transported into mitochondria through the pyruvate transporter to feed OxPhos.

Most cancer cells grown in bi-dimensional cultures, and mature MCTS (a model that resembles early stages of solid tumor) [6, 23], show an accelerated glycolysis compared to non-cancer cells (Table 1), as reported by several research groups [24]. Thus, high glycolysis has been considered as a metabolic hallmark of cancer cells [4].

High glycolysis in cancer cells is associated with an enhanced transcription of genes of several glycolytic pathway enzymes and transporters and enhanced protein synthesis [9]. Several molecular mechanisms have been proposed to explain this activated glycolytic rate in cancer cells. It has been described that the transcriptional factor HIF-1α (hypoxia inducible factor 1α) is overexpressed or its content is elevated in cancer cells [25] and upregulates the expression of specific isoforms of several glycolytic proteins including GLUT, HK, PFK-1, PFK-2, ALDO, GAPDH, PGK, PGAM, ENO, PYK, and LDH [26]. The oncoprotein c-MYC is another transcription factor that regulates the expression of glycolytic proteins [27]. In consequence, the glycolytic flux and the levels of intermediaries are increased. Regardless of the O_2 level, metastatic cancer cells show high levels of HIF-1α, overexpression of glycolytic enzymes, and high glycolysis [25].

In mature MCTS (diameters of 1 mm) glycolysis is the main ATP supplier because their bulk of cells become exposed to partial hypoxia and hypoglycemia for several days. This microenvironment promotes the stabilization of several transcription factors (HIF-1α, c-MYC) and kinases (AMPK) associated to glycolysis activation [27]. Interestingly, although glycolysis predominates as the main ATP supply in these MCTSs, anti-glycolytic treatment has not been successful in inhibiting MCTS growth,suggesting that cells inside the spheroids remain significantly dependent on the alternative ATP supply derived from mitochondria [28].

4.2. High OxPhos rate in cancer cells

For several years the research on cancer mitochondrial metabolism stalled because it was thought that mitochondria were not functional. However, analysis of Warburg's own experimental results revealed that the respiratory rates of cancer cells were as high as those of non-cancer cells [5]. In this regard, an overwhelming amount of data on up-regulated OxPhos genes, respiratory rates and mitochondrial membrane potential from numerous cancer cell lines has become available revealing functional and very active cancer mitochondria [24], correlating with results of the present study (Table 2) and

supporting that cancer mitochondria are not damaged and maintain a functional OxPhos despite enhanced rates of glycolysis [24]. Clinically, the presence of functional mitochondria in cancer cells has led the use of PET-imaging using "mitochondrial probes" such as copper-based lipophilic organic cations [29] or ^{18}-Fluoroglutamine [30] for selective and metastatic cancer (i.e. athymic nude mice bearing U87MG glioma xenografts and xenografts produced in F344 rats by subcutaneous injection of 9L tumor cells and transgenic mice with M/tomND spontaneous mammary gland tumors) detection.

In young MCTS (diameter less than 0.5 mm), the bulk of cells are no limited by nutrients (glucose) and oxygen; thus, normoglycemic and normoxic microenvironment promotes that several transcriptional factors associated to OxPhos gene transcription be overexpressed, which induces that OxPhos may become the main ATP source [6, 27].

The, rigorous experimental analysis of energy metabolism has revealed that cancer mitochondria are not dysfunctional, as Warburg's proposal claims. Perhaps, his proposal was based on the observation that glycolysis was not strongly inhibited by O_2 (i.e. cancer cells maintain an attenuated Pasteur effect) as occurs in non-cancer cells. In normoxia, the Pasteur effect in non-cancer cells derives from the strong inhibition of PFK-1 activity by some OxPhos products expelled out to the cytosol such as citrate, ATP and H^+. However, cancer cells express PFK-1 isoforms with less sensitivity to these metabolites. In addition, tumor PFK-1 shows increased affinity for their activators (Fru2,6BP and AMP) having a net effect of nullifying the inhibitory effects of increased O_2 concentration and mitochondrial activity [31].

4.3. OxPhos is the principal ATP supplier in cancer cells

In the present study, the ATP produced by each energy pathway was calculated for the identification of the principal ATP producer in cancer cells. The contribution of glycolysis to the cellular ATP supply was determined from lactate production, assuming a stoichiometry of 1 mol of ATP produced per 1 mol of lactate produced. The contribution of OxPhos to the cellular ATP supply was determined from the *R-L* net ROUTINE respiration (derived from the oligomycin-sensitive respiration) assuming an ATP/O ratio of 2.5 [10]. This value was chosen because it is in the uppermost range of measured ATP/O values in isolated tumor mitochondria respiring NADH-linked substrates and represents the amount of ATP synthesized (by the ATP synthase) per atom of oxygen consumed (by the cytochrome c oxidase in respiratory electron transfer); the units of the ATP/O ratio would be nmol ATP/ng atom oxygen.

In some studies, greater P/O values are considered for the ATP determination from OxPhos flux. This last P/O range of values is based on the structural model of 8 or 11 c-subunits in the Fo subunit of the bovine heart ATP synthase [32]. From this model, ATP/H^+ ratios of 8/3 [33] or 11/3 [34] are calculated and hence, for a total of 12 H^+ (or positive charges) pumped out by the respiratory chain per atom of oxygen consumed, the resulting P/O ratios are higher. Instead, for the OxPhos ATP quantification in the present study we applied a lower ATP/O ratio directly determined in AS-30D hepatoma mitochondria [10]. The use of this last ratio shows some advantages:

(1) This ratio was determined in functional mitochondria isolated from cancer (hepatocarcinoma) cells, whose cholesterol content is significantly higher than that of mammalian non-cancer cell mitochondria [15, 35], rather than that estimated from an isolated enzyme from bovine heart. As a result of the different lipid

membrane composition, several biochemical functions, associated with membrane passive proton diffusion and respiratory electron transfer, ATP/ADP translocator and ATP synthase activities, are intrinsically involved in the estimated P/O ratio, which cannot be considered in the P/O ratio predicted by the isolated ATP synthase structural model.

(2) The value of the ATP/O ratio used in our calculations was polarographically determined by measuring the oxygen consumption rate in isolated mitochondria rather than the flux of H^+ through the isolated mitochondrial ATPase. A parallel set of experiments performed under similar conditions but measuring the ATPase activity by following the pH change associated with the hydrolysis of ATP was used to determine the amount of ATP involved [36]. Hence, a relationship between moles of oxygen atoms consumed with moles of synthetized (or hydrolyzed) ATP was calculated to be 2.5. This ATP/O value was not determined depending on the number of catalytic subunits found in the Fo subunit of the ATP synthase. Instead, it was determined in function of the ATP synthase capacity to generate ATP coupled to the cytochrome c oxidase activity to consume molecular oxygen. It should be also noted that the P/O value used incorporates the energy expended in the exchange of cytosolic ADP^{3-} by mitochondrial matrix ATP^{4-} catalyzed by the ATP/ADP translocator, and the exchange of cytosolic Pi by mitochondrial matrix OH^- catalyzed by the Pi transporter.

Analysis of the rates of glycolysis and oligomycin-sensitive respiration (Tables 1 and 2) in cancer cells included in our study indicated that OxPhos was the prevalent pathway in the cell supply of ATP (Table 3), under several near-physiological conditions and despite enhanced rates of glycolysis. As a consequence, highly ATP-dependent cancer functions may be controlled by the rate of ATP supplied by OxPhos. Despite the accelerated glycolysis observed in numerous cancer cell types, the glycolytic contribution to the total cellular ATP supply was shown to reach less than 40 % of the total ATP. Then, OxPhos is the pathway that mainly contributes towards the cellular ATP demand. In marked contrast, in other cancer cells forming spheroids with diameters >1 mm or developing point mutations in key transcriptionasl factors like p53, glycolysis accounted for >70 % of the total cellular ATP demand, similar to the value estimated by Warburg [5].

4.4. Blocking cancer cell migration by OxPhos inhibition

The functional (biochemical) determination of cancer energy metabolism may provide mechanistic information on which pathway (glycolysis or OxPhos) is more important for ATP supply to specifically target it as a physiologically essential function.

Data of the present work on glycolysis and OxPhos suggest that many cancer cells have an oxidative type of metabolism, which implies that mitochondria play a central role in supporting ATP-dependent processes such as cancer cell migration, invasiveness, or perhaps proliferation.

Therefore, a follow-up strategy derived from the present results to diminish cancer metastasis would be the use of inhibitors that specifically block OxPhos. The addition of the typical inhibitor of ATP synthase oligomycin, at relatively low doses, abolished the cancer cell migration and, to a much lesser extent, the invasiveness of HeLa cells (Figure 3).

Metabolic Control Analysis (MCA) [17, 18] is a biochemical framework used to elucidate the mechanisms of control and regulation in metabolic and signaling pathways and allows quantifying the degree of control that the activity (*1*) of an enzyme/transporter/biological process (*i*) exerts on a pathway flux (*J*) which is called flux control coefficient (C_{Jai}). The application of MCA to both migration and invasiveness revealed that the C_{Jai} value of OxPhos for cell invasiveness was close to zero, whereas on cell migration, the C_{Jai} was closed to one, indicating the total dependence of cancer cell migration on mitochondrial ATP.

5. Concluding remarks

One of the prevailing dogmas in cancer biology is that glycolysis is the principal energy supplier for cancer cells. Recent studies, including the present work, show a contrasting view based on experimental evidence (measuring both energy metabolism pathways and applying appropriate corrections) to demonstrate that OxPhos is functional in cancer cells, which makes significant contribution to the ATP supply necessary for cancer cell survival, growth, and metastasis. Once OxPhos is proved to be essential for many cancer cells, then it may become an important therapeutic target. MCA analysis provided rational and quantitative basis for understanding how much cancer cell migration depends on OxPhos ATP. Therefore, MCA should be carried out in many more different types of metastatic cancer cells to identifying the dependence of metastasis on glycolysis or OxPhos.

Acknowledgments

The present work was partially supported by CONACyT-México Grants No. 6379 to RMS; No. 377873 to SCPV and No. 283144 to SRE.

References

1. Moreno-Sánchez R, Saavedra E, Gallardo-Pérez JC, Rumjanek FD, Rodríguez-Enríquez S (2016) Understanding the cancer cell phenotype beyond the limitations of current omics analyses. https://doi.org/10.1111/febs.13535
2. Moreno-Sánchez R, Rodríguez-Enríquez S, Marín-Hernández A, Saavedra E (2007). Energy metabolism in tumor cells. https://doi.org/10.1111/j.1742-4658.2007.05686.x
3. Moreno-Sánchez R, Marín-Hernández A, Saavedra E, Pardo JP, Ralph SJ, Rodríguez-Enríquez S (2014) Who controls the ATP supply in cancer cells? Biochemistry lessons to understand cancer energy metabolism. https://doi.org/10.1016/j.biocel.2014.01.025
4. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. https://doi.org/10.1016/j.cell.2011.02.013
5. Warburg O (1956) On the origin of cancer cells. https://doi.org/10.1126/science.123.3191.309
6. Rodríguez-Enríquez S, Gallardo-Pérez JC, Avilés-Salas A, Marín-Hernández A, Carreño-Fuentes L, Maldonado-Lagunas V, Moreno-Sánchez R (2008) Energy metabolism transition in multi-cellular human tumor spheroids. https://doi.org/10.1002/jcp.21392
7. Murphy BJ, Laderoute KR, Vreman HJ, Grant TD, Gill NS, Stevenson DK, Sutherland RM (1993) Enhancement of heme oxygenase expression and activity in A431 squamous carcinoma multicellular tumor spheroids. https://pubmed.ncbi.nlm.nih.gov/8504407/

8. Wu FJ, Friend JR, Remmel RP, Cerra FB, Hu WS (1999) Enhanced cytochrome P450 IA1 activity of self-assembled rat hepatocyte spheroids. https://doi.org/10.1177/096368979900800304
9. Moreno-Sánchez R, Rodríguez-Enríquez S, Saavedra E, Marín-Hernández A, Gallardo-Pérez JC (2009) The bioenergetics of cancer: Is glycolysis the main ATP supplier in all tumor cells? https://doi.org/10.1002/biof.31
10. Nakashima RA, Paggi MG, Pedersen PL (1984) Contributions of glycolysis and oxidative phosphorylation to adenosine 5'-triphosphate production in AS-30D hepatoma cells. https://pubmed.ncbi.nlm.nih.gov/6498833/
11. Rodríguez-Enríquez S, Carreño-Fuentes L, Gallardo-Pérez JC, Saavedra E, Quezada H, Vega A, Marín-Hernández A, Olín-Sandoval V, Torres-Márquez ME, Moreno-Sánchez R (2010) Oxidative phosphorylation is impaired by prolonged hypoxia in breast and possibly in cervix carcinoma. https://doi.org/10.1016/j.biocel.2010.07.010
12. Vanderlinde RE (1985) Measurement of total lactate dehydrogenase activity. https://pubmed.ncbi.nlm.nih.gov/3882046/
13. Gnaiger E (2020) Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis. https://doi.org/10.26124/bec:2020-0002
14. Pacheco-Velázquez SC, Robledo-Cadena DX, Hernández-Reséndiz I, Gallardo-Pérez JC, Moreno-Sánchez R, Rodríguez-Enríquez S (2018) Energy metabolism drugs block triple negative breast metastatic cancer cell phenotype. https://doi.org/10.1021/acs.molpharmaceut.8b00015
15. Rodríguez-Enríquez S, Hernández-Esquivel L, Marín-Hernández A, El Hafidi M, Gallardo-Pérez JC, Hernández-Reséndiz I, Rodríguez-Zavala JS, Pacheco-Velázquez SC, Moreno-Sánchez R (2015) Mitochondrial free fatty acid β-oxidation supports oxidative phosphorylation and proliferation in cancer cells. https://doi.org/10.1016/j.biocel.2015.06.010
16. Plitzko B, Loesgen S (2018) Measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in culture cells for assessment of the energy metabolism. https://doi.org/10.21769/BioProtoc.2850
17. Fell D (1997) Understanding the control of metabolism. Plenum Press, London. ISBN 1 85578 047 X Google Scholar
18. Marín-Hernández A, López-Ramirez S, Gallardo-Pérez JC, Rodríguez-Enríquez S, Moreno-Sánchez, Saavedra E (2014) Systems biology approaches to cancer energy metabolism In: Systems biology of metabolic and signaling networks. Springer, London. https://www.springer.com/series/835
19. Mookerjee SA, Goncalves RLS, Gerencser AA, Nicholls DG, Brand MD (2015) The contributions of respiration and glycolysis to extracellular acid production. https://doi.org/10.1016/j.bbabio.2014.10.005
20. Mookerjee SA, Gerencser AA, Nicholls DG, Brand MD (2017) Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. https://doi.org/10.1074/jbc.M116.774471
21. Froehlich P (1989) Understanding the sensitivity specification of spectroflorometers. https://studylib.net/doc/18133107/the-measurement-of-sensitivity-in-fluorescence-spectroscopy
22. Peterson W (1997) The measurement of sensitivity in fluorescence spectroscopy. https://www.photonicsonline.com/doc/the-measurement-of-sensitivity-in-fluorescenc-0002
23. Kunz-Schughart LA (1999) Multicellular tumor spheroids: intermediates between monolayer culture and in vivo tumor. https://doi.org/10.1006/cbir.1999.0384
24. Zu XL, Guppy M (2004) Cancer metabolism: facts, fantasy, and fiction. https://doi.org/10.1016/j.bbrc.2003.11.136
25. Robey IF, Lien AD, Welsh SJ, Baggett BK, Gillie RJ (2005) Hypoxia-inducible factor-1a and the glycolytic phenotype in tumors. https://doi.org/10.1593/neo.04430

26. Marín-Hernández A, Gallardo-Pérez JC, Ralph SJ, Rodríguez-Enríquez S, Moreno-Sánchez R (2009) HIF-1alpha modulates energy metabolism in cancer cells by inducing over-expression of specific glycolytic isoforms. https://doi.org/10.2174/138955709788922610
27. Rodríguez-Enríquez S, Marín-Hernández Á, Gallardo-Pérez JC, Pacheco-Velázquez SC, Belmont-Díaz JA, Robledo-Cadena DX, Vargas-Navarro JL, Corona de la Peña NA, Saavedra E, Moreno-Sánchez R (2019) Transcriptional regulation of energy metabolism in cancer cells. https://doi.org/10.3390/cells8101225
28. Mandujano-Tinoco EA, Gallardo-Pérez JC, Marín-Hernández A, Moreno-Sánchez R, Rodríguez-Enríquez S. (2013) Anti-mitochondrial therapy in human breast cancer multi-cellular spheroids. https://doi.org/10.1016/j.bbamcr.2012.11.013
29. Zhou Y, Liu S (2011) ^{64}Cu-labeled phosphonium cations as PET radiotracers for tumor imaging. https://doi.org/10.1021/bc200106p
30. Lieberman BP, Ploessl K, Wang L, Qu W, Zha Z, Wise DR, Chodosh LA, Belka G, Thompson CB, Kung HK (2011) PET imaging of glutaminolysis in tumors by 18F-(2S,4R)4-fluoroglutamine. https://doi.org/10.2967/jnumed.111.093815
31. Moreno-Sánchez R, Marín-Hernández A, Gallardo-Pérez JC, Quezada H, Encalada R, Rodríguez-Enríquez S, Saavedra E (2012) Phosphofructokinase type 1 kinetics, isoform expression, and gene polymorphisms in cancer cells. https://doi.org/10.1002/jcb.24039
32. Collinson IR, Runswick MJ, Buchanan SK, Fearnley IM, Skehel JM, van Raaij MJ, Griffiths DE, Walker JE (1994) F_o membrane domain of ATP synthase from bovine heart mitochondria: purification, subunit composition, and reconstitution with F. https://doi.org/10.1021/bi00191a026
33. Brand MD (2005) The efficiency and plasticity of mitochondrial energy transduction. https://doi.org/10.1042/BST0330897
34. Mookerjee SA, Gerencser AA, Nicholls DG, Brand MD (2018) Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. https://doi.org/10.1074/jbc.AAC118.004855
35. Parlo RA, Coleman PS (1984) Enhanced rate of citrate export from cholesterol-rich hepatoma mitochondria. The truncated Krebs cycle and other metabolic ramifications of mitochondrial membrane cholesterol. https://pubmed.ncbi.nlm.nih.gov/6469976/
36. Kaschnitz RM, Hatefi Y, Morris HP (1976) Oxidative phosphorylation properties of mitochondria isolated from transplanted hepatoma. http://doi.org/10.1016/0005-2728(76)90135-3