Experimental and Molecular Pathology 89 (2010) 372–380

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Review

# Cancer metabolism: The Warburg effect today

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ARTICLE INFO

*Article history:*
Received 3 February 2010
and in revised form 22 August 2010
Available online 8 September 2010

*Keywords:*
Cancer
Warburg effect
Metabolism
Microenvironment

ABSTRACT

One of the first studies on the energy metabolism of a tumour was carried out, in 1922, in the laboratory of Otto Warburg. He established that cancer cells exhibited a specific metabolic pattern, characterized by a shift from respiration to fermentation, which has been later named the Warburg effect. Considerable work has been done since then, deepening our understanding of the process, with consequences for diagnosis and therapy. This review presents facts and perspectives on the Warburg effect for the 21st century.



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## Introduction

Metabolism, the sum of all chemical reactions that take place within a cell or organism, is an area of biochemical research with a wide variety of applications in all scientific fields. Understanding its pathways and regulation is somehow to reveal the cells' function and, ultimately, life itself. Cancer is no exception and a profound knowledge of cancer cell metabolism is invaluable in the comprehension of this pathology, as originally proposed by Warburg, who was awarded the Nobel Prize of Medicine in 1931 for his discovery of the oxygen transferring enzyme of respiration, during the 1920s.

One of the first studies on the energy metabolism of a tumour was carried out by this scientist (Warburg, 1923) with slices of living

*Abbreviations:* CL, cardiolipin; EGFR, endothelial growth factors receptor; ETC, electron transport chain; FDG, $^{18}$Fluoro-deoxy-glucose; F2,6BP, fructose 2,6-bisphosphate; GLUT, glucose facilitative transporter; GSK3, glycogen synthase kinase 3; HIF-1, hypoxia inducible factor 1; HK, hexokinase; MCT, monocarboxylate transporter; NHE-1, $Na^+/H^+$ exchanger 1; ODD, "Oxygen Dependent Degradation"; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase; PET, positron emission tomography; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PFK-1, 6-phosphofructo-1-kinase; PPP, pentose phosphate pathway; ROS, reactive oxygen species; SCO2, "Synthesis of Cytochrome c Oxidase 2"; TIGAR, TP53-induced glycolysis and apoptosis regulator; tPMET, trans-plasma membrane electron transporter; UCP2, mitochondrial uncoupling protein 2; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

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0014-4800/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2010.08.006

tissue, where he observed a specific metabolic pattern in tumours: while in normal tissues lactate production occurred almost exclusively during oxygen deprivation (this effect was termed by Warburg the Pasteur effect, in consideration to the work developed by Louis Pasteur during the XIX century (Holmes, 1991)), it was not ablated in tumour slices by the presence of oxygen (Warburg et al., 1924). Based on his first hand knowledge on metabolism, Warburg concluded that cancer cells were performing lactic fermentation, even in aerobic conditions.

There are two major metabolic processes by which mammalian cells obtain energy: lactic fermentation and aerobic respiration. In lactic fermentation, which occurs exclusively in the cytosol, glucose ($C_6H_{12}O_6$) is initially converted to pyruvate ($C_3H_5O_3$) through glycolysis, a nearly universal metabolic pathway. Pyruvate is subsequently reduced to lactate ($C_3H_7O_3$), which is excreted to the bloodstream. Altogether, this process yields two ATPs per glucose molecule. In aerobic respiration, a much more complex energy-yielding process, glucose and other substrates are completely oxidised to carbon dioxide ($CO_2$) and water ($H_2O$). It comprises glycolysis, Krebs cycle and oxidative phosphorylation (OXPHOS), the last two processes occurring inside mitochondria. Aerobic respiration has a much higher energy yield than lactic fermentation (*ca.* 15 times), but it can only occur in the presence of $O_2$, which functions as electron acceptor, whereas fermentation can occur in its absence (Nelson and Cox, 2008).

Warburg's initial findings immediately attracted the attention of the scientific community and were soon followed by several studies by Warburg and others that confirmed and extended them (Cori and Cori, 1925a; Cori and Cori, 1925b; Warburg, 1925; Warburg and Kubowitz, 1927; Warburg et al., 1924; Warburg et al., 1927). Particularly significant were the extensive *in vivo* experiments on the carbohydrate metabolism of tumours carried out by Carl and Gerty Cori (Cori and Cori, 1925a; Cori and Cori, 1925b). Their results confirmed Warburg's previous ones (Warburg et al., 1924), as distinct tumour types showed the lowest free glucose content among all analysed tissues, suggesting an increased glucose consumption compatible with a shift from respiration to fermentation. Although these tumours presented low lactic acid levels, this was found to be due to the bloodstream capacity to remove all excess lactate from these hypoglycaemic tumours. Indeed, high levels of lactic acid were observed in the presence of larger amounts of glucose, which were quite insensitive to insulin administration (Cori and Cori, 1925a). In order to assess the importance of circulating vessels in malignant masses, chickens bearing a tumour of considerable size in only one of their wings were used to compare the compositions of the blood that passed through each wing; tumour veins presented a clear increase in the lactic acid and a decrease in the free glucose and $CO_2$ content (Cori and Cori, 1925b). The *in vitro* Warburg's results (Warburg et al., 1924) had been extended to *in vivo* systems.

In 1925, Warburg described that pieces of cultured tumours not only had a higher rate of lactate production than normal cells, but were even capable of existing during a certain period of time relying exclusively on fermentation (Warburg, 1925). Moreover, tumour masses presented a deficient blood supply of glucose and oxygen, being the percentage of consumed glucose higher than in normal tissues (Warburg et al., 1927). Experiments using virtually pure ascites cancer cells living free in abdominal cavity were later performed, which strongly confirmed the high fermentation quotients of tumour cells in aerated environments.

All these results came in line with Warburg's suggestion that irreversible damage of respiration was on the origin of cancer cells (Warburg, 1929; Warburg, 1956b; Warburg et al., 1924). The causes and the advantages of this specific metabolic pattern, i.e. conspicuous lactate secretion and glucose uptake rates, later termed the Warburg effect (Racker and Spector, 1981), have since then been extensively studied.

## Contestation to Warburg's ideas

Controversy followed the publication of Warburg´s results and various arguments were used to rebuke allegedly doubtful aspects. Already in 1922, when Seigo Minami (Warburg and Minami, 1923), a Warburg´s laboratory technician, measured glycolysis and fermentation rates of slices of Flexner rat carcinoma and rat liver, pancreas, submaxillary gland and connective tissue and found out that anaerobic glycolysis rate was much higher in carcinoma than in liver, some criticisms arose based on suppositions that human tumours might behave differently from those experimental transplanted animal ones and that blood serum could dissolve carcinoma cells (Holmes, 1991).

In November of 1926, Hans Krebs (who was then starting his scientific career and later discovered Krebs and urea cycles, being awarded the Nobel of Medicine in 1953 for his discovery of the first (Holmes, 1991)) was assigned by Warburg to measure glycolysis and respiration rates of human tumour tissues using the new accurate manometrical methods developed by him (Warburg, 1923). At first, Krebs measured both aerobic and anaerobic glycolysis rates of Jensen rat sarcoma tumours in different animals' plasma and sera, salt solutions and in Ringer´s with bicarbonate solution previously used by Warburg in his studies (Warburg et al., 1924), and the values of the sarcoma were higher than normal for all media. Next, he moved to a clinic where he had access to tumour tissue excised from patients and reached the conclusion that anaerobic glycolysis diminished faster in normal and patient serum than in Ringer´s with bicarbonate solution. Nevertheless, in a series of studies done during the beginning of 1927, he found that the differences between glycolytic rate in serum and Ringer's with bicarbonate solution were not systematic and human and animal cancer tissues behaved similarly in almost all the cases. Despite being not new, these results strengthened Warburg's previous conclusions against criticisms (Holmes, 1991).

That lactate levels measured by Warburg and collaborators in 1926 (Warburg et al., 1927) might be exaggerated was first suggested by Sauer and Dauchy sixty years later (Sauer and Dauchy, 1986), after determining these *in vivo* on Jensen sarcoma, the same used by Warburg (Warburg et al., 1927). They showed that both net lactic acid production and utilization could occur in these tumours, depending on the arterial concentrations of ketone bodies and lactate and on whether the animals were fed or fasted, and obtained lactic acid concentrations in tumours' venous blood between 1 and 4 mM (*vs.* 4.4–25.6 mM reported by Warburg) . Two explanations for these conspicuous discrepancies were put forward: the poor specificity of the multistep Clausen method (Clausen, 1922) used by Warburg to determine lactic acid levels (with this method, related molecules like other 2-hidroxy-acids and ketone bodies are also measured as lactate) and the fact that Jensen sarcomas were grown in the peritoneal cavity of the rats, reaching masses of 10 g that may have provoked bowel obstruction and consequent hindrance to the host´s ability to eat.

More recent experiments demonstrated that layers thicker than 100 μm can compromise the homogenous oxygenation of the used excision (Helmlinger et al., 1997), whilst Warburg used tissue sections ranging from 200 to 300 μm (Warburg, 1923). The initially noted high glycolytic rates (Warburg et al., 1924) could then result from hypoxia to some extent.

Albeit the observations above are pertinent, it must be stressed that Warburg also obtained higher-than-normal lactate levels using virtually pure ascites cancer cells living in suspension (Warburg, 1956b).

Immediately after the publication of Warburg's paper “On the Origin of Cancer Cells” (Warburg, 1956b), Weinhouse, based in results obtained in his laboratory showing that neoplasias could have a normal OXPHOS capacity if supplemented with $NAD^+$ (Wenner and Weinhouse, 1953), immediately contested Warburg's ideas, triggering an interesting historical debate (Burk and Schade, 1956; Warburg,

1956a; Weinhouse, 1956). In fact, an increase in respiration of isolated tumour slices after adding succinate or fumarate was already observed (Boyland and Boyland, 1935). These observations led to the hypotheses that Warburg's original method to prepare tumour slices (Warburg, 1923) could have provoked the diffusion of essential electron carriers (Boyland and Boyland, 1935) and that fatty acids and cellular detritus exhibited by the necrotic areas of the tumours could have negatively affected mitochondria integrity during isolation procedures (Weinhouse, 1956), artificially reducing respiration efficiency. Albeit Weinhouse arguments used in the aforementioned discussion ignored new findings signifying that to a higher malignancy grade corresponded higher fermentation and lower respiration rates (Warburg, 1956a), recent work showing that OXPHOS activity can be enhanced in cancer cells (Rossignol et al., 2004) supports Weinhouse results (Wenner and Weinhouse, 1953) and dismisses the possibility of an irreversible mitochondrial impairment in cancer cells.

Contestation to Warburg's ideas in cancer biology persisted to these days. For instance, it is averred in a recent review that, considering the available data, malignant cells are not inherently more glycolytic than normal ones (Zu and Guppy, 2004). These two authors compiled literature data on glycolytic and oxidative ATP contributions to total energy production for different types of normal and malignant cells and concluded that, on average, malignant cells do not rely more on fermentation than normal cells do. However, cell types, such as erythrocytes, reticulocytes and platelets, were considered normal, even though the first two are enucleated, the latter consist of fragments of larger cells called megakaryocytes and all have peculiar energy metabolisms (Balduini et al., 2004). Notwithstanding, these authors did acknowledge that comparisons ought to be made between cells of the same type before and after their transformation to achieve stronger conclusions. Warburg already knew in 1924 that some normal cells were able to grow using solely glycolysis and that there was glycolysis without growth and aerobic glycolysis without abnormal growth (Warburg et al., 1924). Distinct cancers overexpress different numbers of glycolytic genes, ranging from one or two (e.g. cervix) to eleven (e.g. brain) (Altenberg and Greulich, 2004) and normal cells also regulate the expression of these in response to changes in glucose concentration (Roche et al., 1997). Therefore, absolute values of fermentation or respiration can be deceiving if interpreted *verbatim*; Warburg always considered quotients of both processes rates (Warburg, 1956b) to conclude that the respiration of carcinoma tissue was minute relatively to its glycolytic power (Warburg, 1956a).

As we study cancer bioenergetics more in depth, we gradually realize that comprehending the true meaning of Warburg effect implicates resolving a continuously growing puzzle, which spans several fields of scientific research and occupies the mind of thousands of investigators and students. It thus seems wise to present some facts that may greatly improve our capacity of dealing with this issue.

## Glucose's uptake and intracellular fates

An increased glucose uptake in cancer cells was first described by Warburg (Warburg et al., 1927) and it is simultaneously the most used characteristic to identify tumours *in vivo* (by Positron Emission Tomography using $^{18}$Fluoro-deoxy-glucose—FDG-PET (Pauwels et al., 2000)) and a conundrum in cancer cell metabolism, as it feeds multiple pathways (Nelson and Cox, 2008).

Glucose is transported to the intracellular medium mainly via facilitative glucose transporters (GLUTs) (Mueckler, 1994), which comprise six isoforms differing in kinetic behaviour, hexose specificity, tissue localization, etc. It immediately follows that cells belonging to different tissues have distinct capacities to capture glucose and subsequently utilize it in glycolysis and, importantly, in other metabolic pathways.

Once inside the cell, glucose can be used to drive the synthesis of glycogen, as studies performed already in 1925 proved forerunning pathologists' observations that tumours present a notable glycogen content (Cori and Cori, 1925a). One of the enzymes involved in glycogen synthesis is glycogen synthase, which can be inactivated through phosphorylation by glycogen synthase kinase-3 (GSK3), a multi-functional regulatory enzyme (Jope et al., 2007). Recently, there has been an increased interest in its study, as it influences inflammatory response, cell migration and regulation of cell adhesion. Although insulin effect on GSK3 activity is strongly tissue-dependent, it is well established in cancer: insulin and related growth factors activate the PI3K/Akt signalling pathway, which in turn inhibits GSK3. Actually, it is known that GSK3 acts as a tumour suppressor through interference with metastatic potential and apoptosis (Jope et al., 2007) and the aforementioned pathway is often activated in malignancies (Jones, 2009). One of the characteristics of cancers is a reduced GSK3 activity and, as this enzyme slows glycogen synthesis by inactivating glycogen synthase (Jope et al., 2007), this further supports that cancer cells may store glycogen in abnormal quantities. Interestingly, it was found some years ago that raising the extracellular concentration of glucose increased the *ab initio* significant glycogen content of ascites cancer cells, while their glycogenolysis was relatively active (Rodriguez-Enriquez et al., 2000), and that glycogen phosphorylase is overexpressed under hypoxia (Parolin et al., 2000). This additional glucose reserve would therefore constitute a growth advantage to cancer cells when their microenvironment is impoverished in $O_2$ and glucose due to deficient vascularisation (Fukumura and Jain, 2008). Strangely, this scenario is seldom mentioned in literature.

Pentose phosphate pathway (PPP) is another important route for glucose consumption that may lead to the production of lactate, via replenishment of glycolytic intermediates, (Nelson and Cox, 2008). Starting from glucose 6-phosphate, NADPH and ribose 5-phosphate are produced, something that is not attainable neither by glycolysis nor by complete glucose oxidation. NADPH is essential to maintain the high rate of fatty acids synthesis observed in cultured tumour cells (Lazo, 1981) resultant of the overexpression of related enzymes (Brusselmans et al., 2005; Hatzivassiliou et al., 2005) and is a scavenger against toxic reactive oxygen species (ROS) (Ahmad et al., 2005). On the other hand, ribose 5-phosphate is the precursor of nucleic acids bases and its biosynthesis is a constant demand in rapidly proliferating cells (Ramos-Montoya et al., 2006). In fact, studies using MCF10 cells showed that the amount of glucose shunted through the PPP progressively increased with carcinogenic transformation (Richardson et al., 2008).

FDG-PET hence gives a narrow contribution to the study of cancer bioenergetics: it reveals that some tumours have a higher glucose uptake but not its fate. FDG is converted to $^{18}$Fluoro-deoxy-glucose 6-phosphate, which is not further metabolized and remains trapped within the cells (Pauwels et al., 2000), impeding us to know which path would it follow. In addition, the presence of inflammatory cells with high glucose uptakes, such as tumour associated macrophages (Kroemer and Pouyssegur, 2008), can become a significant distractive factor (Kubota et al., 1992).

## Lactate production and induced acidosis

A physiological consequence of an increased lactate production is the diminution of interstitial pH observed in tumours since the 1920s (Warburg et al., 1927).

Several studies corroborate an acid-mediated tumour invasion. Lactate stimulates the production of hyaluronan, a glycosaminoglycan, and the expression of CD44, its main transmembrane receptor. When hyaluronan binds CD44 at cell surface, it enhances malignant progression by reducing cell adherence and providing spaces through each cells can move thanks to its notable water of hydration (Stern

et al., 2002). Cytotoxic T lymphocytes are highly glycolytic cells and rely in monocarboxylate transporters (MCTs) to co-transport lactate and $H^+$ to the milieu in order to maintain their intracellular pH and produce cytokines (soluble signalling proteins (Nelson and Cox, 2008)). This makes lactic acid an imunosupressor, for its accumulation near tumours disrupts T cells metabolism and function (Fischer et al., 2007). Low pH induces several matrix remodelling systems, namely metalloproteinases, lysosomal proteases and hyaluronidase (Robey et al., 2009). In fact, treatment of cancer cells with acid enhanced experimental metastases (Rofstad et al., 2006), while orally administered bicarbonate effectively reduced both tumour densities and their colonization at distant sites by diminishing the acidic boundaries of tumours without affecting their intracellular pH (Robey et al., 2009). Lactate production can be a positive feedback control mechanism, as its augmentation further depletes NADH pool and inhibits pyruvate dehydrogenase (PDH), while increasing its flux through MCTs by locally lowering pH (Bongaerts et al., 2006); if lactate were overproduced relatively to its secretion, the decrease in intracellular pH would inhibit 6-phosphofructo-1-kinase (PFK-1) and consequently glycolysis (Erecinska et al., 1995). It was also observed that silencing the expression of CD147, a molecular chaperone associated with MCT1 and MCT4, inhibited ATP production, cell migration, extracellular acidification and vascular endothelial growth factor (VEGF, a key player in angiogenesis) release, indicating the preponderant role of lactate homeostasis in cancer (Su et al., 2009).

Nowadays, the focus in cancer metabolism is turning to anabolic processes, as malignancies comprise aggressively proliferating cells (Richardson et al., 2008). The Warburg effect establishes a paradox: why is carbon wasted to secrete conspicuous amounts of lactate? Considering the available evidence, it seems that lactate production and consequent induced acidosis in healthy tissues is one of the *raisons d´être* of this recurrent phenotype.

However, lactic acid may not account alone for tumour acidosis. Newell originally established a role for $CO_2$ in extracellular acidification using glycolysis-impaired cells (Newell et al., 1993), being this confirmed by posterior *in vitro and in vivo* experiments (Helmlinger et al., 2002): although glycolysis-impaired cells presented visibly lower glucose consumption and lactate production rates than parental cells, both had the same extracellular pH, and assessments in solid tumours unveiled a strong lactate-independent inverse correlation between pH and $CO_2$ pressure, justifying the effectiveness of bicarbonate, a weak acid, in buffering tumour interstitial fluid (Robey et al., 2009). Another mechanism of lactate-independent extracellular acidification in endothelial and cancer cells is the extrusion of protons via surface $F_1F_o$ ATPases (Kroemer and Pouyssegur, 2008).

The regulation of Ehrlich ascites tumours' lactate production by intracellular ADP was assessed by Racker in a classical study (Racker and Spector, 1981). Although frequently underestimated, ATP hydrolysis and transphosphorylation steps of glycolysis have been attributed pivotal roles in the cross-talk between fermentation and respiration (Racker and Spector, 1981; Weinhouse, 1972). Using inhibitors specific for different ATP consuming processes (mitochondrial ATPase, $Na^+/K^+$-ATPase, etc.), Racker established that ATP hydrolysis was directly proportional to lactate formation from glucose; the notorious fermentation rates observed by Warburg in suspended ascites cancer cells (Warburg, 1956b) could then be due to defective $Na^+/K^+$ pumps, which would increase glycolytic flux by squandering ATP during membrane potential maintenance. More recent studies (James et al., 1996) showed that muscle cells present increased lactic fermentation rates in fully oxygenated conditions due to over activity of $Na^+/K^+$-ATPase, whose energy consumption represents a significant fraction of glycolytic ATP and causes intracellular alkalinisation through $Na^+/H^+$ exchange. The latter effect proved to be crucial since an early stage of tumorigenesis: NHE-1, a member of the $Na^+/H^+$ exchanger protein family, increases its activity in response to transformation-competent oncoproteins, inducing a glycolytic shift and other cancer hallmarks (Reshkin et al., 2000).

## Hypoxia

According to Bertout, the first unequivocal observation of cancer cells living in a hypoxic environment was carried out by Thomlinson and Gray in 1955 (Bertout et al., 2008). Apparently, sections of lung tumours presented bands of viable malignant cells surrounding necrotic regions (due to oxygen and nutrients deprivation) and neighbouring normal ones, being these hypoxic regions implicated in tumour radioresistance. Hypoxia is able to produce different effects in cultured cells, depending on whether they are oncogene-transformed or not. It seems that an anaerobic medium provokes $G_1$ arrest in normal cells, but an enhanced proliferation in tumour cells when uncoupled from acidosis, through downregulation of p53, a tumour suppressor gene (Schmaltz et al., 1998). Low pH drives the contrary effect, being considered the primary trigger of cell apoptosis rather than hypoxia *per se* (Schmaltz et al., 1998). Albeit this discrepancy might seem artificial, hypoxic regions of tumours are frequently nonacidotic, existing therefore a lack of correlation between hypoxia and low pH *in vivo* (Helmlinger et al., 1997). Moreover, Burgman found out that hypoxia stimulates FDG uptake in cancer cells, but no concomitant increase in GLUT-1, GLUT-3, or HK II was observed (Burgman et al., 2001). In fact, experiments using simultaneously FDG and $^{18}$Fluoro-misonidazole, a hypoxia probe, demonstrated that FDG uptake and hypoxia do not always correlate *in vivo* (Cherk et al., 2006).

Respiratory quotient, defined as the ratio between $CO_2$ output and $O_2$ uptake that takes the value one for total oxidation of carbohydrates (Vaupel, 2006), was found to be lower than unit in tumour slices many decades ago (Dickens and Simer, 1930). Still, recent experiments yielded high respiratory quotients for tumours *in vivo*. This behaviour, however, may not reflect an active respiration, but rather an augmented non-metabolic tumour $CO_2$ extrusion (Vaupel, 2006). Solid tumours present high levels of hypoxia inducible factor (HIF)-1, a heterodimeric protein responsible for sensing oxygen levels which induces the overexpression of glycolytic enzymes, lactate dehydrogenase and carbonic anhydrase IX, a membrane-bound ectoenzyme that converts bicarbonate to $CO_2$ in response to low extracellular pH, whilst inhibiting pyruvate dehydrogenase (PDH) (Semenza, 2009), thus accounting for the elevated $CO_2$ pressures found in some tumours (Yamagata et al., 1998). Under normal conditions, a group of prolyl and asparagyl hydroxylases trigger HIF-1 degradation via the ODD ("oxygen dependent degradation") region of the VHL (von Hippel–Lindau) protein. During hypoxia, however, $O_2$ scarcity inhibits these reactions, thus stabilizing HIF-1 (Klimova and Chandel, 2008). Curiously, some tumours display significant HIF activity even when properly oxygenated: for instance, the dysfunction of succinate dehydrogenase and fumarate hydratase observed in some cancers allows their accumulated substrates to leak from mitochondria to cytosol and inactivate a family of prolyl hydroxylases, thus allowing activation of HIF-1 and all its effects in an oxygen-independent manner (King et al., 2006). Mutations in VHL gene also induce HIF-1 (Semenza, 2009). If the Warburg effect was a mere Pasteur effect, then reoxygenation of hypoxic tumours would not fail to revert the metabolic shift and unequivocally improve patients' survival (Fyles et al., 1998).

Resistance to severe hypoxia is not a special attribute of cancer cells. Cardiomyocytes, for example, are well known for also doing so by increasing their glycolytic ATP production (Casey et al., 2002). The notorious difference consists in the fact that, unlike the latter, whose rates of RNA and protein synthesis are downregulated due to energetic stress, neoplasias continue proliferating. Some reports actually show that cells belonging to more hypoxic regions of tumours have bigger

malignant potentials when transplanted into nude mice (Yu et al., 2001).

Although most literature focuses on acute and chronic hypoxia, it is now perceived that the most relevant form of oxygen deprivation in tumours is intermittent hypoxia. Warburg first proposed in 1956 that the repeated exposure of cancer cells to periods of hypoxia rendered them more aggressive and resistant, while continued hypoxia *per se* could ultimately kill them (Warburg, 1956b). Short periods of hypoxia are sufficient to stabilize HIF-1, while long hypoxic periods actually decrease its stability (Berra et al., 2001)). As this timing doesn´t allow the translation of its targets, stress granules containing those transcripts are formed, being depolymerised during reoxygenation. This might explain in part the pseudohypoxia found in some cancers (King et al., 2006). Furthermore, hypoxia–reoxygenation cycles enhance tumour cells' survival and proliferation through ROS-activated proteins produced in reoxygenation phase, inhibition of apoptotic proteins and increased resistance of endothelial cells that are associated with neoplastic ones (Toffoli and Michiels, 2008). This point of view has a sound physiological basis, as tumour blood flow is neither constant nor evenly distributed (Fukumura and Jain, 2008).

## Impairment of mitochondrial function

Although OXPHOS represents a clear advantage in energetic terms, it can also damage cells through ROS formation. When these free radicals are overproduced relatively to cell´s arsenal of antioxidants, damage to biomolecules and cellular signalling pathways are activated, triggering oncogenic transformation (Valko et al., 2006).

In 2008, Lopez-Lázaro proposed that cancer cells have a dysoxic metabolism: instead of being reduced to water via OXPHOS, oxygen is transformed into the ROS superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$), inducing glycolysis; $O_2$ inhibition of fermentation (Pasteur effect) is bypassed simply because it is not used for ATP production (Lopez-Lazaro, 2008). In fact, superoxide provokes intracellular alkalinisation without compromising cell integrity, consuming protons from the reduction of $O_2$ to $H_2O_2$ (Ikebuchi et al., 1991) and stimulating PFK-1, a key enzyme in glycolysis (Erecinska et al., 1995). Several hematopoietic growth factors also proved to selectively trigger $O_2^-$ release in human monocytes in order to induce cellular proliferation (Yuo et al., 1992). On the other hand, $H_2O_2$ increases $Na^+/H^+$-ATPase activity within its physiological range of concentrations (González-Flecha et al., 1996), enhances proto-oncogenes expression (Li and Spector, 1997) and is an important messenger in Endothelial Growth Factor Receptors (EGFR) family and HIF-1α activation (Liu et al., 2006). Furthermore, a recent study (Rodriguez-Enriquez et al., 2000) confirmed that ascites cancer cells present high rates of glucose into lactate conversion, even in the presence of a $O_2$ concentration that greatly exceeded OXPHOS needs, in accordance with Warburg's original results (Warburg, 1956b).

Lipidomics studies (Kiebish et al., 2008) with purified mitochondria samples from different types of brain tumours grown *in vivo* and respective host strains established a clear connection between cardiolipin (CL) content and composition and electron transport chain (ETC) activity. In brief, all brain tumour lines presented CL abnormalities (although specific for each line) and corresponding ETC defects, being restoration of CL molecular speciation not reasonable. Thus, despite the variety of genetic, epigenetic and environmental issues that may interfere with a cell´s CL composition (Kiebish et al., 2008), intrinsic mitochondrial defects play an important role in cancer metabolism. Mitochondrial DNA mutations were found to interfere with mitochondrial function in some cancers, inducing essentially low respiratory rates and elevated ROS productions (Bonora et al., 2006), but their relevance is still controversial due to studies pointing out that they may often remain silent (Meierhofer et al., 2006).

According to Samudio, Feodor Lynen had put forward already in 1951 the possibility that behind cancer cells' increased glycolytic rate was mitochondrial uncoupling rather than a permanent respiratory impairment, rendering them unable to synthesize ATP via mitochondrial proton gradient (Samudio et al., 2009). This alternative to part of Warburg's hypothesis gained visibility with the recent finding that UCP2 (a mitochondrial uncoupling protein) is overexpressed in several chemoresistant cancer cell lines (Samudio et al., 2009). Despite its name, there is no consistent data yet ensuring that it is actually a mitochondrial uncoupler, as its abundance is low in all tissues under normal conditions and it was identified with UCP1 (which exists in brown adipose tissue and is involved in thermogenesis (Nelson and Cox, 2008)) through sequence homology (Baffy, 2010). Notwithstanding, these evidence endorse the importance of other carbon sources besides glucose, such as glutamine (Kovacevic and McGivan, 1983), in maintaining mitochondrial function, and metabolic reprogramming by mitochondrial uncoupling in increasing some malignancies' chemoresistance (Samudio et al., 2009). Furthermore, recent experiments (Derdak et al., 2008) revealed that UCP2 overexpression visibly diminished apoptosis in malignant cells subjected to different anticancer agents, while increasing oxygen consumption and maintaining ATP levels, possibly through decreased mitochondrial transmembrane potential and thus reduction of ROS production or by inhibiting p53 oxidative stress-dependent activation of apoptosis.

There is a strong correlation between glucose deprivation-induced apoptosis and mitochondrial ROS production both in cancer and in normal cells. When treated with ETC blockers, glucose-deprived prostate cancer cells were found to be unable to produce enough glutathione to balance $O_2^-$ and $H_2O_2$ overproduction, resulting in oxidative stress, the disruption of redox signalling and control (Ahmad et al., 2005). Similarly, hypoglycaemia in PC12 cells was responsible for an increase in ROS production and ATP depletion, which led to mitochondrial dysfunction and ultimately to cell death (Liu et al., 2003). As during glucose scarcity the oxidative metabolism is preferred and indeed cells lacking functional mitochondria (rho 0 cells) were far more resistant to citotoxicity under these conditions, mitochondrial ROS thus trigger cancer cell death when glucose availability is compromised. An increase in glycolysis rate in detriment of OXPHOS may serve as a faster way to produce energy and antioxidant molecules (pyruvate and NADH (Ahmad et al., 2005)), which further explains the deleterious effects of glucose deprivation in tumours *in vivo* demonstrated by Warburg during the 1920 s (Warburg et al., 1927).

Mitochondria are responsible for detecting decreases in oxygen concentration and initiate HIF-1α activation and VEGF release from tumour cells (Klimova and Chandel, 2008). Although cell cycle arrest and apoptosis may occur during $O_2$ and nutrients scarcity before angiogenesis (Li et al., 2007), certain mutations, HIF-dependent or not, can render tumour cells resistant to the latter (Akakura et al., 2001). Interestingly, ROS produced by the complex III of respiratory chain seem to be involved in HIF-1α stabilization during hypoxia, as hypoxia increases ROS generation and treatments with antioxidants block HIF-1α stabilization, making this complex part of the cell oxygen sensing machinery (Klimova and Chandel, 2008). Activation of complex III diminishes conversion of pyruvate in acetyl-CoA, decreasing OXPHOS rate, and switches cytochrome c oxidase to a more efficient form, limiting electron leak. This is therefore a feedback mechanism used by cancer cells to survive under hypoxic conditions (Klimova and Chandel, 2008). Mitochondrial dynamics comprehension is thus essential to understand carcinogenic transformations.

## Tumour microenvironment

New methods are available for the imaging of tumours' microenvironment (Fukumura and Jain, 2008). Although the use of cell cultures to study bioenergetics is utmost important to advance our knowledge on cancer behaviour, it cannot mimic *in vivo* tumour-host interactions and may introduce artefacts. For example, the simple change from $Na^+$ to $K^+$ in culture medium rendered visible

differences in glucose, glycogen and lactate contents in both normal hepatic and hepatoma cells (Ashmore et al., 1958). Tumours develop integrated in living tissues and, as suggested by Warburg, consist of a complex mixture of proliferating, quiescent and dead cells (Pyaskovskaya et al., 2008; Warburg et al., 1927).

Tumour growth beyond certain dimensions is dependent on angiogenesis and may be controlled by microvascular endothelial cells (Folkman, 2003). Cancer cells produce several oncogene-triggered angiogenic proteins whose overexpression, and hence tumour growth and metastases, can be provoked by hypoxia and low pH, the two main attributes of tumours' microenvironment (Fukumura, 2005). Furthermore, VEGF release is activated in a non-cooperative manner: while hypoxia increases HIF-1α stability, which binds to VEGF promoter, enhancing its transcription, low pH induces interleukin 8 and nitric oxide synthase via NFkB, leading to VEGF upregulation (Fukumura et al., 2001). The reason for this lack of synergism remains unclear. Interestingly, a gradual diminution in vascular demand is observed in tumours. It is known that an angiogenic switch happens during tumour development, perhaps resulting from an imbalance of positive and negative regulators of angiogenesis produced by cancers, which contributes to a highly heterogeneous tumour neovasculature that is ineffectual in delivering nutrients and removing waste products (Folkman, 2003).

Although depletion of mitochondrial DNA to create rho 0 cells usually results in ablation of oxygen uptake (Li et al., 2003), new evidence may provide a partial explanation to high glycolysis rates without a visible decrease in oxygen consumption in cancer cells and the low pH of tumours *in vivo*. A study published in 2007 draws our attention to the implication of a trans-plasma membrane electron transport (tPMET) system in attenuating reductive stress in highly glycolytic cells (Herst and Berridge, 2007): using several cell lines in comparison with their rho 0 counterparts, the authors found that cell surface oxygen consumption significantly accounts for total oxygen consumption by various tumorigenic cell lines and is proportional to glycolysis rate, challenging the paradigm that all bioenergetic oxygen consumption in mammalian cells is mitochondrial. Therefore, the measurement of oxygen used by Weinhouse fifty years ago to test mitochondrial function in neoplasias (Weinhouse, 1955) or, more recently, the proposal of Guppy and Zu to use this same aspect to assess oxidative metabolism (Zu and Guppy, 2004) could be intrinsically error prone. Furthermore, it was shown that tPMET also extrudes protons, agreeing with experiments that found lactic acid production insufficient to explain malignancies low pH (Newell et al., 1993).

Importantly, tumour microenvironment has not clear-cut properties, for its hallmarks, increased glucose uptake, hypoxia and acidosis, are not always intuitively distributed (Cherk et al., 2006; Helmlinger et al., 1997). As wisely pointed out by Helmlinger, the activation of different metabolic pathways in tumours may produce similar characteristics in their microenvironment (Helmlinger et al., 2002).

## Proliferating *versus* cancer cells

In 1929, Crabtree first described that glucose addition inhibited oxygen consumption in proliferative cells, a phenomenon now called Crabtree effect (Crabtree, 1929) that was established in a specific cell line only sixty years later (Guppy et al., 1993). It was accepted that oxygen uptake diminished due to ADP diversion from OXPHOS by hyperglycaemia-driven glycolysis upregulation (Krebs, 1972), whereas the sparing of a still unknown endogenous fuel and the duplication of ATP turnover without visibly increased ROS production were seen as benefits of this phenotype (Guppy et al., 1993). The importance of glucose in proliferation is unquestionable, as this substrate represents the starting point for more than 80% of all the phosphorus containing compounds in cells (Pyaskovskaya et al., 2008). In fact, malignant cells are more responsive to glucose depletion than to cell density fluctuations, promoting a transition from proliferating to resting state to improve their resistance to hypoglycaemia (Pyaskovskaya et al., 2008).

One of the main regulators of glycolysis is fructose 2,6-bisphosphate (F2,6BP) (Vanschaftingen et al., 1980), as it activates PFK-1, a key enzyme in glycolysis (Erecinska et al., 1995), being its concentration dependent on the bifunctional 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) enzyme activity (Yalcin et al., 2009). One of the isozymes of the PFKFB family, PFKFB3, is markedly expressed both in malignancies and in non-transformed proliferating tissues with a high kinase relatively to phosphatase activity, ensuring high levels of F2,6BP (Yalcin et al., 2009). PFKB3 thus participates in the coordination of cellular proliferation and glycolysis rate in both proliferative and cancer cells.

Some cancer cell lines were recently found to rapidly present visible morphological adaptations to substrate availability changes: when glucose was not in the medium, synthesis of OXPHOS components, cristae content and ramification of the mitochondrial network all increased (but not total mitochondria mass). Similar but less significant structural changes took place in nontransformed fibroblasts, establishing another possible link between proliferating and cancer cells (Rossignol et al., 2004). The role of dedifferentiation in tumorigenesis is often overlooked, but Warburg's original hypothesis already established a connection between cancer metabolism and cell structure, implying mitochondrial impairment in malignant dedifferentiation (Warburg, 1956b) (oxygen was the creator of differentiation for him (Warburg, 1969)) and the disordered growth of cancer cells relatively to normal proliferation in their altered metabolism (Warburg, 1930). This relation was taken into account by other authors during those years: it was observed in a classical review on cell division control (Swann, 1958) that, during division, a large proportion of cell's mass consists of the nucleus and mitotic apparatus, while the amount of cytoplasm is almost halved. The damage to respiration as the prime cause of neoplasias proposed by Warburg (Warburg et al., 1924) could then be in line with the selection of cells with reduced non-essential protein synthesis and high demand for rapid energy production in an anaerobic microenvironment. Differentiated functions, such as mitochondrial respiration, are thus severely affected during the early stages of carcinogenesis, which creates a selective pressure that favours cancerous cells with conspicuous proliferation rates and glucose consumptions to outgrow normal cells (Gillies and Gatenby, 2007). A parallel between proliferating and cancer cells can thus be established. Warburg effect has also been observed at some levels in non-transformed proliferating cells (Lopez-Lazaro, 2008), but a frontier between both ought to be defined. It is known for many decades that neoplasias and proliferating cells have distinct properties besides their division rates, as studies carried out with two cell lines displaying similar division capacities proved that the malignant one presented objectively greater aerobic and anaerobic fermentation quotients (Kidd et al., 1944), in line with previous findings showing that, although aggressive tumour cells overexpressed isozymes only present in embryonic tissues (Weinhouse, 1972), the latter did not produce lactic acid under aerobic conditions (Warburg et al., 1924).

Most importantly, normal proliferating cells come back to an oxidative metabolism in the resting state, unlike tumour masses (Warburg et al., 1924). The first work establishing an irrefutable connection between p53 and the glycolytic pattern observed in some malignancies was published more than a decade ago (Mathupala et al., 1997), and src and ras oncogenes were found to trigger GLUTs expression even earlier (Flier et al., 1987). In fact, p53 has a recognized intervention in both main energy-yielding processes: it directly regulates the expression of the SCO2 ("Synthesis of Cytochrome c Oxidase") gene, which facilitates the assembly of cytochrome c oxidase complex in ETC (Matoba et al., 2006), while it represses glycolytic flux by promoting the synthesis of TIGAR ("TP-53 induced glycolysis and

apoptosis regulator"), which dephosphorylates F2,6BP back to fructose 6-phosphate and hence diminishes glycolytic flux (Bensaad et al., 2006). It is becoming evident that cancer metabolic reprogramming, unlike normal resting to proliferative state transition, occurs in the sequence of permanent mutations, from which p53 is just an example, that render tumour cells incredibly resilient to changes in their microenvironment through the constitutive expression of cytokines (Argilés et al., 2003) and mutated oncogenes involved in metabolic regulation (Jones, 2009; Kroemer and Pouyssegur, 2008).

## More on cancer bioenergetics - integration of metabolism

Several studies call the attention to the diversity of carbon sources besides glucose used (Lazo, 1981; Richardson et al., 2008; Rodriguez-Enriquez et al., 2000) and the altered protein expression pattern found during malignant transformation (Rempel et al., 1994; Weinhouse, 1972), urging the need of extensive metabolomics and proteomics studies to fully characterize cancer bioenergetics. In fact, some cancers overexpress GLUT-5, a fructose transporter (Pauwels et al., 2000), perhaps to be less affected by glycolytic regulation (Zamora-León et al., 1996), and lactate is not always resultant of fermentation, as cells can also obtain pyruvate via alanine transamination or glutamine-derived malate conversion (Frezza and Gottlieb, 2009). The recognition of the importance of these metabolic networks led to the use of several spectroscopic techniques, such as $^{13}$C-Nuclear Magnetic Resonance and Mass Spectrometry, in modern cellular metabolic profiling (Richardson et al., 2008).

That increased activity of a key enzyme in a given pathway augments its flux is a classical misconception. However, overexpression of hexokinase (HK) and PFK-1 does not increase glycolytic flux, as their regulatory mechanisms, allosteric or product inhibition, are also exacerbated, thus reducing their activity in a directly proportional way (Moreno-Sanchez et al., 2008). The reason for the increased glycolytic flux in cancers which overexpress HK may be more subtle and not readily assessed by studies using purified enzymes *in vitro*. Different HK isoenzymes are able to bind to the outer mitochondrial membrane, becoming less sensitive to feedback inhibition by glucose 6-phosphate and proteolytic degradation (Rempel et al., 1994). Curiously, experiments show that the percentage of bound HK (mainly the type IIb) activity increases visibly during the process of malignization (Rempel et al., 1994). A broad enzymatic study carried out in 1990 with metastatic cells yielded some interesting results: the maximum activity of PDH greatly exceeded the glycolytic flux, diverting pyruvate from mitochondria; pyruvate kinase was overexpressed relatively to HK and PFK-1, whose levels were similar to those in normal glycolytic tissues (e.g. muscles (Zammit et al., 1978)); Krebs cycle enzymes were all functional, but the flux through the ones used in glutaminolysis (from oxoglutarate to malate) was much higher, greatly exceeding nitrogen demand for nucleotides synthesis; glutaminase maximum activity often surpassed that of HK (Board et al., 1990). The information presented above not only confirms that pyruvate fate decision is a crucial checkpoint (Fantin et al., 2006), the high lipogenesis rates of cancer cells (Brusselmans et al., 2005; Hatzivassiliou et al., 2005; Lazo, 1981) and the role of PEP in growth arrest (Zeitouni et al., 2002), but also suggests that glutamine may be used for energy purposes. In fact, it has been observed since the 1980s that glutaminolysis rate increases with malignancy grade (Kovacevic and McGivan, 1983). This pathway allows replenishment of Krebs cycle for anaplerotic reactions (Frezza and Gottlieb, 2009) and yields ammonia, which was recently found to be a diffusible autophagy regulator, thus mediating resistance to chemotherapy and oxidative stress in nutrient-poor regions of solid tumours (Eng et al., 2010).

The importance of fat in cancer patients was first described by Budwig in 1952 (Warburg, 1969). Some fatty acids have indeed a well recognized role in enhancing cell proliferation (Kim et al., 2009) and represent an important energy source in slow-growing hepatomas (Weinhouse, 1972). Moreover, cancer cells present high rates of cholesterologenesis, being the inhibition of its principal enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and consequent decrease in Ras oncoprotein levels (Cox and Der, 1997) an efficient strategy in cancer therapeutics (Wachtershauser et al., 2001). However, palmitoylcarnitine and carnitine induce apoptosis in cultured transformed cells by increasing synthesis of ceramide, a proapoptotic lipid, and inducing glucose and fatty acid oxidation, leading to mitochondrial ROS generation (Wenzel et al., 2005). The latter modification is achieved via carnitine/acetyl transferase (delivers free CoA to the matrix, enabling pyruvate uptake by mitochondria) and via acylcarnitine/carnitine translocase (allows an increased fatty acid import and subsequent β-oxidation) (Wenzel et al., 2005). As some tumour cells present low free carnitine levels (Peluso et al., 2000) and conspicuous lipogenesis rates (Brusselmans et al., 2005; Hatzivassiliou et al., 2005; Lazo, 1981), diminution of fatty acid oxidation in cancer could be another protective mechanism against ROS-induced apoptosis.

Substrate availability can also shape cancer metabolism, being proline an elucidative example (Rossignol et al., 2004). Albeit its contribution to PPP is modest under physiological conditions (Phang et al., 2008), it may be relevant for neoplasias energy generation: although proline supply is limited by the inefficient tumour vasculature (Fukumura and Jain, 2008), the proline dehydrogenase gene is upregulated under genotoxic, inflammatory and nutrient stress (Phang et al., 2008), and its availability increases substantially during metastases and invasion, as extracellular matrix degradation (of which 80% is collagen) releases free proline; this amino acid is indeed conspicuously consumed in breast cancer cells (Richardson et al., 2008)).

Finally, some symptoms of cancer patients, namely cachexia (loss of body fat and muscle mass (Bongaerts et al., 2006)), can also be explained using a metabolic point of view. Tumours rely heavily on glucose uptake to generate ATP and lactic acid through glycolysis, inducing hepatic gluconeogenesis: as the surplus of lactic acid and glucose deficit increase, the first is transported through the blood stream to the liver, where it is converted into glucose, being the energetic cost of this process covered by the release of amino and fatty acids from tissues (Bongaerts et al., 2006). The Cori cycle (Cori and Cori, 1929) is then also involved in cancer bioenergetics *in vivo*. It is now known that these metabolic adaptations are achieved through cytokines action: although the host produces several of these factors under normal and pathological conditions, the focus is turning to tumour-derived cytokines, as they are expressed constitutively and act both in the tumour itself and in the surrounding tissues (Argilés et al., 2003).

Altogether, these data clearly show that studies on the energy metabolism of cancer cells can no longer focus solely on glycolysis and OXPHOS. Integration of metabolism is essential to unravel tumours' behaviour, which is conditioned by several factors, including location, cell type, and malignancy grade. This complexity greatly hampers an accurate definition of cancer metabolome.

## Perspectives

It is quite impressive that, after all these scientifically glorious decades since Warburg´s first observations, the metabolism of cancer cells is such a remarkably exciting issue in so many ways. The Warburg effect, as a description of the metabolic particularities of malignant tissues observed by Otto Warburg in his pioneering experiments, can be objectively defined as an increased glucose uptake and lactate extrusion by tumours, with the consequent pH decrease in surrounding tissues, even in presence of ample oxygen. Any statement that goes beyond this is hence a conjecture about the topic and shall not be confused with incongruence of the phenomenon itself.

A complete comprehension of cancer is not foreseen in the near future. Nevertheless, by exploring cancer metabolism, we are now able to design new therapeutic methods, effective at the molecular level. For instance, a myriad of drugs targeting metabolic pathways (Kroemer and Pouyssegur, 2008; Lopez-Lazaro, 2008) and cytokines' synthesis and action (Argilés et al., 2003) is continuously being developed for cancer therapy. More innovative and effective approaches are burgeoning due to metabolomics and systems biology thriving (Moreno-Sanchez et al., 2008). Although molecular biology methods didn´t convince all biochemists of the 20th century at first (Warburg, 1969; Weinhouse, 1972), several findings brought these two sciences together in order to study the numerous interplays between cancer cell biology and biochemistry (Racker and Spector, 1981): it was found that infection of cells with retroviruses could result in neoplasia (Bishop, 1985), being Rous sarcoma a famous example, as it proved to have the same metabolism of carcinomas in Warburg's laboratory (Warburg, 1925); Weinhouse acknowledged cancer's dysfunction of gene control (Weinhouse, 1972) and Warburg recognized that damaged respiration was hereditary in cancer cells (Warburg, 1930). Thanks to this thought revolution, it is common nowadays to address cancer diagnostics and treatments with an effective metabolic point of view (Kroemer and Pouyssegur, 2008), much in line with Warburg's suggestions during his brilliant research career (Warburg, 1969).

## Conflict of interest statement

None declared.

## Acknowledgments

Thanks are due to Prof. Dr. Ana M. Urbano for critical input and several helpful discussions during preparation of the manuscript.

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