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The Warburg effect: 80 years on

Michelle Potter, Emma Newport and Karl J. Morten

Nuffield Department of Obstetrics and Gynaecology, The Women Centre, University of Oxford, John Radcliffe Hospital, Oxford, U.K.

Correspondence: Karl J. Morten (karl.morten@obs-gyn.ox.ac.uk)

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Influential research by Warburg and Cori in the 1920s ignited interest in how cancer cells' energy generation is different from that of normal cells. They observed high glucose consumption and large amounts of lactate excretion from cancer cells compared with normal cells, which oxidised glucose using mitochondria. It was therefore assumed that cancer cells were generating energy using glycolysis rather than mitochondrial oxidative phosphorylation, and that the mitochondria were dysfunctional. Advances in research techniques since then have shown the mitochondria in cancer cells to be functional across a range of tumour types. However, different tumour populations have different bioenergetic alterations in order to meet their high energy requirement; the Warburg effect is not consistent across all cancer types. This review will discuss the metabolic reprogramming of cancer, possible explanations for the high glucose consumption in cancer cells observed by Warburg, and suggest key experimental practices we should consider when studying the metabolism of cancer.

The Warburg effect

Despite decades of research and countless financial investments, cancer continues to elude our complete understanding and more importantly our therapies. Pivotal research in the 1920s by Warburg and Cori demonstrated that cancer avidly consumes glucose and excretes lactate [1,2]. When oxygen is present, normal cells use mitochondria to oxidise glucose, but in the absence of oxygen, glucose is converted into lactate. Otto Warburg first described in the 1920s that cancer cells utilised higher levels of glucose in the presence of oxygen with an associated increase in lactate production. The phenomenon of aerobic glycolysis, termed the Warburg effect, has been observed in a variety of other tumour types, including colorectal cancer [3], breast [4], lung [5] and glioblastoma [6,7]. From his observations, Warburg concluded that the mitochondria were dysfunctional [8,9]. The Warburg effect has been confirmed in previous studies including those of DeBerardinis et al. [10], where cells were incubated under oxygenated conditions in 10 mM C-13-labelled glucose. Cells were then perfused using 4 mM glucose prior to metabolomics analysis and even in the presence of oxygen, high levels of glycolytic metabolites were observed supporting Warburg's hypothesis. In addition, Fantin et al. [11] made the observation that inhibiting lactate dehydrogenase preventing the conversion of pyruvate to lactate reduced tumourigenicity. These data were interpreted as tumourigenicity being dependent on high levels of energy derived from glycolysis. Another study by Schulz et al. [12] showed that when mitochondrial oxidative phosphorylation is up-regulated by overexpression of frataxin, malignant growth and tumourigenic capacity are decreased. The authors suggest that rather than an increase in glycolysis being the main cause of malignant tumour growth, it is the efficiency of mitochondrial energy conversion that is the key metabolic factor.

Over the past couple of decades, advances in technology have allowed mitochondrial function to be studied in a far greater detail, and it is now realised that cancer cells have active and functional mitochondria, contrary to Warburg's theory [13,14]. In the last decade, research has shown that different tumour types (and indeed subpopulations within a tumour) have different bioenergetic alterations. This was shown as early as 1967, when Weinhouse reported that slow-growing rat hepatoma cells were oxidative, whereas the more proliferative hepatomas were glycolytic [15]. The Warburg effect is not consistent across all tumours, and the phenomenon of aerobic glycolysis has now been challenged by several groups with many cell lines reported as having mitochondrial function [16–18]. In a

tumour, it is likely that a dynamic interplay exists between oxidative metabolism and glycolysis. Metabolic flexibility has now been observed in a range of cancers, including cervical, breast and pancreatic cancer (see ref. [16] and reviewed in ref. [19]). In 2004, Zu and Guppy reported that adenosine triphosphate (ATP) derived through glycolysis in various cancers and cell lines accounts for only 17% of the total ATP. In fact, the ATP generated through glycolysis was highly dependent on the cell type and could be as low as 0.31% (fibrosarcoma) or as high as 64% (hepatoma), with the remaining ATP being derived from mitochondrial oxidative phosphorylation [20]. In addition to metabolic flexibility linked to environmental conditions, there is also the influence of various cancer-associated mutations, many of which have an impact on metabolism. Mutations in the mitochondrial tricarboxylic acid cycle and respiratory chain component succinate dehydrogenase, for example, can cause phenochromocytoma and paraganglioma, where neuroendocrine tumours arise in the adrenal medulla and paraganglia in the autonomic nervous system [21,22]. Mutations in isocitrate dehydrogenase 1 are associated with adult cases of glioblastoma and appear to have a major role in the development of the tumour by a gain-of-function effect [23,24]. Understanding how cancer cell environment and mutations affect metabolism will be of fundamental importance in selecting appropriate metabolic drug combinations to impact on patients' cancer cell growth.

Cancer hallmarks and metabolic reprogramming

Cancer cells show complex, dynamic behaviour allowing survival even in the most unfavourable conditions of substrate and oxygen stress. Advances in technology have helped in furthering our knowledge of the underlying molecular processes underpinning cancer, but there are still many unanswered questions. In 2000, Hanahan and Weinberg published a highly cited review article identifying six cancer hallmarks [25]. These included uncontrolled proliferative signalling, resistance to apoptosis, initiating angiogenesis, acquiring replicative immortality, activating invasion and metastasis and evading growth suppressors. Over the last decade, research has increased our knowledge of cancer, and in 2011, Hanahan and Weinberg extended the list of cancer hallmarks to include metabolic reprogramming/deregulated cellular energetics as an emerging hallmark and potential cancer target [26]. Uncontrolled proliferation is one of the essential characteristics of cancer. It has been proposed that reprogramming energy metabolism is essential to fuel and maintain such behaviour [26]. The exact reasons behind the metabolic switch are not known, but likely reasons include: (i) sustaining high proliferative rates in hypoxia [27] and (ii) evading apoptosis as a result of reduced mitochondrial function [28]. Increases in glycolysis have been linked to invasiveness, with changes in glycolysis identified in several studies [29,30]. However, in all studies listed above, cancer cells were grown on cell culture media containing high levels of glucose between 10 and 25 mM. This is considerably higher than plasma glucose, which lies between 4 and 6 mM. Levels in a rapidly dividing tumour with poor vasculature are considerably lower. The same is true of studies investigating the role of hypoxia in down-regulating mitochondrial respiration and increasing glycolysis, where 25 mM glucose is used in the culture media of key publications [31–34]. The impact of high levels of glucose on the above findings is a key consideration for future studies, where it is crucial to test new drugs targeting cancer cell metabolism under physiologically relevant conditions. Metformin, for example, a drug currently being investigated as an anticancer agent in a wide range of cancers [35], has recently been shown to be more effective in enhancing chemotherapy sensitivity of oesophageal squamous cancer cells under reduced glucose conditions [36]. Although its mode of action on cancer cells *in vivo* is not entirely clear, mitochondrial studies suggest that metformin can directly impair complex I of the respiratory chain [37,38]. The effect observed by Yu et al. is probably due to a greater reliance of the cancer cells on mitochondrial respiration for energy production when cultured on reduced glucose conditions. Previous studies have shown that high levels of glucose in the culture media can significantly reduce levels of mitochondrial respiration, with reduced glucose conditions showing much higher rates of mitochondrial respiration, as cells use other substrates for cellular ATP production [39,40]. Similar results are shown in Figure 1, where the oxygen consumption rates (OCRs; mitochondrial respiration) and extracellular acidification rates (ECAR; glycolysis) of a range of cancer cell lines are compared under high (25 mM) and low (1 mM) glucose conditions. Under high glucose (25 mM) conditions, cancer cell lines either show the Warburg effect (low OCR and high ECAR), high rates of OCR and low ECAR or something in between high/moderate OCR with high/moderate ECAR. A finding highly relevant to the situation *in vivo* is that when cultured under low glucose (1 mM) conditions, all cancer lines tested show high–moderate OCR with very little ECAR (glycolysis).
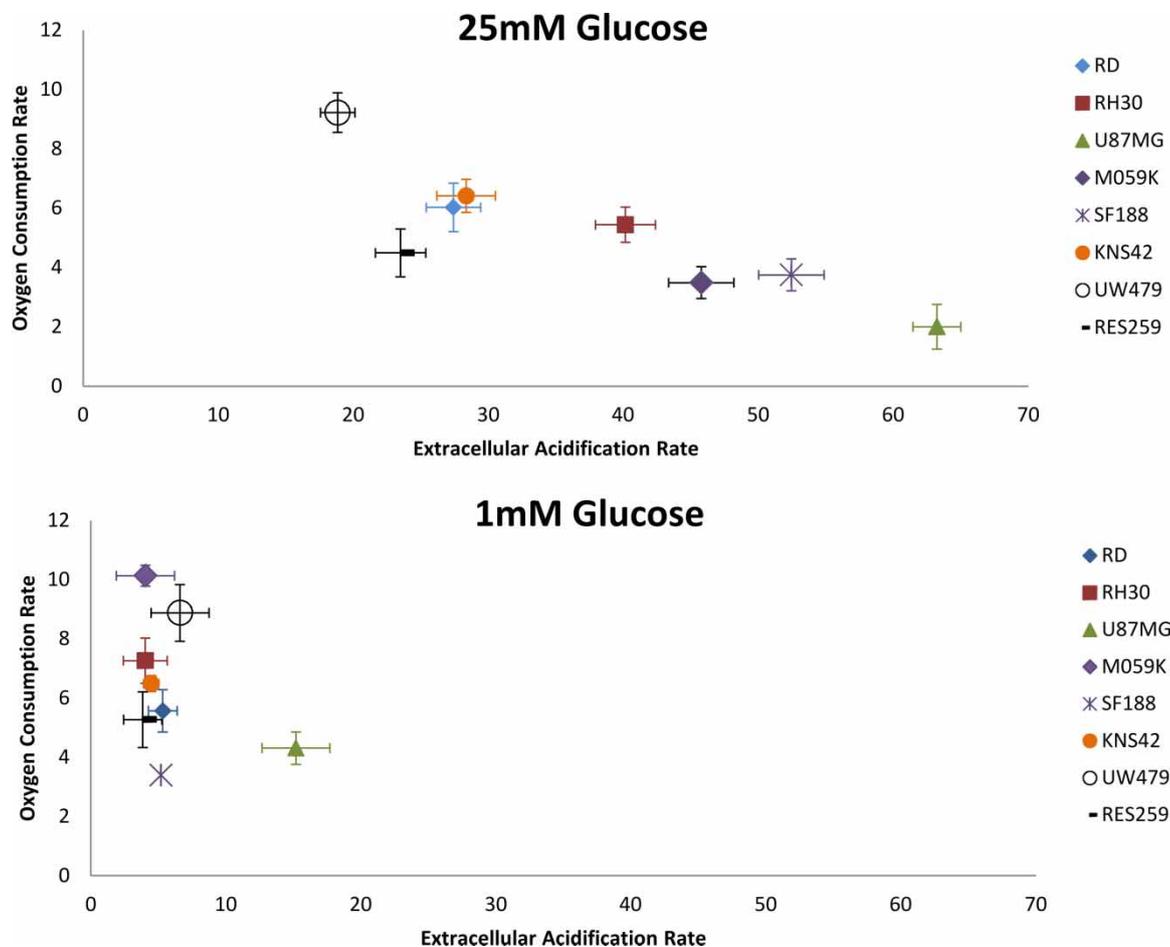
Figure 1. Bioenergetic profiles of cancer cell lines RD, RH30, U87MG, M059K, SF188, KNS42, UW479 and Res259.
OCR and ECAR are plotted to quantify mitochondrial respiration and to give an indication of glycolysis rates. OCR and ECAR
are expressed as changes in fluorescence life time/h/75 000 cells ($n = 3$). Assays were set up in black 96-well plates with
pre-incubation in 1 mM and 25 mM glucose media carried out for 16 h. Oxygen and glycolysis sensing probes: MitoXpress xtra
and pH xtra from Luxcel Biosciences were used to determine OCR and ECAR (http://luxcel.com/).

Positron emission tomography imaging and the reverse Warburg effect

Positron emission tomography (PET) imaging uses a radioisotope-labelled glucose tracer, ^{18}F-fluorodeoxyglucose (^{18}F-FDG), to identify areas of high glucose uptake/metabolism in the body. ^{18}F-FDG is transported into cells by glucose transporters (GLUTs) and phosphorylated by hexokinase (HK) to ^{18}F-FDG-6-phosphate (^{18}F-FDG-6-P). Once inside the cell, ^{18}F-FDG-6-P cannot be further metabolised through the glycolytic pathway and due to its high polar nature becomes trapped. Tumours above a certain size label strongly with this approach, and it is used to identify the presence of solid tumours and the effectiveness of treatments. Other highly metabolically active tissues, such as the brain and heart, also label strongly. It is believed that PET scans show an increased uptake of glucose in tumours due to overexpression of GLUTs [41–43]. Historically, increased glucose uptake has been associated with supporting the Warburg effect [20]. However, high glucose uptake does not automatically equate to increased glycolysis and reduced mitochondrial metabolism. An increased PET signal could be due to a general increase in glucose oxidation with increased glycolysis and mitochondrial respiration or a high demand for lipids derived from glucose. In addition, over-expression of GLUTs cannot be assumed to correlate with increased metabolic flux. A high PET signal would be obtained if glucose entered the cancer cell and was not metabolised. Many tumours are characterised by

increased levels of GLUTs (particularly GLUT 1 and GLUT 3) and high expression of HK (I and II), which is associated with the increases in ^{18}F-FDG signal compared with surrounding tissues as reviewed in ref. [44]. However, not all cancers are easily detected by PET imaging, including renal clear cell carcinoma, which is a prototypical Warburg cancer [45]. Conversely, not all ^{18}F-FDG avid tissue is malignant; inflammation can also lead to a positive PET signal. Interestingly, Hodgkin's lymphoma responds well to PET imaging [46]. This tumour is less than 10% cancer cells, and the remaining cells are stromal/inflammation cells [47].

Although PET imaging is undeniably an extremely useful and an important clinical technique, there can be issues with the interpretation of the image. It is a common problem that the PET images can often overestimate the actual size of the tumour. A possible reason for this is that the microenvironment of the tumour is glycolytic in a phenomenon that has been called the reverse Warburg effect. First postulated by Pavlides et al. in 2009 [48], the reverse Warburg effect describes how oxidative stress in the cancer-associated fibroblasts (CAF) induces mitophagy and autophagy. The hypothesis is that hydrogen peroxide secreted from the cancer cells leads to oxidative stress in the CAF. The fibroblasts then undergo cellular catabolism, which results in a loss of mitochondrial function and ultimately a switch from aerobic metabolism to glycolysis [49]. This glycolytic switch results in increased lactate production by CAF, which is then exported into the extracellular space by monocarboxylate transporter 4 (MCT4). The lactate is ultimately taken up by the cancer cells via MCT1 and used to fuel oxidative metabolism [50,51]. In ref. [50], the authors demonstrate support for the reverse Warburg effect by culturing human breast cancer cell lines with human fibroblasts. Both MCF-7 and MDA-MB-231, when co-cultured with fibroblasts, show reduced mitochondrial function in fibroblasts with increased activity in the cancer cell lines. As in the studies of the Warburg effect and the impact of hypoxia described earlier, studies to date on the reverse Warburg effect use high concentrations of glucose 25 mM. If glucose is rapidly removed from the media by the cancer cells, this would tend to drive the cancer cells to use mitochondrial respiration (see Figure 1). Under high glucose conditions, this property of cancer cell lines will be held back by glucose inhibition of mitochondrial respiration.

Lactate was discovered in the late 1700s and was traditionally thought of as a waste product of glycolysis. In reality, lactate is an extremely efficient fuel and also an important signalling molecule [52,53]. It is constantly turned around in our cells, regardless of oxygenation state. Lactate is a key metabolite in the body, capable of replacing glucose as an energy source. Lactate is also capable of stabilising hypoxia-inducing factor and increasing vascular endothelial growth factor expression [53]. The fibroblast–tumour metabolic coupling proposed to exist in the reverse Warburg effect is analogous to the metabolic symbiosis seen in the brain (Figure 2). The brain is a metabolically demanding organ that gives high PET signals. Its avidity for glucose was historically attributed to the neurons; however, it is the astrocytes that are glucose hungry and glycolytic. The lactate secreted by the astrocytes then fuels the neurons, which use oxidative phosphorylation to generate ATP [54]. The Warburg effect is not a universal feature of cancer, and similarly the reverse Warburg is not universal in all tumours. Yoshida, in 2015, showed that tumours expressing high levels of MCT4 do no exhibit the reverse Warburg effect [55]. The microenvironment of cancer is ever changing, and cancer cells can and do vary in their metabolic phenotype even within the same tumour mass [56]. Although hard to generalise in solid tumours with a hypoxic core, perhaps the Warburg effect most probably predominates with reduced oxygen levels driving the cells to make the most of all available glucose. The more actively proliferating cells in the periphery may perhaps use the lactate excreted in the hypoxic region and oxidise it [57], leading to a symbiotic relationship between the hypoxic and aerobic cell populations. *In vitro* metabolism studies are useful tools, but it only serves to hinder therapeutic translation when non-physiological glucose concentrations up to and including 25 mM glucose are used instead of a physiological concentration of 5 mM (plasma) or <5 mM (tissue).

In summary, we have sought here to revisit the Warburg effect and review its significance in cancer based on recent advances in our knowledge and understanding of the complex biology underlying this disease. It is indisputable that certain features of cancer are indeed hallmarks that are essential for most types of cancer. However, looking to the future, the role of aerobic glycolysis needs further elucidation, as it is not a consistent feature in all cancers. Recent research has shown there to be a broad spectrum of bioenergetic phenotypes displayed by cancer both *in vivo* and *in vitro*, with many cancer types displaying a surprising degree of mitochondrial activity. When investigating the role of aerobic glycolysis *in vitro*, it is pertinent to use physiologically relevant concentrations of nutrients, in particular glucose. The excessive glucose often found in cell culture media can decrease mitochondrial respiration, allowing aerobic glycolysis to predominate. Reducing the glucose in the media to physiological levels will give a truer picture of the complex metabolic processes at work.
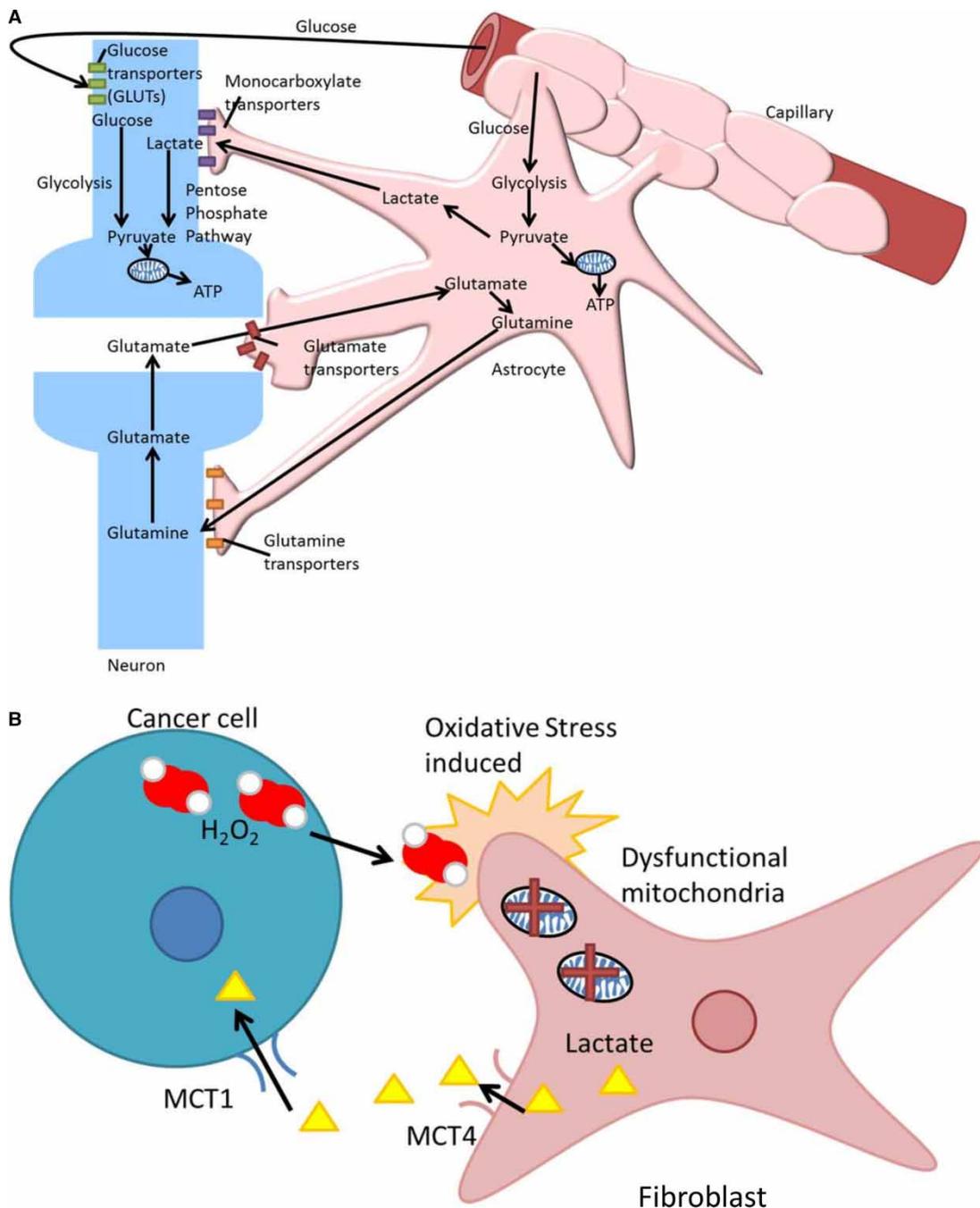
Figure 2. The astrocyte–neuron shuttle (A) and the reverse Warburg effect (B).

(**A**) Glutamate is released from activated synapses and taken up by astrocytes triggering an increase in glycolysis and lactate production. The lactate can be oxidised by the neurons in response to their increased energy requirement to produce ATP.

(**B**) nIn the proposed reverse Warburg effect, hydrogen peroxide is secreted by cancer cells leading to oxidative stress in the associated fibroblasts. The resulting loss of mitochondrial function acts as a switch from aerobic metabolism to glycolysis.

Abbreviations

^{18}F-FDG, ^{18}F-fluorodeoxyglucose; ^{18}F-FDG-6-P, ^{18}F-FDG-6-phosphate; ATP, adenosine triphosphate; CAF, cancer-associated fibroblasts; ECAR, extracellular acidification rate; GLUT, glucose transporter; HK, hexokinase; MCT4, monocarboxylate transporter 4; OCR, oxygen consumption rate; PET, positron emission tomography.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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