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Metabolomics and cancer drug discovery: let the cells do the talking

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Recent developments in cancer research have led to reconsiderations regarding metabolic dysfunctions in cancer cell proliferation and differentiation. The original concept stemmed from the observation that, even in presence of oxygen, highly proliferating cells tend to generate energy strictly from the glycolytic pathway, through a process called aerobic glycolysis, also known as the Warburg effect. More recently, advances in the field of metabolomics applied to cancer research enabled the documenting of the generality of the Warburg effect in a broad variety of tumors.
Through metabolomics, cancer cells told us that oxidative stress, while representing one leading cause of genetic instability underpinning carcinogenesis, could also deliver a window of probable therapeutic opportunities that is worth opening.

Omics and drug discovery

In recent years, the pharmaceutical industry has 'upped the ante' in the application of 'omic' sciences to the biology-driven search for novel targets in cancer drug discovery [1]. In this context, genomics, transcriptomics and proteomics represented the perfect trifecta. These disciplines were thought to transform the entire process by providing enough information to advance compounds more efficiently and to reduce drug attrition. Despite the constant efforts made so far, practical use of these technologies is still limited to discovery applications [2].

Failures in the attempt to fulfil the promises made by pharmacogenomicists and pharmacoproteomicists mainly stem from the intrinsic difficulty in translating biologically interesting signatures into actual therapeutic targets.

As Wishart summarized [3], 'many of these failures could be prevented or the risks (financial and personal) could be reduced if there were better ways of screening drug targets (at the discovery stage), tracking drug toxicity (at the development and clinical trial stage) and monitoring adverse drug reactions (at the prescription/physician stage)'. Metabolomics is rapidly becoming one of those 'better ways'. Metabolomics is the lesser-known cousin of genomics and proteomics. Embracing the omics philosophy, this discipline seeks to measure the concentrations of nominally all of the small molecular weight (MW) (i.e. below 1.5 kDa) metabolites in a particular system, for example, a body fluid such as serum or an ensemble of cells [4,5]. However, a more restricted subset is measured in practice. This is mainly owing to the huge chemical diversity of metabolic compounds, especially in terms of polarity [6]. The number of human metabolites estimated via genomics (i.e. approximately 3000) and the number measured experimentally have been rapidly converging over the past few years [6]. Nevertheless, the measured metabolome is greater than that encoded by the genome, because it will include molecules acquired exogenously as drugs, foods or food additives, and will also include molecules derived from the microflora of the host [6].

Directly profiling metabolites (i.e. metabolic profiling or metabolomics) has distinct advantages over other omics approaches regarding building knowledge of biological status efficiently. Biologically relevant information includes the genesis of metabolic biomarkers, the progress of a disease and the modes of action, efficacy, off-target effects and toxicity of pharmaceutical drugs [6].

Indeed, intermediary metabolism is proximal to phenotype, and the possibility to measure metabolites quantitatively and semi-comprehensively

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enables almost immediate screening of biological matrices, which is often difficult to obtain with other omics approaches [7].

At the same time, metabolomics is also 'clinical biochemistry on steroids' [3]. In clinical biochemistry, most metabolites are typically identified and quantified using colorimetric chemical assays. In metabolomics, large numbers (i.e. tens to hundreds) of metabolites are measured within minutes using non-chemical, non-colorimetric methods such as chromatography, mass spectrometry (MS) and NMR [3–5]. The close relationship to clinical biochemistry, which is routinely used in everyday screening of patients worldwide, might enable easier translation of novel findings from discovery science, this being a crucial step for all of the other omics fields so far. In clinical practice, the extension of actual protocols to those biomarkers discovered through genomics and proteomics has not taken proportional advantage of the wealth of data available. For example, in the USA the rate of introduction of protein tests approved by the FDA has declined to less than one new protein diagnostic marker per year [8].

Advancements in metabolomics

In the late 1940s Williams *et al.* introduced the concept that individuals might be characterized by a unique 'metabolic profile' (e.g. the composition of their biological fluids) that could reflect their health condition [9]. However, it was only upon the optimization of extraction

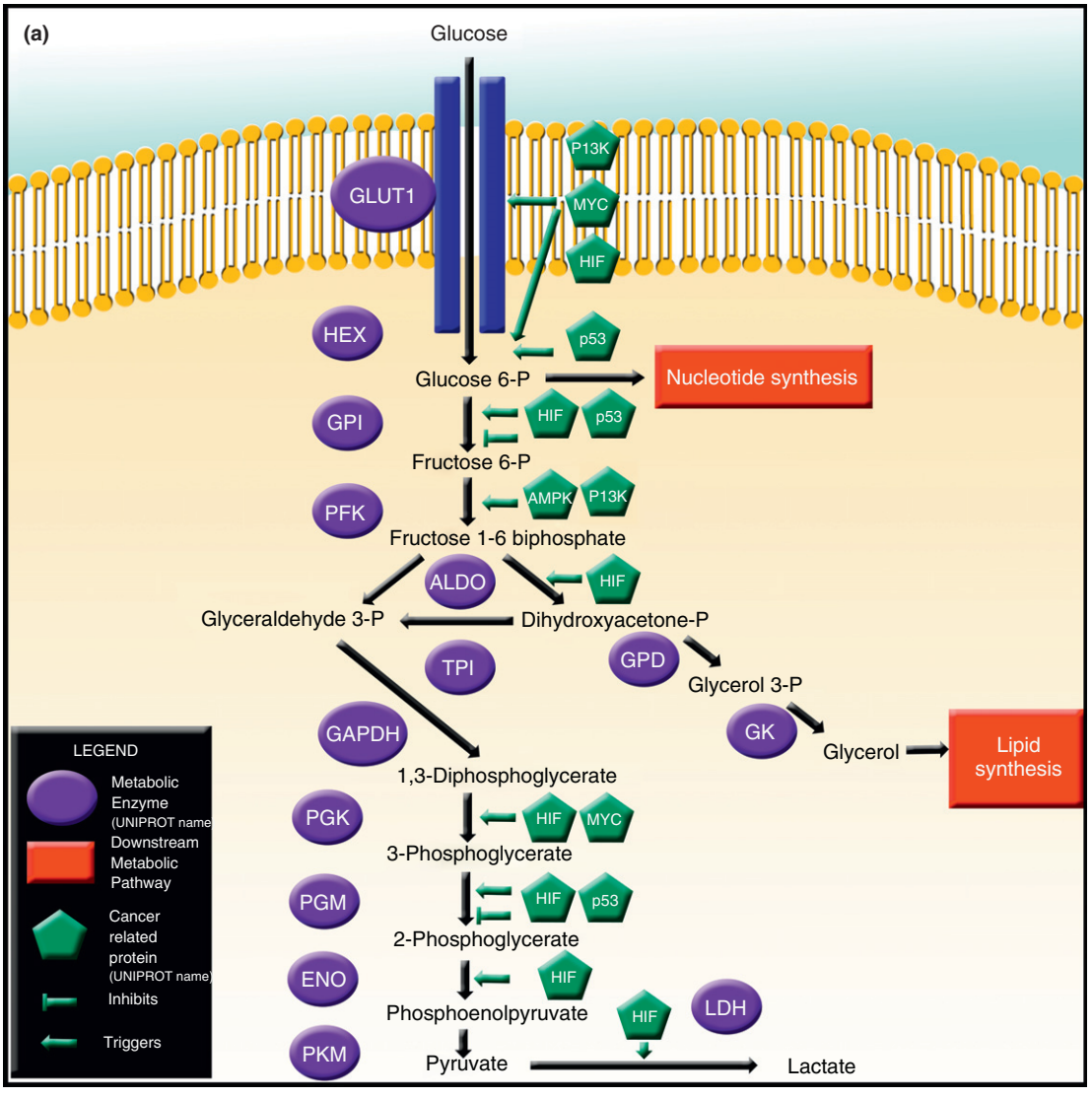

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FIGURE 1

An overview of cancer cell metabolism. In **(a)** an overview of the glycolytic pathway and its crosstalk with oncogenes and tumor suppressor genes is shown. Shifts from the main glycolytic pathway towards precursors for lipid synthesis via the glycerol phosphate shuttle are evidenced as well. In **(b)** an overview of the glucose/glutamine downstream metabolic pathways and the interconnections with the Krebs cycle and oncogenes and oncosuppresors in tumour cells is represented. Part (b) also provides a simplified overview of the possible interactions between Krebs overactivation, anomalous ROS production via the electron transport chain and the homeostasis of glutathione. In **(c)** a detail of the Krebs cycle and its correlation to ROS production through the electron transport chain and apoptosis is shown. ROS tackle proteins and DNA (other than lipids), producing structural and functional alterations that activate an apoptotic fate. Antioxidant defences might counteract ROS-triggered cascades, whereas heat shock proteins (HSPs) protect proteins from external insults through their chaperone role. Enzymes (blue circles) are reported with Uniprot names. In Box 1 protein abbreviations are defined. Metabolic intermediates are shown in black and green pentagons have been used to represent oncosuppressors and oncogenes [7].

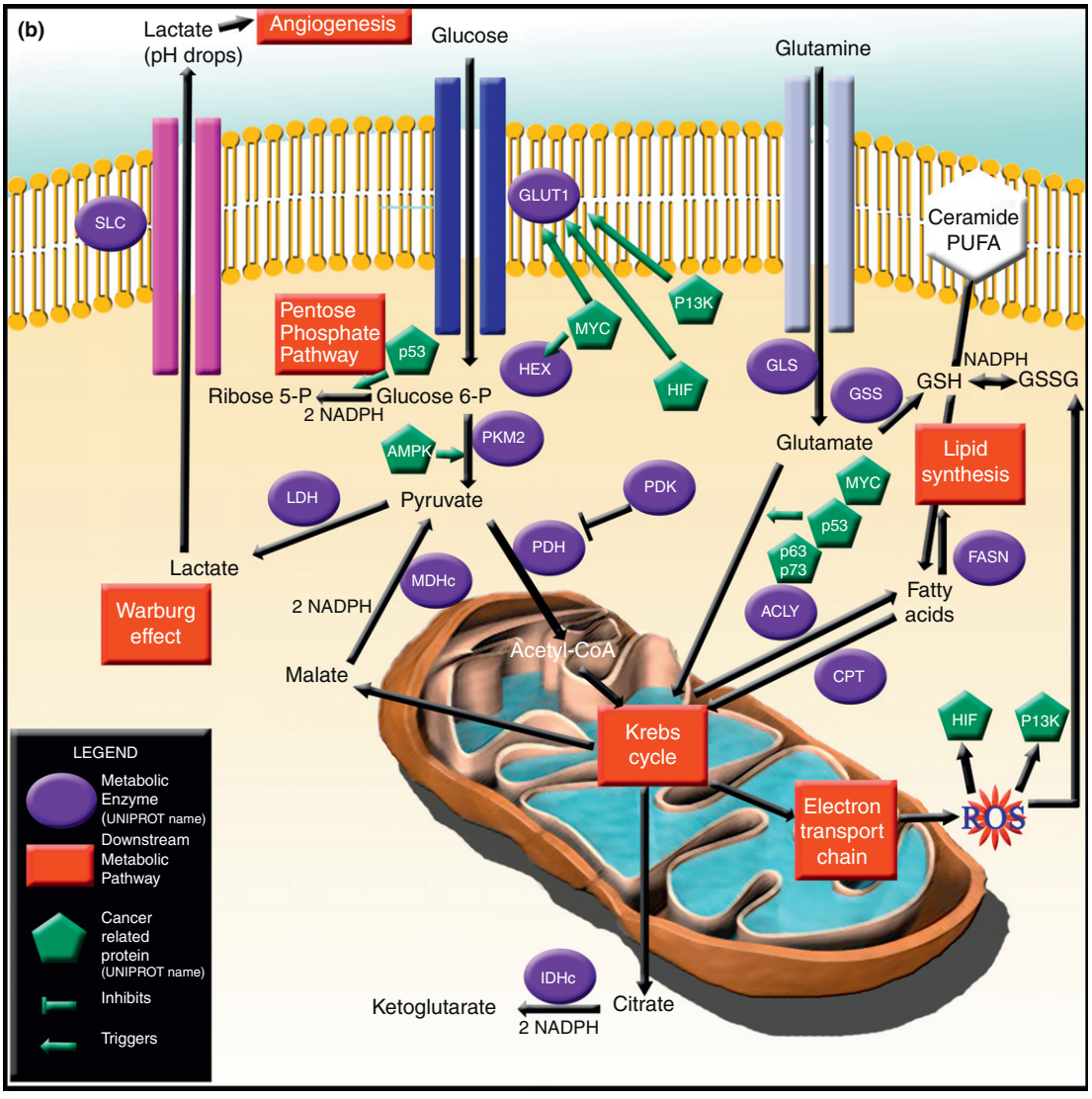

Figure 1. (Continued)

methods [10] and the introduction of more-sensitive analytical platforms, such as novel NMR and MS, that metabolomics began to flourish.

The earliest approaches to metabolomic investigations mainly relied on NMR, which was favored by machine accessibility, established data handling and the non-destructive nature of the analysis [11]. Nevertheless, NMR has been gradually complemented by MS because both technologies hold several advantages.

A brief list of the 'pros' of using NMR includes easy sample preparation, no required derivatization, safe metabolite identification and quantification, non-destructive nature of analysis (both on intact tissue and biofluids), easy sample automation and the possibility to translate applications to *ex vivo* or *in vivo* samples.

MS provides higher sensitivity, improved metabolite discrimination, full coverage of the metabolome space and is characterized by modularity to perform compound-class-specific analyses, other than for a dramatically reduced demand for starting material necessary to perform an extensive analysis [12].

NMR- and MS-based metabolomics have some limitations as well, including the timing or temporal relationship of biological intermediates, the rapidity of enzymatic kinetics and variability across individuals (i.e. biological variability).

MS is often coupled to pre-analytical approaches, such as gas chromatography, liquid chromatography and capillary electrophoresis. Gas chromatography is hindered by poor discrimination against large intermediates such as nucleotides, flavines and coenzyme A derivatives. Liquid chromatography holds several advantages, *viz* widespread coverage, sensitivity, ease-of-use, robustness to matrix and robustness in routine operation. Capillary electrophoresis is equivalent to liquid chromatography in terms of separation and sensitivity, although it lacks robustness, which is pivotal for routine analysis of biological extracts [13].

Recent advances are not only inherent to the analysis itself but also include the creation of *ad hoc* freely available databases (e.g. such as METLIN [14]) or the introduction of specific bioinformatic tools. Software advances now enable the production (and computer-readable encoding such as SBML) of metabolic network models reconstructed from genome sequences, as well as experimental measurements of much of the metabolome [6]. In a similar fashion to functional enrichment of proteomics data (pathway analyses, gene ontology term enrichment, protein–protein interaction modeling [2]),

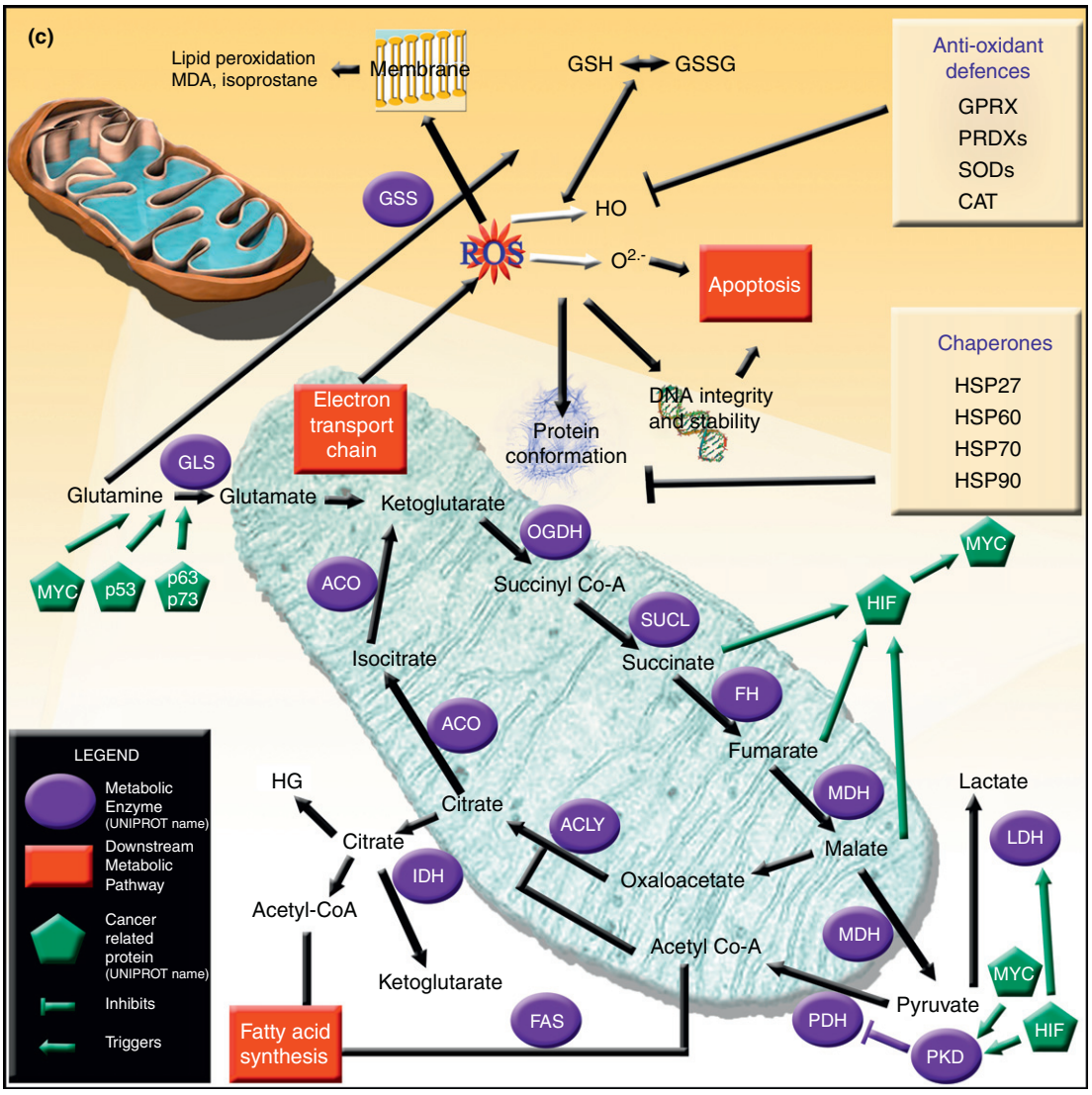

Figure 1. (Continued).

metabolic modeling exploits metabolic networks or logical graphs, and resources such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) [15].

As is the case for proteomics [2], one of the main challenges for metabolomics is to cope with the diverse range of metabolites found within the cell (either hydrophilic or lipophilic), in conjunction with the large dynamic range of metabolite concentrations [16].

Conversely, unlike signaling pathways [6], metabolic networks are subject to strict stoichiometric constraints. In other terms, slight changes at the protein level might be amplified at the metabolite level in theory [17] as well as in practice [18]. Besides, changes at the metabolic level might be caused by disease or pharmaceutical intervention [19].

MS-based metabolomics also offers the potential to perform targeted analyses through selection, isolation and fragmentation of precursor ions, and subsequent isolation of the product ions (i.e. features) of interest. This 'targeted' metabolomics strategy is known as selected/multiple reaction monitoring (SRM or MRM) [5].

However, in discovery science metabolomics-only 'untargeted' strategies enable individuating molecules where concentrations are unexpectedly fluctuating in the experimental matrix under investigation.

As an extension of clinical biochemistry, the metabolome is chemical pathology writ large. Therefore, it is realistic enough to assume that measuring small molecule concentrations in biofluids will prove to be of significant use in various kinds of diagnoses, with obvious consequences in the fields of drug design, development and testing [19].

Cancer metabolism: the Warburg effect and the (old) new deal

Recent developments in cancer research have led researchers to reconsider the strong relationships of metabolic dysfunctions in cancer cell proliferation and differentiation [20] (Fig. 1a–c). The original concept stemmed from the observation that, even in presence of oxygen, highly proliferating cells tend to generate energy strictly from the glycolytic pathway (Fig. 1a). This process is referred to as aerobic glycolysis or Warburg effect, as to acknowledge the name of the first researcher evidencing this basic biochemical behavior [20]. However,

BOX 1

Uniprot protein name and general abbreviations from Fig. 1.

ACLY = ATP-citrate synthase
ACO = aconitase
ALDO = aldolase
AMPK = AMP-activated protein kinase
CAT = catalase
CPT = carnitine *O*-palmitoyltransferase
ENO = enolase
FAS = tumour necrosis factor receptor superfamily member 6
FASN = fatty acid synthase
FH = fumarate hydratase
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GK = glycerol kinase
GLS = glutaminase
GLUT1 = glucose transporter 1
GPD = glycerol-3-phosphate dehydrogenase
GPI = glucose-6-phosphate isomerase
GPRX = glutathione peroxidase
GSH = glutathione
GSS = glutathione synthetase
GSSG = oxidized glutathione
HEX = hexokinase
HIF = hypoxia-inducible factor
HSP27 = heat shock protein 27 or beta-1
HSP60 = heat shock protein 60 kDa
HSP70 = heat shock protein 70 kDa
HSP90 = heat shock protein 90 kDa
IDH = isocitrate dehydrogenase
IDHc = isocitrate dehydrogenase cytosolic
LDH = lactate dehydrogenase
MDH = malate dehydrogenase
NADPH = reduced nicotinamide adenine dinucleotide phosphate
OGDH = 2-oxoglutarate dehydrogenase
PDH = pyruvate dehydrogenase
PDK = pyruvate dehydrogenase kinase
PFK = phosphofructokinase
PGK = phosphoglycerate kinase
PGM = phosphoglucomutase
PI3K = phosphatidylinositol-bisphosphate 3-kinase
PKM = pyruvate kinase isozyme M1/M2
PRDXs = peroxiredoxins
PUFA = polyunsaturated fatty acids
SLC = solute carrier
SODs = superoxide dismutases
SUCL = succinyl-CoA ligase
TPI = triosephosphate isomerase

despite early encouraging observations, the Warburg hypothesis was rapidly set aside upon appreciation of the part played by sequential acquisition of mutations to oncogenes and tumor suppressor genes in triggering malignant transformation [21]. Besides, a further obstacle hindering the way to clinical application of the concepts deriving from the Warburg hypothesis was represented by the inability to come up with therapeutic interventions (i.e. drugs) that could change energetics in the tumor without leading to devastating changes in the metabolism in the host.

More recently, advances in the field of metabolomics applied to cancer research documented the generality of the Warburg effect in a broad variety of tumors [22]. Rapidly proliferating mammalian cells, including cancer cells, almost always exhibit aerobic glycolysis even under normoxic conditions, characterized by an increased lactate excretion rate relative to the respiration rate [22] (Fig. 1b).

From the standpoint of metabolic efficiency this has been a surprising observation because aerobic glycolysis is far less efficient than mitochondrial respiration in terms of moles of adenosine triphosphate (ATP) generated per mole of glucose [23]. Implicit to this result, however, is the assumption that glucose uptake is a limiting factor of cell metabolism [22]. It has indeed been observed that, at low glucose uptake rates, mitochondrial respiration was the most efficient pathway for ATP generation, whereas, above a threshold of glucose uptake rate, a gradual activation of aerobic glycolysis and slight decrease of mitochondrial respiration resulted in the highest rate of ATP production [22]. This process is also modulated by additional physicochemical constraints, such as the solvent capacity of the cell's cytoplasm [22]. At high glucose uptake rates, cytosol solvent capacity becomes a limiting factor. Excessive uptake of glucose can thus be diverted towards a pathway that is less efficient in terms of ATP yield per mole of glucose but is characterized by a higher rate of lactate (and thus ATP) production per its own [i.e. glycolytic and lactate dehydrogenase (LDH) enzymes] mass than the rate of ATP production by respiration per mitochondrial mass [22].

Analogous observations have been reported for pre-implantation embryos. Mammalian preimplantation embryos experience a crucial switch from an oxidative to a predominantly glycolytic metabolism [24]. In the study by Chi *et al*. embryos exposed to elevated glucose conditions experienced significantly lower fructose biphosphate levels, which was suggestive of a decreased glycolytic rate. By contrast, significantly higher levels of pyruvate were detected, which was suggestive of an inability to oxidize the pyruvate and, thus, slow the Krebs cycle rate [24].

The integration of metabolic observations and the genetic theory of cancer stimulated the broader concept that a 'metabolic transformation' represents an inevitable stage during tumorigenesis and, conversely, that oncosuppressor activity might benefit from the activation of the aerobic metabolism passing through the Krebs cycle via a bypass of the ordinary glycolysis through alternative metabolic pathways. The biochemistry behind these processes envisages the consumption of aminoacids (such as glutamic acid) or, probably, fatty acid substrates [25]. This mechanism represents a physiological process that is activated through the participation of oncosuppressor genes, such as p53 and its closely related p63 and p73 families [26,27] (Fig. 1).

Mitochondrial regulation of apoptosis might not only follow canonical pathways [28], such as voltage-dependent anion channel (VDAC), Bax/Bak and Bcl-2/Bcl-$_{XL}$ pro- and anti-apoptotic balance, but it could also be linked to the accumulation of Krebs cycle intermediates, because it emerges from recent integrated omics and targeted studies [25–27]. A retrograde signaling mechanism might imply the crosstalk between accumulated Krebs cycle intermediates and transcription factors, in a similar fashion to the observed indirect interaction of succinate and fumarate with the hypoxia-inducible factor (HIF) [29] (Fig. 1c). A body of literature has recently reviewed the connections between the Krebs cycle and the cell cycle [29].

Recently, we could monitor the biological events leading to apoptosis of pancreatic adenocarcinoma human cells (PACA-44 cell line) upon administration of an n-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA; 22:6 n-3) [25]. Through an integrated proteomics, interactomics and metabolomics approach it emerged that pancreatic cancer cells treated with DHA (200 μM for 48 hours), while undertaking an apoptotic fate, ended up overexpressing Krebs-cycle-related enzymes, which have been related to an increased accumulation of Krebs cycle intermediates and diminution of glycolytic counterparts. Furthermore, through metabolomics we could detect a diversion from the main glycolytic pathway towards the pentose phosphate pathway. This shift resulted in accumulation of NADPH, an essential coenzyme in oxidized-glutathione (GSSG) reduction to GSH [25] (Fig. 1b).

Anomalies to oxidative phosphorylation coupling and accumulation of reactive oxygen species (ROS) might underpin the mechanisms by which overactivation of the Krebs cycle can trigger apoptotic cascades.

Oxidative stress in cancer treatment: a matter of balance

There is an equilibrium between ROS formation and endogenous antioxidant defence mechanisms. Disturbances to this balance result in oxidative stress conditions. Accumulation of oxidative stress can result in injuries to all of the important cellular components such as proteins, DNA and membrane lipids, therefore promoting cell death. In recent years, increasing quantities of experimental and clinical data have provided compelling evidence for the involvement of oxidative stress in a large number of pathological states including carcinogenesis [30,31]. However, the recently postulated involvement of aerobic metabolism in the modulation of the cell cycle through ROS signaling [29] and feedback interaction with upstream oncosuppressor genes [26,27] seems to suggest that oxidative stress is not always detrimental.

Selective oxidative stress is sometimes desirable and can be exploited therapeutically. There are numerous drugs that are known to promote pro-oxidant cascades such as chloroquine, quinine, mefloquine, primaquine, artemisinin and ciprofloxacin [30]. Alternatively, and gaining momentum, there are new therapeutic strategies that take advantage of the inhibition of endogenous antioxidant defences (such as superoxide dismutases (SODs); Fig. 1c), hence producing a state of oxidative stress selectively in cancer cells [30].

Concluding remarks

Over the past 35 years of cancer research, Hanahan and Weinberg brilliantly pointed out that 'several lines of evidence indicate that tumorigenesis is a multistep process' and 'these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives' [30]. The so-called 'hallmarks of cancer' included: (i) self-sufficiency in growth signals; (ii) insensitivity to growth-inhibitory signals; (iii) evasion of programmed cell death; (iv) limitless replicative potential; (v) sustained angiogenesis; and (vi) tissue invasion and metastasis [30]. Although anomalies to normal cell metabolism upon transformation to cancerous cells has been a widely held view over the past 50 years, it would be pivotal to further our understanding of these changes to relate, if possible, a specific hallmark of cancer to a peculiar metabolic pattern. Questions like 'at which point in the transformation of the normal cell that changes in energetics occur?' and 'which if any of the hallmarks proposed by Hanahan and Weinberg are essential for a change in energy?' still lie unanswered. Resolving these queries would enable early diagnosis on the one hand and prompt intervention on the other.

Cancer cell answers to the metabolomic questions suggested a relationship between overactivation of the Krebs cycle and induction of apoptosis, although it is yet to be assessed whether this relationship is either causative or consequential.

Nevertheless, it is perhaps worth exploring the possibility of tackling cancer through forcing aerobic metabolism either directly (i.e. overexpression of lactate dehydrogenase B forced cancer gastric cells through Krebs cycle and inhibited cancer cell progression [32], Fig. 1b) or via a blockade of glycolysis: evidence has been reported of 3-bromopyruvate inhibiting hexokinase (Fig. 1a) and arresting colon cancer cell growth [33]. Another possibility is through triggering alternate catabolic pathways (e.g. aminoacids and fatty acids), either through drugs or nutraceuticals – polyunsaturated fatty acids (PUFAs) for example [25].

Pharmacological reduction of lactate production and excretion could indeed reduce the invasive potential of tumor cells or make them more susceptible to apoptosis [22]. However, the metabolic state of tumor cells is not in itself a unique target for cancer chemotherapy, although individual, tumor-specific isozymes could be targeted [22].

These approaches might prove useful in complementing therapeutic options that have been available for a long time as well as recent advances (i.e. antibodies towards protein targets such as hormones, receptors or heat shock proteins [1]), or rather pave the way for a whole new deal in cancer treatment.

Through metabolomics, cancer cells have told us that oxidative stress, even if it represents one leading cause of genetic instability underpinning carcinogenesis [21], could also deliver us a window of probable therapeutic opportunities that it is worth opening and looking into.

Conflict of interest

The authors disclose no conflict of interest.

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ADA and LZ would like to dedicate this article to the retiring Professor Alessandro Finazzi Agrò, former Magnificent Rector of the Tor Vergata University of Rome (Italy), for his brilliant and inspiring career in the field of Biochemistry.

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