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# Lactic dehydrogenase and cancer: an overview

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## 1. ABSTRACT

Despite the intense scientific efforts made, there are still many tumors that are difficult to treat and the percentage of patient survival in the long-term is still too low. Thus, new approaches to the treatment of cancer are needed. Cancer is a highly heterogeneous and complex disease, whose development requires a reorganization of cell metabolism. Most tumor cells downregulate mitochondrial oxidative phosphorylation and increase the rate of glucose consumption and lactate release, independently of oxygen availability (Warburg effect). This metabolic rewiring is largely believed to favour tumor growth and survival, although the underlying molecular mechanisms are not completely understood. Importantly, the correlation between the aerobic glycolysis and cancer is widely regarded as a useful biochemical basis for the development of novel anticancer strategies. Among the enzymes involved in glycolysis, lactate dehydrogenase (LDH) is emerging as a very attractive target for possible pharmacological approaches in cancer therapy. This review addresses the state of the art and the perspectives concerning LDH both as a useful diagnostic marker and a relevant molecular target in cancer therapy and management.

## 2. INTRODUCTION

With respect to other diseases, there is not a universal cure for cancer; the different type of tumors, their size and the location, the nature of the cells and its development and the health condition of the subjects represent important factors to take in consideration in order to address a possible best treatment. The approaches most often used are surgical interventions, radiation therapy, chemotherapy and, where applicable, targeted therapies. In most tumors, these treatments are used either simultaneously or in an appropriate sequence (1-3). Recently, combination chemotherapy has received more attention in order to find compounds that could increase the therapeutic index of clinical anticancer drugs. In this regard, dietary supplements, phytotherapeutic agents and naturally occurring molecules with antitumor activity and with the least toxicity to normal tissues are proposed as possible intriguing candidates to be investigated for their synergistic efficacy in combination with antineoplastic drugs (4,5). In addition, other treatment strategies adopt new approaches deriving from the concept of removing tumor nutrients (6). Moreover, great therapeutic opportunities are expected from nanomedicine (7).

However, to date, despite decades of intensive efforts, the cancer remains a disease extremely difficult to treat, especially when the diagnosis is delayed and the tumor rooted. By recording large differences in the success rate of treatment depending on the type of cancer, cancer cure rate (absence of the recurrence of the tumor after 5 years) can reach 70% for breast cancer

or prostate cancer, whereas for tumors of the lung, pancreas, esophagus, the percentage of survival does not exceed 20%. In total, the cancer survival rate after 5 years is approximately of 60% (8). Thus, novel strategies for the treatment of cancer are warranted. Thanks to the basic research, in the past decade considerable advances have been made in the understanding of cancer. In particular, one relevant aspect of the recent research is the ability of cancer cells to change their energy metabolism. Notably, energy metabolism is considered an emerging hallmark of cancer and a very attractive area for developing innovative therapeutic strategies in cancer therapy (9, 10). In fact, the metabolic properties of cancer cells diverge significantly from those of normal cells, so that targeting cellular metabolism is considered an intriguing approach to obtain specific antitumor effects without or minimally affecting normal cells (10, 11).

In particular, the majority of the tumor cells decreases the mitochondrial oxidative phosphorylation (OXPHOS) and instead increases the amount of glucose consumed and lactate production in a manner completely independent of the availability of oxygen (Warburg effect), so that the energy production in cancer cells is abnormally dependent on aerobic glycolysis (see below) (12). Thus, targeting aerobic glycolysis remains attractive for therapeutic intervention in cancer. At this regard, glycolytic inhibitors serve as a classical example of cancer metabolism targeting agents. Several preclinical investigations have indeed demonstrated the effectiveness of this therapeutic approach (13, 14). Among the enzymes involved in glycolysis, lactate dehydrogenase, LDH, which belongs to the class of oxidoreductases and converts pyruvate to lactate at the end of glycolysis, is regarded as one of the most interesting molecular targets for the development of new glycolytic inhibitors to possibly use in cancer therapy (15). The LDH appears to be the main metabolic enzyme whose inhibition can lead to a block of aerobic glycolysis of the tumor cells without damaging healthy cells which, in condition of normal activities and of sufficient supply of oxygen, usually degrade completely the glucose to $CO_2$ and $H_2O$ (via the tricarboxylic acid cycle, TCA/Krebs cycle) and do not require this enzyme (16).

## 3. GLYCOLYSIS AND AEROBIC GLYCOLYSIS

The uncontrolled and sustained cell proliferation is one of the major hallmark that characterizes virtually all types of cancer (9). Tumor initiation and progression require selection for the most aggressive and resilient cells to drive and increase proliferation and survival. To fuel growth, cancer cells must adapt their metabolism according to the new energy needs, and the metabolism of glucose, that provides the most efficient method of generating energy within the cell, is primarily affected (17).

The glucose metabolism can be divided into two major phases (18). In the first part, namely glycolysis, common to both normal and tumor cells, one molecule of glucose is metabolized in the cytoplasm with the formation of two residues of reduced NADH and two molecules of pyruvate and the concomitant production of two molecules of ATP, the molecule that conveys the energy to all cellular processes. This process can occur in the absence of oxygen and for this reason it is a very exploited process in nature for the production of energy. In the second step, pyruvate is imported in the mitochondria and, after the decarboxylation to acetyl-CoA, enters the Krebs cycle where is degraded to $CO_2$ and $H_2O$ while the NADH is reoxidized by the mitochondrial respiratory chain. In the overall process (glycolysis, Krebs cycle and respiratory chain) a single molecule of glucose produces 36 molecules of ATP.

There are circumstances where pyruvate entry to the mitochondria is prevented and it is converted by the enzyme lactate dehydrogenase into lactate with the simultaneous re-generation of NADH, thus allowing the continuous flow of the first stage of the reactions of glycolysis (which require NADH) and leading to an accumulation of lactate in the extracellular environment. Through this pathway, just two moles of ATP per mole of glucose metabolized are obtained. In normal cells, this pathway is active only in particular conditions such as embryonic development, or in conditions of insufficient supply of oxygen, for example in the hyper-working skeletal muscle (18). On the contrary, cancer cells reprogram their metabolism to use mainly glycolysis even in the presence of oxygen, through the so called “aerobic glycolysis” or “Warburg effect” (12, 19). Why does a cell that proliferates more and faster use the glycolytic metabolism, which guarantees a lower energy production? Despite its low energy yield and wasteful of glucose, the aerobic glycolysis is considered advantageous to the cancer cells. This metabolic pathway produces ATP far quicker than the slow route of oxidative phosphorylation and results in the generation of crucial precursors for biomass production such as NADPH which is not produced at such levels via oxidative phosphorylation. It is believed that cancer cells utilize the rapid generation of ATP and the increase in de novo fatty acid synthesis to grow and divide quickly (20). Moreover, the Warburg shift not only allows rapid generation of ATP, fatty acids and nucleotides whilst glucose is abundant, but the consequent accumulation of lactic acid produces the acidification of the microenvironment that could induce degradation of the extracellular matrix and facilitate angiogenesis, tumor invasion and a protection against immune attack (21).

On the other hand, it is known also that specific changes in the tumor microenvironment and hypoxia can enhance glycolysis through activation of specific transcriptional factors, such as Myc proto-oncogene

protein or the hypoxia inducible factors HIF-1 and HIF-2, which regulate the transcription of genes involved in glucose metabolism (22). Notably, aerobic glycolysis was described for the first time about a century ago by Otto H. Warburg who showed that cancer cells metabolize glucose differently than normal cells (Warburg effect) and that tumors derive energy mainly from the conversion of glucose to lactic acid and minimally via cellular respiration involving oxygen (12, 23). The observation that tumors produce massive amounts of the aerobic glycolysis waste product, lactic acid, was central to the concept of deregulated metabolism, so that cancer was even termed "disorder of metabolism" (12). Although cancer is now more accurately defined in terms of genomics, it appears clear that there are substantial modifications to metabolic pathways as a consequence of genetic and epigenetic changes and also that an impaired mitochondrial function is strongly involved in (9, 24-26).

Coming back to the Warburg effect/aerobic glycolysis, it is a very hallmark of cancer, so widespread and common to the cancer cells, that it is the principle, for example, at the basis of the largely used diagnostic techniques such as Positron Emission Tomography (PET) for the identification of primary and metastatic lesions. The patient takes a radioactive molecule, glucose analogue, which is incorporated mainly by tumor cells. PET identifies the tumor cells revealing increased radioactivity, indicative of a higher rate of metabolic consumption of glucose (27).

As far as the molecular mechanisms underlying the Warburg shift concerned, they are not fully clarified and include mitochondrial defects and malfunction, adaptation to hypoxic tumor microenvironments, abnormal expression of metabolic enzymes, and oncogenic signaling (28). At this regard, increasing evidence indicates that a central role on the shift to aerobic glycolysis occurring in cancer cells is played by the nuclear receptor superfamily. This large family of receptors, including steroidal, retinoids and peroxisome proliferator-activated receptors, is significantly involved in sensing environmental cues and controlling decisions on proliferation, differentiation and cell death for example, to regulate glucose uptake and metabolism and to modulate the actions of oncogenes and tumour suppressors (29).

By the way, whatever the mechanisms, the general increased dependence of cancer cells by aerobic glycolysis has been recently regarding as a useful biochemical basis for the development of novel anticancer strategies (10, 11, 13, 14, 30, 31).

At this regard, lactate dehydrogenase (LDH) is involved in the critical step of inter-conversion of pyruvate to lactate and plays a central role in the Warburg effect. Consistently, LDH is considered very relevant to cancer and a highly promising therapeutic target for new anticancer treatments.

## 4. LACTIC DEHYDROGENASE (LDH): FEATURES OF THE DIFFERENT ISOZYMES

Lactate dehydrogenase (LDH, L-lactate, NAD+ oxidoreductase, EC1.1.1.27) is an ubiquitous enzyme present in mammals, yeast, plants and microorganisms. LDH interconverts pyruvate and lactate at the end of the glycolytic pathway using NAD+ as a cofactor. LDH was isolated many years ago from cell free muscle extracts and it is one of the best characterized enzymes in the scientific literature (32, 33). Its molecular characteristics have been well studied and recently the structures of the different isoforms of the human LDH enzyme have been characterized (34, 35). LDH is a 140 kDa tetrameric molecule that exists in five major isoenzymes, numbered LDH-1 through LDH-5, formed by the association of two different types of approximately 35 kDa subunits, M (Muscle) and H (Heart), encoded by two different genes: *ldh-a* and *ldh-b* which are respectively located on chromosomes 11p15.4 and 12p12.2-p12.1 (36). In addition, it is known also a sixth, minor LDH isoenzyme, containing a third subunit X. Such third subunit, encoded by the *ldh-c* or *ldh-x* gene, which is located on chromosome 11p15.5-p15.3 and is likely a duplicate of LDH-5, forms testis-specific isoform known as LDH-6 or LDHC (37, 38).

Both human *ldh-a* and *ldh-b* genes are comprised of eight exons encoding proteins of 331 and 333 amino acids, respectively (39, 40). Several amino acid substitutions were demonstrated in human LDH variants by genetic analysis (41-43). These variants are of interest for investigation of the relationships between the LDH structure and function. Although the structure of the five isoforms of LDH is very similar, there are instead differences in the tissue (and cellular) distribution of the enzyme and in its kinetics and regulation (18, 34, 44).

On the basis of combination of M and H monomers, two homotetrameric isoforms, LDH-1 (H4) and LDH-5 (M4), and three heterotetrameric isoforms, LDH-2 (M1H3), LDH-3 (M2H2), LDH-4 (M3H1) can be obtained. To note, LDH-1 and LDH-5 are commonly named also LDHB and LDHA, respectively. LDH isoforms are present in all tissues at different ratios, so that the isoenzymatic profile is tissue-specific. LDH-5 and LDH-4 isoforms, containing exclusively (LDH-5) or mainly (LDH-4) M subunits, are expressed in most of tissues subject to anaerobic conditions such as skeletal muscle, liver and tumor tissues. On the other hand, LDH-1 and LDH-2 isoforms, composed exclusively (LDH-1) or mainly (LDH-2) by H subunits, are prevalently present in tissues subjected to high aerobic metabolism, such as the heart, the spleen, the kidney and the brain. LDH-3 isoform, characterized by a balanced subunit composition (two

M and two H subunits), is expressed mainly in lymphoid tissues.

As far as the intracellular localization concerned, LDH engaged in glycolysis is located in the cytosol; however, the enzyme is also present in mitochondria, peroxisomes and nuclei, where it exerts some peculiar functions (18, 34, 45). Moreover, the intracellular localization of each isoform may be different among different tissues. For instance, in the heart LDH-1 is present in the mitochondrion whereas LDH-5 is equally distributed both in the cytosol and mitochondrial matrix. In liver, LDH-5 isoform is mostly present within the mitochondrion, whereas in cancer cells it is mainly localized to the cytoplasm (18, 34). The different distribution of LDH isoforms is thought to be linked to its fundamental role in intracellular lactate shuttle mechanism, that is especially present in liver and muscle tissues. According to this mechanism, the lactate produced by glycolysis is transported from cytosol into the mitochondrial intermembrane space where, after its oxidation to pyruvate by a mitochondrial LDH, is released into the mitochondrial matrix in order to enter the Krebs cycle (21, 46). The different tissue and cellular distribution of LDH is probably linked to the different affinity of the various isoforms toward the substrates. LDH isoforms that contain predominantly M subunits (LDHA), display a higher affinity for pyruvate and catalyze preferably the reaction of conversion of pyruvate into lactate. Conversely, LDH isoforms that are constituted mostly by H monomers (LDHB), show higher efficiency in the conversion of lactate to pyruvate. This observations confirms Kaplan's theory (33). According to this theory, LDH-5/LDHA, that has the highest efficiency to catalyze the conversion of pyruvate to lactate, is favored in tissues with a low level of oxygenation, whereas LDH-1/LDHB, that is more able to produce pyruvate and favors the conversion of pyruvate to acetyl-CoA for entry into the citric acid (Krebs) cycle, is instead favored in tissues with a strong aerobic metabolism. Relevantly, normal and tumor tissues have basically similar levels of LDHB/LDH 1; whereas, LDHA/LDH-5 is primarily expressed in cancer cells (see below) (47). As anticipated and extensively discussed below, the interest toward this enzyme in the field of cancer is continuously increasing.

## 5. LDH AND CANCER

### 5.1. LDH expression in tumor maintenance, progression and metastasis

The key role played by LDHA in the Warburg effect and the prevalence in cancer cells of this metabolic pathway independently from the presence of oxygen, highlights the importance of the LDHA function in human tumors (47).

LDHA is elevated in many types of cancers and has been linked to tumor growth, maintenance and invasion. Importantly, there is clear evidence showing that knocking down LDH-A expression by RNA interference, the malignant behaviour of cancer cells is severely affected *in vitro* and *in vivo* (48). In a recent study, RNA interference mediated by lentiviral vectors was applied to investigate the role of LDHA in tumor growth and metastasis of hepatocellular carcinoma (HCC) (49). In this investigation, it was clearly shown that, first, HCC cell lines over-express LDHA and, second, LDHA inhibition increased apoptosis through the production of reactive oxygen species. Moreover, the knockdown of LDHA resulted in a significant reduction of the metastatic potential in a xenograft mouse model. Authors showed also that FAK, MMP-2, VEGF and E-cadherin are involved in (49). Relation between clinic-pathological factors of breast cancer and LDHA also have been analysed, with particular regard whether LDHA silencing could suppress breast cancer growth and to its potential mechanisms (50). Specimens of breast cancer were collected to study the correlation between the expression of LDHA and clinic-pathological characteristics. Moreover, short hairpin RNA (shRNAs) were applied to silence the expression of LDHA in breast cancer cell lines. The expression of LDHA strongly correlated with tumour size and showed to be independent from other clinic-pathological factors. Down-regulation of LDHA led to an inhibition of cancer cell proliferation accompanied by a strong Ki67 decrease, elevated intracellular oxidative stress and induction of mitochondrial pathway apoptosis. On the other hand, the tumorigenic capability of LDHA deficient cancer cells was significantly limited in breast cancer xenografts (50).

Currently, it has been reported that the LDHB/LDHA ratio reflects (very likely more than the level of LDHA protein alone) the metabolic capacity of breast cancer cells (51). Authors propose a new measurement, the "*Glycolytic Index*," which quantitates the LDHB/LDHA ratio in cancer cells as a possible biomarker of breast cancer aggressiveness (51). To note, an estimation of the LDH subunit ratio has been even suggested in the past to be useful as an indicator of neoplastic transformation in cultures of normal human fibroblasts, too (52).

In contrast to LDHA expression, LDHB is highly expressed in non-malignant tissues relative to tumors (53). In malignant tumors, LDHB appears to be silenced by promoter hypermethylation; this occurs at a high frequency in primary breast tumors and in primary prostate tumors (54, 55). However, although LDHB expression is commonly believed to be decreased in tumors (53-55), it has been described to be partially up-regulated in some cancer cells (56, 57).

A crucial role in the process of tumour initiation and progression is played by microenvironment. Tumor-associated stroma (TAS), for instance, plays an important role in tumour growth, invasion and metastasis (58).

Changes of stromal constituents levels like loss of Caveolin-1 have been linked to tumor aggressiveness. Caveolin-1 is a principal structural protein of the plasma membrane expressed in normal and hyperplastic fibroblasts and it is closely related to malignant growth (59). Other works indicated that Caveolin-1 to facilitate tumor invasion, can favor cell elongation and promote force-dependent contraction, matrix alignment and microenvironment stiffening (60). Interestingly, the relevant costituents of Tumor-Associated Stroma (TAS), Caveolin-1 and thymidine phosphorylase (TP, whose overexpression has been linked to the aggressiveness of tumours, too), have been investigated in prostatic cancer together with the expression of the isoenzymes LDHB and LDHA (61). TAS was found to play a primary role in the growth and development of prostatic cancer. Indeed, the loss of Caveolin-1 and the overexpression of TP were markers of TAS. Furthermore, compared to cancer cells, the tumour stroma is characterized by the overexpression of LDHB and downregulation or lack of LDHA. These behaviors are present in the majority of cancer cases independently from Caveolin-1 or TP expression, and highlight the metabolic co-operation between the aerobic stroma and the anaerobic cancer cells (58, 61).

Recently, Xie *et al.* have shown that the inactivation of LDHA in mouse models of non-small cell lung cancer (NSCLC) induced by the oncogene K-RAS or EGFR led to a decrease of established tumors and regression of disease. In addition, these authors also showed that LDHA down-regulation was accompanied by reactivation of mitochondrial function (62).

These data are in agreement with previous ones, showing that via immunohistochemistry (IHC) almost 90% of NSCLC were immunopositive for LDHA, whereas all non-neoplastic lung tissues appeared LDHA immunonegative. Moreover, the staining intensity of LDHA was found to correlate highly with the histological type of lung carcinomas and lymph node metastases, indicating that the tissue level of LDHA can have prognostic value in NSCLC (see also below) (63). LDHA has been shown to be upregulated also in esophageal squamous cell carcinoma, pancreatic cancer and oral squamous cell carcinoma (64-66). Interestingly, also in such cases it was proposed that LDHA correlate with metastases, tumor stage, recurrence of the tumor and patient survival (see also below). Taken together, these above observations indicate that LDHA is central to tumor proliferation and malignant behaviour and that survival of tumor cells is highly dependent on LDHA activity in a hypoxic environment.

### 5.2. LDH as diagnostic marker, prognostic factor and predictive marker for response to cancer therapy

As largely known and also reported above, LDH is present inside the cell, but when an injury occurs and cells are damaged, the enzyme is released into the bloodstream where its concentration increases. In medicine, for many years the interest for LDH has been mainly due to its importance as a diagnostic test in human diseases. An increase of LDH activity in serum is a consequence of massive cell death, which causes the release of the intracellular LDH (and that of other intracellular components, including other enzymes) into the circulation, and is associated to acute diseases. Reference values are dependent on many factors, including patient age, sex, sample population, test method, and numeric test results can have different meanings in different labs. Normal serum value range is 105-333 IU/L (International Units per Liter) (67). An increase of LDH can be determined measuring total LDH activity or that one of single isoenzymes (67, 68).

As above described, although some overlap might occur, each of the five LDH isoenzymes appear to be more concentrated in a specific tissue (34). Therefore, evaluation of each isoenzyme concentration can be used, together with other tests, to evaluate the disease condition or the condition that determines cell damages and the compromise of organ or tissue. As anticipated above, the dosage of LDH in serum of patients is largely used and is commonly done when there is a suspect of damage and/or disfunction of a specific organs such as heart, liver, muscle. Interestingly, it is also well known that serum LDH levels increase during neoplastic diseases, as a consequence of tissue destruction caused by the neoplastic growth; accordingly, the serum LDH measurement has an important clinical significance in cancer (47). Serum LDH is commonly increased in patients with hematopoietic malignancies, such as Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NHL) or multiple myeloma. LDH is one of the risk factors included in the International Prognostic Index (IPI) and it is believed a strong predictor of survival in patients with aggressive lymphoid cancers (69-74).

As above described, LDHA is upregulated in some types of human cancers and is associated with aggressive tumor outcomes. Kolev *et al.* investigated the expression levels of LDHA and HIF-1 in a group of patients with gastric carcinoma. Authors showed that overexpression of LDHA correlates with intratumoral angiogenesis, hypoxia and with several clinic-pathological parameters including prognosis. Relevantly, patients with overexpression of LDHA showed far lower disease-free (63.5% vs 82.7%) and overall lower (56.3% vs 78.4%) survival rates compared with patients with low LDHA expression (75). In addition, more recently, it has been shown that increased expression of LDHA that positively correlated with VEGF expression characterized almost 60% of gastric adenocarcinomas, suggesting the potential use of LDHA as a biomarker for response to VEGF-targeted therapy (76). In a prospective study in which several cancer types, symptoms, signs and other

serological variables were included, the value of LDH as a predictor of survival time in terminal cancer patients were evaluated. The results obtained indicated that the serum LDH level was a useful predictor for terminally ill cancer patients (77). Hepatocellular carcinoma (HCC) is a common cancer of liver and one of the most frequent and aggressive cancer in the world. Recently, a systematic review of large number of studies regarding the prognostic indicators in HCC has been reported (78). Interestingly, from such review, LDH was included among the most strong predictors of death in patients affected by HCC. Moreover, a study by Faloppi *et al.* reported that LDH was an important predictive factor in HCC patients evaluating the pre-treatment serum LDH level and its variation during treatment in HCC patients receiving the tyrosine kinase inhibitor, sorafenib. Remarkably, LDH appeared able to predict clinical outcome in terms of progression free survival (PFS) and overall survival (OS) for HCC patients in response to sorafenib (79). In addition, pretreatment serum LDH levels have been linked to the prediction of clinical outcome also for HCC patients undergoing trans-arterial-chemo-embolization (TACE) (80).

The prostate-specific antigen (PSA) is present in small quantities in serum and increases in some cases, including prostate cancer and benign prostatic hypertrophy. Very recently, Santotoribio *et al.* have evaluate the utility of LDH dosage in combination with free-to-total serum prostate specific antigen ratio (%fPSA) determination, in the diagnosis of prostate cancer; authors conclude that LDH in combination with %fPSA improved diagnostic performance for detection of prostate cancer compared to using %fPSA alone (81). It was also shown that pretreatment serum LDH levels might serve as a significant prognostic factor in high-risk patients with metastatic renal cell carcinoma (RCC) and a predictive factor associated with the response and survival benefit of the mTOR complex-1 (mTORC1) inhibitor temsirolimus (82). To note, there is evidence of the diagnostic significance of lactate dehydrogenase isoenzymes in urogenital tract tumours also from old studies (83, 84). Additionally, in patients with metastatic colon cancer or locally advanced nasopharyngeal carcinoma treated with radiotherapy combined with neoadjuvant chemotherapy, high-serum LDH levels were an independent unfavorable risk factor for overall survival (OS) (85-87).

Osteosarcoma is a a very aggressive tumor of bone and its treatment remains a challenge (88). A study has reviewed records from patients diagnosed with conventional high-grade osteosarcoma treated with chemotherapy over a 25-year period and has analysed the prognostic significance of LDH, showing that the pre-treatment serum LDH level had an independent prognostic value for both progression-free survival (PFS) and overall survival (OS) in these patients (89). Another multicenter retrospective study has evaluated the clinicopathological characteristics and prognostic factors in 240 Turkish patients with osteosarcoma from March 1995 to September 2011. By multivariable analysis, high LDH level (and also the presence of metastasis at diagnosis) were associated with poor overall survival in this study, too (90).

Pancreatic cancer is a fatal malignancy, with a median survival of 6 months and a very low percentage of long-term surviving patients (91). Due to poor progress provided from chemotherapeutics in the pancreatic cancer therapy, recent studies aimed to improve in selection of patients with poor prognosis to be treated only with supportive care and would avoid unnecessary adverse effects and complication of systemic chemotherapy.

At this regard, a retrospective recent study investigated the impact of pretreatment serum LDH along with CA19-9, CEA levels on the prognosis of 196 metastatic pancreatic cancer (MPC) patients, treated with gemcitabine-based chemotherapy. Interestingly, patients with normal serum levels of all three tumor markers had better outcome than others ($p = 0.002$) and those with normal serum LDH and CEA levels (whatever CA19-9) levels had associated with better survival compared with other possible alternatives ($p < 0.001$) (92).

Moreover, Zhao *et al.* have found that acetylation at lysine-5 of LDHA was reduced in pancreatic cancer (93). Lysine acetylation, in addition to phosphorylation, appears as an important modification of LDHA, although it has been poorly addressed so far. It has been involved in the control of its activity. Acetylated LDHA can be recognized by a cytosolic chaperone and it is easily degraded by lysosomal proteolysis. Interestingly, LDHA lysine-5 acetylation has been shown to be reduced and accompanied with increased LDHA protein levels in both early and late stages of pancreatic cancers (93). On the other hand, Authors failed to detect a correlation between decreased lysine-5 acetylation and LDHA-linked liver cancer development (94). Therefore, given the fact that LDHA lysine acetylation can be readily detected by specific antibody, it has been suggested that it might serve as a potential early diagnostic marker in pancreatic cancer (93, 94).

As far as LDH and lung cancer concerned, above we have already reported that the tissue level of LDHA can have prognostic value in non-small cell lung cancer (NSCLC) (60). In addition, it has been previously shown that serum LDH levels inversely correlated with the survival of patients with small cell lung cancer (SCLC) and allowed the selection of very high-risk patients. In addition, a significant relationship between high levels of LDH and a higher incidence of liver and bone metastases was found in the case of advanced SCLCs (95). Moreover, it has been described for a long time that

elevated levels of LDH are a predictor of poor prognosis in malignant pleural effusion (MPE), very likely reflecting a higher degree of necrosis in the pleural cavity (96). Recently, it has been shown for the first time a significant association between high LDH, low pleural glucose levels and overexpression of HER-2 in lung cancer (97). This relation allowed to hypothesize the possible use of low pleural glucose and high LDH levels as a screening tool for finding HER2-positive cases of lung cancer (97).

Concerning LDH as a possible predictive marker for assessing the response of tumor cells to therapeutic agents, a recent study describes that serum LDH levels could be used as a predictive marker useful to elaborate a possible algorithm for clinical use in patients with metastatic melanoma to be sequencially treated with BRAF inhibitors and immune checkpoint inhibitor, monoclonal antibody ipilimumab (98).

Additionally it has been shown that serum LDH levels could be also valuable to predict hypersensitivity reactions in colorectal cancer patients treated with the platinum anticancer agent, oxaliplatin (L-OHP) (99). In addition, Koukourakis *et al.* reported that tissue LDHA was associated with resistance to standard chemotherapy, poor progression-free survival and high performance status in patients with advanced colorectal cancer (CRC) (100).

Moreover, an investigation on acquired Taxol resistance in a number of human breast cancer cell lines found that Taxol-resistant cells expressed more LDHA compared to Taxol-sensitive cells and that their sensitivity could be increased by downregulating LDHA. It was also described that Taxol-resistant cells could be resensitized by specific LDHA inhibition (101, 102). Overall, these above observations strongly indicate that LDH can be considered as a relevant diagnostic marker, a prognostic factor and a predictive marker for response to therapy in cancer.

### 5.3. LDH as drug target for cancer therapy

There is a very large number of data indicating that the isoform 5 of the LDH (LDH-5 or LDHA) plays undoubtedly a key role in tumorigenesis. First of all, LDHA expression is directly targeted by oncogenes (such as Myc and HIF-1), which are critical factors in tumor development (103). Accordingly, as above described, LDHA is consistently up-regulated in tumors and has been found also to correlate with tumor size and prognosis. Relevantly, down-regulating LDH-A expression by antisense approaches has been shown to result in inhibition of cell growth, migration and *in vivo* tumorigenesis in many cancer models (48, 49, 50, 62, 64, 65, 101, 104, 105). On the contrary, it has been observed that silencing LDH-A expression in non cancer cultured cells, proliferation and protein synthesis were not impaired (106, 107).

Interestingly, patients with homozygous absence of M subunits (also referred as A subunits) do not show significant clinical symptoms under ordinary circumstances. Individuals lacking M subunits only complain of muscle rigidity and sudden myoglobinuria after intense exercise, when an increase in aerobic glycolysis and in ATP synthesis is required (108, 109). Homozygous gene mutations causing complete deficiencies of LDH H subunits (also known as B subunits) have been described, too (110). Also in this case, such patients do not show relevant symptoms. The H subunit containing isoform is the major isoenzyme found in red blood cells, which do not have mitochondria and obtain all their ATP by aerobic glycolysis. In spite of a strongly reduced LDH activity in erythrocytes, individuals with genetic deficiency of the H subunits do not suffer from anemia and just show signs of mild hemolysis in some case (110). Notably, the above reported data suggest that LDH inhibition (including LDHA inhibition) could be well tolerated by normal cells. Therefore, inhibitors of LDHA might have a potential antitumor action accompanied by a relatively modest systemic toxicity.

By the above encouraging considerations, they are largely justified the increasing interest towards LDHA as a specific anticancer target and the efforts in the search of small molecule metabolic inhibitors directed to LDHA. Recently, some drug-like inhibitors selective for human LDHA have been reported to exhibit promising anticancer activity both *in vitro* and *in vivo* (111). These LDHA inhibitors include analogues of gossypol, such as FX11; quinoline 3-sulfonamides; a series of malonic derivatives (Mal), including AZ-33; a group of heterocyclic derivatives, the N hydroxyindoles (NHI), bearing a carboxylic acid group in the 2-position; a salt of oxalic acid, oxamate; and the recently identified gallic acid derivative galloflavin (GF) (62, 112-115 ). All of them act as competitive inhibitors of LDHA with respect to both the substrate (pyruvate) and the cofactor (NADH). To note, most of these drug-like molecules inhibiting LDHA have been straightforwardly reviewed in two very recent and exhaustive articles (116, 117).

Although various LDHA inhibitors have been proposed as possible drugs, they only show a moderate efficacy and selectivity when administered alone. However, some of them exhibit high potential when used in combination with current drugs. Recently, Maftouh *et al.* have showed that NHI-1 and -2 in combination with gemcitabine potentiated the antiproliferative and anti-invasive effects induced by the chemotherapeutic drug in pancreatic ductal adenocarcinoma (PDAC) cell lines (118). Moreover, it has been also shown that NIH 2, combined with the redox-sensitive anticancer drugs synergistically induced apoptosis via a novel p53/NAD (H)-dependent mechanism (119). In addition, oxamate used in combination with phenformin, a biguanide anti-diabetic drug recently recognized to reduce cancer risk,

was shown to have a synergistic anti-cancer effect in a syngeneic mouse model (120). However, none of these inhibitors has been shown in clinical trials so far. Very recently, another promising LDH-5 inhibitor, GNE-140, which is a piperidine derivative generated by researchers at Genetech, Inc., has been demonstrated to be effective in inhibiting MiaPaCa-2 pancreatic cell proliferation with an EC50 of 0.25 μM (117). In addition, the high-throughput screening (HTS) of the Roche and Genentech chemical archives, with the use of a fluorescence based assay which monitored the disappearance of the NADH cofactor during enzymatic conversion of pyruvate to lactate, allowed to identify a novel class of 3-hydroxy-2-mercaptocyclohex-2-enonecontaining inhibitors of human LDHA that effectively interact with its active site, by imitating the pyruvate substrate, and exhibit good pharmacokinetic properties after oral administration to rats. (116, 117, 121).

Finally, numerous natural product extracts have been tested for their ability to inhibit LDHA, too (117, 122). Among these, the results of fractionation and purification of bioactive compounds from the crude Spatholobus suberectus (SS) extract identify the epigallocatechin (EGC) as the most potent compound with anti- LDHA activity under both normoxia and hypoxia conditions (117, 123). Currently, there is continuously new information on novel LDHA inhibitors that are being actively identified, designed and synthesized (116, 117, 124-126). Therefore, it could be expected that effective anti-LDHA agents will soon be successfully developed for clinical use.

## 6. SUMMARY AND PERSPECTIVES

Recently, there is an increasing interest in the bioenergetic features of cancer cells regarded as a very attractive area for developing novel therapeutic strategies in cancer therapy. At this regard, several preclinical investigations have indeed demonstrated that targeting aerobic glycolysis is an effective therapeutic approach. Among the enzymes involved in glycolysis, LDH is emerging as one of the most interesting molecular targets for the development of glycolytic inhibitors to possibly use in cancer therapy. In addition, LDH is considered as an important diagnostic marker, a prognostic factor and a predictive marker for response to therapy in cancer. LDHA is considered very relevant to cancer due to its role as a metabolic checkpoint in the cancer glycolytic pathway, its associations with the activation of some proto-oncogenes and the maintenance of invasiveness and metastatic potential, and its associations with resistance to chemo- and radiotherapy of cancer cells. Importantly, patients with complete lack of LDHA, due to homozygous gene mutations, have been described and do not show significant clinical symptoms under ordinary conditions, strongly suggesting that LDHA inhibition could be well tolerated in healthy cells and not accompanied by relevant systemic toxicity in patients.

A number of effective LDH inhibitors have been identified. Although LDH is considered an intricate target for the development of inhibitors due to the characteristic of its active site (poorly accessible and highly polar) and the involvement of its cofactor NADH in several other enzymatic activities, pharmaceutical industries and academic institutions recently succeeded in identifying promising small molecules inhibitors. However, most of the active LDH inhibitors need to be extensively investigated on human tumor models to evaluate the complete therapeutic potential by LDHA inhibition in cancer treatment. Overall, the possible introduction of these compounds into the clinical practice will hopefully provide new opportunities for the treatment of cancer patients.

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