Dissertation

Expression of Gluconeogenesis and Glycolysis Markers in Non-Small Cell Lung Cancer

Das Verteilungsmuster von Gluconeogenese- und Glycolyse-Markern im nicht-kleinzelligen Lungenkarzinom

submitted by

Dr.med.univ.

Elisabeth SMOLLE

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Division of Pulmonology, Department of Internal Medicine

under the Supervision of

Priv.-Doz.in Dr.in med.univ. Katharina LEITHNER, PhD

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1. Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice”.

Authors’ Contributions:
Conception and design: K. Leithner, E. Smolle, H. Olschewski
Development of methodology: K. Leithner, P. Leko, A. El-Heliebi
Acquisition of data: E. Smolle, H.H. Popper, K. Leithner, A. El-Heliebi, L. Hofmann
Analysis and interpretation of data (statistical analysis, biostatistics, computational analysis): F. Quehenberger, K. Leithner, E. Smolle
Writing, review, and/or revision of the manuscript: E. Smolle, K. Leithner, P. Leko, E. Stacher, L. Brcic, A. El-Heliebi, L. Hofmann, F. Quehenberger, A. Hrzenjak, H.H. Popper, H. Olschewski
Administrative, technical, or material support (*i.e.*, reporting or organizing data, constructing databases): K. Leithner, E. Smolle, F. Quehenberger, L. Hofmann, L. Brcic, E. Stacher, A. El-Heliebi
Study supervision: K. Leithner, H. Olschewski, A. Hrzenjak

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2. Disclosures

All co-authors have agreed to the inclusion of their published data in the dissertation, and permission to reproduce illustrations and figures from own or third-party publications has been granted.

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There are no conflict of interest to declare.

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3. Foreword

Glycolysis and abbreviated gluconeogenesis, an alternative pathway operating under low glucose conditions, have been considered as potential therapeutic targets in certain cancers. This study was conducted to examine in-depth the interplay of both pathways in non-small cell lung cancer (NSCLC). A large proportion of NSCLC showed a mixed glycolytic and gluconeogenic phenotype, with adenocarcinoma (AC) subtypes being more prone to gluconeogenesis and squamous cell carcinomas (SCC) showing elevated glycolysis. Metastases largely resembled primary tumors with respect to glycolysis markers but showed slightly higher levels of gluconeogenesis. Among AC, gluconeogenic tumors were associated with improved survival compared to mixed or glycolytic tumors. Taken together, the results of our study reveal that gluconeogenesis is active in a large proportion of NSCLC, and that the co-occurrence of both, glycolysis and gluconeogenesis is frequent. This study outlines that efficient targeting of the central carbon metabolism in NSCLC may only be achieved by inhibiting both glycolysis and gluconeogenesis.

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6. Abbreviations and Definitions

5-FU	5-fluouracil
AC	adenocarcinoma
ALK	anaplastic lymphoma kinase
ATF4	activating transcription factor 4
ATP	adenosine triphosphate
BAX	BCL2 associated X, apoptosis regulator
BAD	BCL2 associated agonist of cell death
BCL-2	B-cell lymphoma 2
BRAF	B-Raf protooncogene, serine/threonine kinase
CD31	cluster of differentiation 31
cDNA	complementary deoxyribonucleic acid
CHOP	C/EBP-homologous protein
CO_2	carbon dioxide
CT	computed tomography
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DHA	docosahexaenoic acid
DRP 1	dynamin 1 like
EGFR	epidermal growth factor receptor
EPO	erythropoietin
ER	endoplasmic reticulum
$FADH_2$	flavin adenine dinucleotide, reduced form
FBPase	fructose-1,6-bisphosphatase
FBP1	fructose-1,6-bisphosphatase, isoform 1
FBP2	fructose-1,6-bisphosphatase, isoform 2
FCS	fetal calf serum
FDA	food and drug administration
FDG	fluorodeoxyglucose
FDG-PET	fluorodeoxyglucose-positron emission tomography
FFA	free fatty acids
FFPE	formalin-fixed, paraffin-embedded
FH	fumarate hydratase
FSA	fibrosarcoma
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate

GEO	Gene Expression Omnibus
GLUTs	members of the glucose transporter family
GLUT1	glucose transporter type I
GTP	guanosine triphosphate
H2O2	hydrogen peroxide
HCC	hepatocellular carcinoma
HE	hematoxylin-eosin (stain)
HER2	human epidermal growth factor receptor 2
HIF1	hypoxia-inducible factor-1
HIF2	hypoxia-inducible factor-2
HK2	hexokinase 2
IDH	isocitrate dehydrogenase
IHC	immunohistochemistry
KDMs	Nε-lysine demethylases
KL	KrasG12D; Lkb1-null (mouse model)
KRAS	KRAS protooncogene, GTPase
LAT1	large neutral amino acid transporter
LC3	microtubule associated protein 1 light chain 3
LDH	lactate dehydrogenase
LDHA	lactate dehydrogenase A
LDHB	lactate dehydrogenase B
LUAD	lung adenocarcinoma
LUSC	lung squamous cell carcinoma
MCPyV-MCC	merkel cell polyomavirus skin cancer
MET	MET proto-oncogene, receptor tyrosine kinase
MPA	3-mercaptopicolinic acid
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MYC	MYC protooncogene, BHLH transcription factor
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NFAT	nuclear factor of activated T cells
NSCLC	non-small cell lung cancer
NTRK	neurotrophic tropomyosin receptor kinase
OAA	oxaloacetate

PARP	poly (ADP-ribose) polymerase
PCK1	phosphoenolpyruvate carboxykinase, cytoplasmic isoform
PCK2	phosphoenolpyruvate carboxykinase, mitochondrial isoform
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PD-1	programmed death 1
PDK	pyruvate dehydrogenase kinase
PDK3	pyruvate dehydrogenase kinase 3
PD-L1	programmed death ligand 1
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PFK1	phosphofructokinase 1
PKM-2	pyruvate kinase, mitochondrial isoform 2
PPP	pentose phosphate pathway
PVDF	polyvinylidene fluoride
pO2	partial pressure of oxygen
qPCR	quantitative polymerase chain reaction
RAS	rat sarcoma
RET	rearranged during transfection
ROS	reactive oxygen species
ROS1	ROS protooncogene 1 receptor tyrosine kinase
RPMI	Roswell Park Memorial Institute (medium)
rRNA	ribosomal ribonucleic acid
rtPCR	real-time PCR
SCC	squamous cell carcinoma
SDH	succinate dehydrogenase
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SIRT5	sirtuin 5
SLC2A1	solute carrier family 2 member 1 (encoding GLUT1)
SOD2	superoxide dismutase
STR	short tandem repeat
TCA	tricarboxylic acid
TCGA	The Cancer Genome Atlas
TET	ten eleven translocation; family of enzymes

TIC	tumor-initiating cell
TMA	tissue microarray
TP53	tumor protein P53
TP63	tumor protein P63
tRNA	transfer ribonucleic acid
UCSC	University of California Santa Cruz
VHL	von-Hippel-Lindau

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9. Abstrakt

Hintergrund der Studie. Die Stoffwechselwege der Glykolyse und Gluconeogenese sind maßgeblich für anabole Stoffwechselvorgänge in Tumorzellen verantwortlich. Wie Glykolyse und Gluconeogenese jedoch im Detail zusammenspielen, speziell in Bezug auf solide Tumore, ist noch nicht hinlänglich bekannt.

Experimentelles Design. Die Expression des GLUT1 Proteins, als primärer Vertreter der Glukosetransporter, sowie der Enzyme PCK1 und PCK2, wurde anhand von 450 Proben von nicht-kleinzelligem Lungenkarzinom und 54 Proben von Lungenkarzinommetastasen auf Tissue Microarrays (TMAs) analysiert. Ergänzend wurden Analysen von kompletten histologischen Schnitten durchgeführt. Die Lokalisation der immunhistochemischen Expression in Bezug auf gewisse Tumorareale wurde mittels automatisierter Datenanalyse ermittelt. Weiters wurde die Genexpression des Glykolyse- versus Gluconeogenese-Pathways anhand von Daten aus der Cancer Genome Atlas (TCGA) Datenbank untersucht.

Resultate. PCK2 war vorwiegend in Adenokarzinomen exprimiert (69% positive Fälle), während GLUT1 höhere Expressionsprofile in Plattenepithelkarzinomen zeigte (94% positive Fälle). In über 40% aller Tumoren wurde ein gemischter Phänotyp festgestellt, bestehend aus teils glykolytischen und teils gluconeogenetischen Tumorzellen. Gluconeogenetische Adenokarzinome waren mit einem besseren Gesamtüberleben assoziiert, verglichen zu glykolytischen oder gemischten Tumoren. Hingegen zeigte sich der gluconeogenetische Phänotyp vermehrt in Metastasen, verglichen zu Primärtumoren. Die PCK2 Expression war in KRAS mutierten Tumoren vermindert, weswegen wir annehmen, dass der metabolische Phänotyp teilweise durch bestimmte Mutationen beeinflusst wird. Die Tumor-Mikroumgebung und der 3-dimensionale Kontext spielen eine wichtige Rolle bei der Modulation beider Pathways, wie die vorwiegend am Tumorrand liegende PCK2 Expression, sowie der Einfluss von Hypoxie *in vitro* auf Glykolyse und Gluconeogenese im Lungenkarzinom verdeutlichen.

Conclusio. Unsere Studie zeigt ein komplexes Zusammenspiel der zentralen Stoffwechselwege im nicht-kleinzelligen Lungenkarzinom, mit einer heterogenen Aktivierung von Glykolyse und Gluconeogenese. Weiters kommen deutliche Histologie-assoziierte Unterschiede, sowie eine inter-individuelle Variabilität und die Assoziation mit der Prognose der Patienten zur Darstellung.

10. Abstract

Purpose. Glycolysis and gluconeogenesis feed anabolic metabolism of tumor cells from carbohydrate and non-carbohydrate precursors, respectively. Their interplay in solid tumors is still poorly understood.

Experimental Design. Protein abundance of GLUT1, the prime glucose transporter, and of the gluconeogenesis enzymes PCK1 and PCK2 was analyzed in 450 non-small cell lung cancer (NSCLC) specimens and in 54 NSCLC metastases by means of tissue microarrays (TMAs), as well as in whole tumor sections. Distribution of gene expression in the tumor core and -margin was analyzed by automated image analysis. In addition, gene expression profiling of gluconeogenic and glycolytic genes was evaluated, using 'The Cancer Genome Atlas' (TCGA) database.

Results. PCK2 was predominantly expressed in lung adenocarcinoma (69% positive cases), while GLUT1 expression was higher in squamous cell carcinoma (94%) compared to adenocarcinoma (41%). A mixed phenotype harboring both, glycolytic and gluconeogenic cancer cells was found in more than 40% of all cases. Gluconeogenic adenocarcinomas were associated with improved overall survival compared to glycolytic or mixed tumors, however gluconeogenesis gene expression was enhanced in metastases compared to primary tumors. PCK2 expression was reduced in KRAS mutant tumors, indicating that metabolic phenotype is influenced by particular mutations. The tumor microenvironment and the 3-dimensional context play an important role in modulating both pathways, as suggested by preferential PCK2 expression at the tumor margin and by a differential impact of hypoxia on glycolysis and gluconeogenesis in NSCLC cells *in vitro.*

Conclusion. Our data demonstrates, that the metabolic pattern in NSCLC is complex and heterogeneous. Expression of glycolysis and gluconeogenesis markers is differential, depending on histology. A considerable inter-patient variability and correlation with outcome is observed.

11. Introduction

11.1 Lung cancer

Lung cancer is the number one matter leading to cancer-associated deaths globally (2). More than 1.7 million individuals decease, directly related to lung cancer each year (3). Non-small cell lung cancer (NSCLC) makes up approximately 85% of the entirety of lung carcinoma cases. New treatment strategies have improved the mean overall survival of NSCLC within the last few decades. However, the 5-year survival is still poor with just about 24% for the entirety of patients with NSCLC, and 5.5% for patients with metastatic disease (4).

Mortality rate from lung cancer has been going down continuously in men during the last two decades, while mortality rate in women has increased within the last twenty years, and has recently stabilized (5). The highest mortality rate from lung cancer in men worldwide is reported in Eastern Europe, and in the female population highest mortality rates are observed in Northern Europe and in North America. Notably, in the United States, the black ethnicity is more affected by the disease when compared to the white, Asian, or other ethnic groups (6). In low- and middle income countries, lung cancer prevalence and mortality is lower when compared to first-world countries (7), however, lung cancer incidence is expected to rise also in these countries, because active smokers are an ever-increasing group in the developing world (8). The most prevalent risk factor for lung cancer development is tobacco smoking, accounting for 90% of lung cancers (9, 10). 15% of lung cancer cases occur in non- and never-smokers. Still, it has to be kept in mind that passive smoking is also a major risk factor, and beyond that, air pollution, radon gas exposure, asbestos and genetic risk factors account for a proportion of lung cancer cases as well (11). Lung cancer diagnostics comprises chest X-ray, sputum test, computed tomography (CT) scan, functional lung assessment, bloodwork and, of course, tissue biopsy (7, 12-14). Depending on tumor stage and grade, as well as the patients' performance status, lung cancer treatment consists of surgery, chemotherapy, radiation- and photodynamic therapy and – recently of increasing importance – immunotherapy with novel targeted agents (15). Because of ever-increasing costs of novel treatment methods, studies on cost-effectiveness are continuously performed in order to analyze the effect size (*i.e.* prolongation of overall-survival) of various treatment regimens (7). According to recent analyses on cost-effectiveness in lung cancer treatment, novel targeted agents such as crizotinib, atezolizumab or pembrolizumab have proven very cost-effective and thus beneficial for patients (16-18). Yet, cost-effectiveness of every treatment may vary from country to country, depending on the respective health care system. Regarding possible lung cancer screening methods, routine screening in patients at risk via CT scan has failed to demonstrate effectiveness, given the high costs and especially, high radiation exposure rates for the

subjects (6). In recent years, the standard of care in first-line treatment of advanced or metastatic lung cancer has changed (19). Whilst for the past decades, most patients with non-resectable NSCLC have received platinum-doublet chemotherapy (20), only modest improvements with regards to outcome have been achieved about 15 years ago with the adjunction of the antiangiogenic agent bevacizumab and pemetrexed-based chemotherapy for non-squamous NSCLC (21, 22). Various targeted therapeutics have consecutively been engineered, aiming to address tumor-promoting factors on the molecular level. For instance, oncogene addiction can be directly targeted in a selected subset of NSCLC harboring particular driver mutations. Among these agents count epidermal growth factor (EGFR)-, anaplastic lymphoma kinase (ALK)-, ROS protooncogene 1 receptor tyrosine kinase (ROS1)-, B-Raf protooncogene, serine/threonine kinase (BRAF)-, rearranged during transfection (RET)-, human epidermal growth factor receptor 2 (HER2)-, and neurotrophic tropomyosin receptor kinase (NTRK) inhibiting drugs, all of them being associated with substantial improvements in outcome (to various extents), but also with an inevitable emergence of drug resistance over the disease course (23-25). In recent years, monoclonal antibodies aiming at programmed death 1 (PD-1) or its ligand programmed death ligand 1 (PD-L1) are increasingly used as a standard of care in everyday clinical routine in patients with non-operable NSCLC (26, 27). PD-1 occurs on the surface of cytotoxic T cells and T-regulatory cells, and is expressed when T cells become activated in response to inflammation or infection (28, 29). Cancer cells express PD-L1, so they can practically hide from attacks of the immune system. Anti-PD-1 therapies disrupt this mechanism of immune-escape, leaving activated cytotoxic T cells available for the direct attack of malignant cells (29). PD-1 and PD-L1 inhibitors have led to a marked improvement in outcome, most notably in patients with tumors that highly express PD-L1. Because of proven effectiveness, PD-1 inhibitors like pembrolizumab have also been approved for the first-line treatment of advanced NSCLC (30).

The above-mentioned novel targeted treatment modalities have undoubtedly led to an improvement in the survival of patients suffering from NSCLC. Yet, lung cancer remains one of those cancers with a particularly bad outcome, especially so when it is diagnosed at an advanced stage. More research is warranted to find additional molecular targets for novel treatment modalities.
With our research project, we aimed to shed light on the aspects of cancer cell metabolism in NSCLC.

11.2 Metabolism in malignant tissues

As tumors increase in size over the disease course, they need to form new blood vessels in order to keep up a constant nutrient supply. Yet, the process of *de-novo* blood vessel formation

is highly disorganized, which ultimately leads to nutrient and oxygen deprivation in certain parts of the tumor (31). In essence, the physiology of tumors is unique when comparing to the corresponding healthy tissue: Due to the rapid growth it is characterized by oxygen deprivation, extracellular acidosis, high levels of lactate, glucose and energy deprivation, and enhanced interstitial liquid flow (32-34). At large part, the overall hostile tumor microenvironment is due to dysfunctional tumor microcirculation.

As a result from rapid tumor growth and little perfusion, metabolic pathways in cancer cells are rewired, to allow for sustained growth and cancer cell proliferation. Angiogenesis is enhanced in tumors, yet, the demand for nutrients usually exceeds availability. Continuous adaptation to fluctuations in nutrient and oxygen supply is mandatory in order to keep up cancer cell metabolism (35-37). The first observation of an aberrant metabolism in cancers as compared to the corresponding normal tissue was made almost a century ago, yet, in recent years the interest in this topic is growing, aiming at outlining certain anchor points for therapeutic targets (38). New biochemical and molecular biological tools are used to study in-depth the complex metabolic rewiring of cancer energy metabolism, leading to a better understanding of these alterations, *i.e.* with respect to different stages of a tumor and the process of metastasis. It has become particularly evident that 1) the influx of metabolites is altered in cancers, allowing for a preference of certain nutrients, 2) nutrients are assigned to metabolic pathways in a very specific fashion and 3) this affects cell growth, proliferation and ultimately metastasis in the long term (38). Not only tumor cells *per se* are affected by the above-listed changes, but also the tumor stroma, *i.e.* tumor microenvironment, is critically modified. Since tumors have to keep up with increased metabolic demands, tumor cells must increase nutrient influx from the environment.

11.2.1 Angiogenesis

Angiogenesis commences by the induction of endothelial sprouting from venules, recruiting of existing blood vessels, *de-novo* blood vessel generation, splitting of the lumen of one blood vessel into two, and even the formation of pseudovascular channels lined by tumor cells rather than endothelial cells (this phenomenon being termed “vascular mimicry”) (39, 40). Furthermore, steep gradients of glucose, oxygen and pH occur within tumors with increasing distance from vessels (31). Newly formed microvessels in solid cancers exhibit an abnormal morphology. For instance, they may be dilated, tortuous, abnormally stretched or saccular (31). Arteriovenous shunts are often found, and the overall chaotic vascular organization lacks any regulation or structure and is not matched at all to the metabolic and oxygen-demands of tumor cells. As tumor microvessels often feature excessive branching, blind endings of blood vessels commonly occur. Moreover, the endothelial lining is at large part incomplete or even missing,

leading to an increase in vascular permeability, extravasation of blood plasma and even erythrocytes, which stream into the interstitial space, consecutively increasing interstitial hydrostatic pressure (31). Viscous resistance to blood flow in solid tumors thus results from increased hemoconcentration, resulting from the above mentioned high vascular permeability. The high viscosity of blood circulating in tumor vessels contributes to inadequate perfusion in some parts of the growing tumor. Inequal distribution of blood vessels in a given tumor leads to relatively good blood supply in one part, while other parts are underperfused, resulting in a lack of tissue oxygenation and nutrient supply (41). A considerable intratumoral heterogeneity in the properties that make up the metabolic microenvironment is observed. However, even if blood flow can vary significantly between different microareas of the same tumor, tumor-to-tumor heterogeneity, *i.e.* between cancers at different sites or between patients, are usually much more pronounced (42).

11.2.2 Hypoxia

Hypoxic tissue parts significantly reduce their protein synthesis as a response mechanism. This usually results in a stop of tumor cell proliferation, and sometimes cell death (43). Sustained hypoxia impacts the cell cycle and may change the number of quiescent cells in the tumor tissue. These alterations are the cause for a different response to radiotherapy and various chemotherapeutic agents. Mainly, hypoxic cells transition from their current cell cycle phase into G1/S-phase arrest (31, 44). Yet, the role of hypoxia in cancers is pivotal. While hypoxia can lead to cell cycle arrest and apoptosis, tumor cells may also undergo hypoxia-induced epigenetic changes which enable them to keep up proliferation. Hypoxia is enhancing the antiapoptotic potential of tumor cells and may cause therapy resistance. Some of the hypoxia-induced changes involve cell energy metabolism, allowing cancers to escape also nutrient deprivation. These changes in underperfused tumors ultimately result in a clinically even more aggressive phenotype (45, 46). Summing up these findings, irregular and insufficient tumor microcirculation, hypoxia and nutrient deprivation are closely intertwined. Although cancer cells with insufficient blood supply may undergo cell death, certain genotypic and phenotypic changes lead to the opposite effect, rendering cells resistant to oxygen- and nutrient shortage and ultimately rendering them even more aggressive.

While in healthy tissues oxygen pressure is considerably high, ranging between 40 and 60 mmHg, in most solid tumors oxygen pressure only amounts to 10 mmHg (47). Tumor hypoxia alters pro-survival gene expression, results in genomic instability, formation of new blood vessels, enhancement of tyrosine kinase receptor downstream signaling pathways, and promotes epithelial-to-mesenchymal transition (48). Thus, hypoxia may promote tumorigenesis, and become a major obstacle in the treatment of solid cancers (49). Four

different types of tumor hypoxia are currently known: acute and chronic (diffusion-limited) hypoxia, toxic hypoxia and anemic hypoxia (44). Acute hypoxia arises due to a rapid and transient collapse of blood vessels, while in chronic hypoxia, tumor-angiogenesis cannot keep up with the rapid proliferation of cancer cells. Most tumor cells reside at a distance from blood vessels, and oxygen can diffuse only short distances (100 to 180 μm) from a capillary. Since many cancer cells are located further away from blood vessels, they remain in a state of chronic hypoxia (49). Toxic hypoxia is caused, for example, by excessive smoking or carbon monoxide intoxication, because of the higher affinity of carbon monoxide to hemoglobin, than oxygen. The fourth kind of hypoxia, anemic hypoxia, is often caused as a side effect of chemotherapy, or can result from tumor anemia, causing a reduced oxygen transport capacity of the blood (44). Reactive oxygen species (ROS) are required for the destruction of cancer cells by radiotherapy and certain chemotherapeutic agents, like bleomycin. Upon hypoxic conditions, the oxygen depletion gradually leads to a decrease of ROS, thus resulting in chemo- and radiotherapy resistance, although this effect may be context dependent and in some tissues hypoxia leads to an increase in ROS. On the other hand, deoxyribonucleic acid (DNA) damage afflicted by ionizing radiation may be reconditioned by the presence of oxygen (50). Overexpression of hypoxia-inducible factor-1 (HIF1) is often a result of chronic tumor hypoxia. However, HIF1 overexpression has been found to cause chemotherapy- and radiotherapy resistance as well (51). In recent years, research has focused on using hypoxia as a tool for anticarcinogenic therapy. While conventional chemotherapeutic agents are somewhat nonspecific, damaging the DNA of healthy and tumor cells alike, a new approach would be to exploit hypoxia as a unique feature in tumors (52). While normal chemotherapeutics impact predominantly tissues with rapid proliferation, slowly growing tumor entities are not affected as much, while rapidly proliferating healthy tissues (*e.g.* the small and large intestine) are damaged as a side effect. Hypoxia would be a feature unique to slowly dividing tumors, where necrosis and apoptosis are commonly found. Until today, two methods have been outlined to specifically target tumor hypoxia. First, combining radio- and chemotherapy, alongside an improvement of the oxygenation of hypoxic tumor areas. This is accomplished by a combination of erythropoietin (EPO) and chemotherapy, or by combining radiotherapy and nicotinamide (53). Another option is targeting the cancer microenvironment with recombinant, HIF1 targeting hypoxia-activated prodrugs, hypoxia-specific gene therapy or hypoxia-targeting fusion proteins (54-56).

However, these new therapeutic approaches have not been implemented in everyday clinical practice yet, and clearly more research is warranted in the field of tumor hypoxia. What remains clear is the fact, that hypoxia is a unique feature in most solid tumors, and may in the future be used as a therapeutic target, rendering these future hypoxia-targeting agents more cancer-specific and thereby avoiding side effects on healthy tissues.

11.2.3 Glucose and glutamine

Glucose and glutamine are the number-one sources of cellular energy generation in mammalian species. Catabolizing of these important nutrient sources allows cells to create and maintain pools of diverse carbon intermediates, and to use them as building bricks for the consecutive generation of intermediate substrates of the glucose metabolism. Besides, a variety of macromolecules can be generated utilizing glucose and glutamine as well. Glutamine and glucose undergo oxidization, which in turn leads to the creation of reducing equivalents in the form of nicotinamide adenine dinucleotide, reduced form (NADH) or else, flavin adenine dinucleotide, reduced form ($FADH_2$) in the tricarboxylic acid (TCA-) or Krebs cycle. Both glucose and glutamine have the ability to transfer electrons to the electron transport chain, thereby endorsing the formation of adenosine triphosphate (ATP). Using the above-mentioned mechanisms, cancer cells are maintaining their redox capacity (38).

Glutamine, as opposed to glucose, does not only deliver carbon, but also nitrogen, which is essential for *de-novo* generation of nitrogen-containing cellular compounds. Glutamine thus supplies nitrogen for purine- and pyrimidine-nucleotide synthesis, glucosamine-6-phosphate, and nitrogen-containing amino acids. Synergistically, glutamine has also been demonstrated to facilitate and alleviate the uptake of essential amino acids in general (57). Non-essential amino acids can be produced *de-novo* in tissues of mammals, while essential amino acids need to be obtained exclusively from external sources. The influx of the essential amino acid leucine through the plasma membrane-localized amino acid antiporter, large neutral amino acid transporter (LAT1) was proven to take place simultaneously with glutamine influx (57). The assumption, that intracellular glutamine facilitates the import of a large variety of LAT1 substrates, namely leucine, isoleucine, valine, methionine, tyrosine, tryptophan and phenylalanine, was made based on this observation (58). The high demand of proliferating tumor cells for glutamine was originally demonstrated in the 1950ies, when Harry Eagle showed that HeLa cells in culture require a 10- to 100-fold molecular excess of glutamine in the cell culture medium, relative to the requirement of other amino acids (59). The TCA cycle, also known as Krebs cycle, constitutes one of the fundamental metabolic pathways in healthy, as well as in malignant tissues. Function of this pathway is frequently impaired in cancers, with glutamine playing a central role in rewiring and changing how the TCA cycle normally works (60). In short, the TCA cycle acts as a common pathway for catabolizing a variety of key nutrients, *i.e.* sugars, fatty- and amino acids (61). Furthermore, electrons that are a prerequisite of oxidative phosphorylation are generated by the TCA cycle, hence, the majority of the energy required by cells under normoxic conditions is generated via this pathway (61). Of note, the TCA cycle functions as a metabolic hub as well, supplying cells with precursor substrates for the biosynthesis of different amino acids and nucleotides. Mutations of the TCA cycle have

been outlined in several familial cancer types (62). In particular, the TCA cycle enzymes succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase (IDH) are mutated in certain cancers, leading to an aberrant function of the TCA cycle (36). As already mentioned, not only glucose is essential for cancer cell proliferation, but also glutamine is used as a key source of energy and biosynthetic precursor molecules in malignant tissues. A certain “glutamine dependency” of cancer cells has been described, meaning that they predominantly rely on glutamine as fuel, contrary to non-malignant cells. Generally, in malignant cells a strong dependency on several nutrients – especially to feed the TCA cycle – is observed. It has even been reported that cancer cells go into cell death in the absence of exogenous glutamine (63). Upon sufficient glutamine supply, cancer cells use glutaminolysis to acquire glutamine-derived alpha-ketoglutaric acid as a fuel for the TCA cycle. In renal cell cancer, for instance, with disturbed TCA cycle function, tumor cells relied on reductive carboxylation of glutamine-derived citrate for the production of acetyl-CoA aside from other precursor molecules. Without acetyl-CoA, which serves as a main fuel for lipid synthesis, cancer cells are not viable. In general, TCA intermediates are essential for the generation of a variety of cellular building blocks, and therefore cancer cells become strongly dependent on glutaminolysis as a result of genetic alterations affecting oxidative mitochondrial function (64). Furthermore, it was found that expression of the transcription factor HIF by tumor cells led to maintenance of a low level of intracellular citrate to keep up constant adequate lipogenesis. Renal cancer cells deficient of the VHL gene will consecutively express HIF1 alpha and / or hypoxia-inducible factor-2 (HIF2) alpha and became utterly dependent on glutamine for proliferation (65). Glutamine addiction was also found in glioma cells harboring a mutation of the enzyme isocitrate dehydrogenase 1 (IDH1). In non-malignant cells, IDH1 catalyzes the conversion of isocitrate to alpha-ketoglutaric acid. However, the mutated isoform of IDH1 converts alpha-ketoglutaric acid into the oncometabolite D-2-hydroxyglutarate which inhibits cellular differentiation through epigenetic alteration (66). Due to the aberrant function of mutated IDH1, glioma cells become dependent on glutamine-derived generation of alpha-ketoglutaric acid, rendering them glutamine addicted (67). Mounting evidence supports the fact, that dependence of cancer cells on glutamine is even more frequent than primarily assumed. Fluorodeoxyglucose-positron emission tomography (FDG-PET) scanning detects cancers with a high glucose metabolism. Yet, PET-negative cancers might rely on glutamine as their prime energy source instead – and in the future glutamine-based imaging might be developed as a feasible alternative for the detection of PET-negative lesions (60, 68). Importantly, glutamine is also one of the most important precursors for gluconeogenesis, the reverse pathway of glycolysis.

Summing up this data about glutamine, it becomes clear that cancers continuously develop new survival mechanisms if deprived of their primary energy and carbon sources. In-depth

understanding and further research on cancer metabolism is necessary to find new and ever-more specific anti-tumor treatments.

11.2.4 Glycolysis versus gluconeogenesis

In solid neoplasms, extracellular fluids exhibit average glucose levels that lie well below those of the adjacent healthy tissue, usually containing 50% less glucose (69). This observation is in line with a generally reduced content of glucose in malignant tissues reported by a variety of metabolomics studies (70). Hence, in solid, rapidly growing cancers like NSCLC, it is mandatory that cancer cells develop adaptation mechanisms to conquer glucose deprivation. In part, this is realized when bypassing the glycolytic pathway and instead going through the early stages of gluconeogenesis, utilizing non-carbohydrate precursors to overcome metabolic stress (71-75). The prime enzyme which mediates gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK), has been recently discovered to stock up glycolytic pools in cancer cells of different origins, thereby mediating an abbreviated version of gluconeogenesis (71-76). In a variety of studies, PEPCK was shown to enhance tumor cell metabolic flexibility under conditions where the glucose supply becomes insufficient (for review see (76)). Figure 1 illustrates the pathways of glycolysis and gluconeogenesis, showing PEPCK to be exclusive for the gluconeogenesis pathway.

The process of gluconeogenesis, which is in essence the generation of glucose from non-carbohydrate precursor molecules like amino acids or lactate, primarily takes place in the liver and kidney (77). Abbreviated gluconeogenesis, by contrast, rather aims at creating different intermediate substrates, *e.g.* glycerol phosphate (76, 78). The first step of gluconeogenesis is mediated by PEPCK, which catalyzes the reversible conversion of oxaloacetate (OAA) + guanosine triphosphate (GTP) to phosphoenolpyruvate (PEP) + guanosine diphosphate (GDP) + carbon dioxide (CO_2). Two isoforms of PEPCK exist: first, the cytoplasmic isoform PCK1, and second, PCK2, the mitochondrial isoform (79, 80). PCK1 is abundantly expressed in healthy and differentiated liver cells, the small intestine, kidney cortex and adipose tissue (75, 80). By contrast, PCK2 occurs ubiquitously in a different cell- and tissue types, including hepatocytes, T- and B-cells, pancreatic beta-cells and neurons (75).

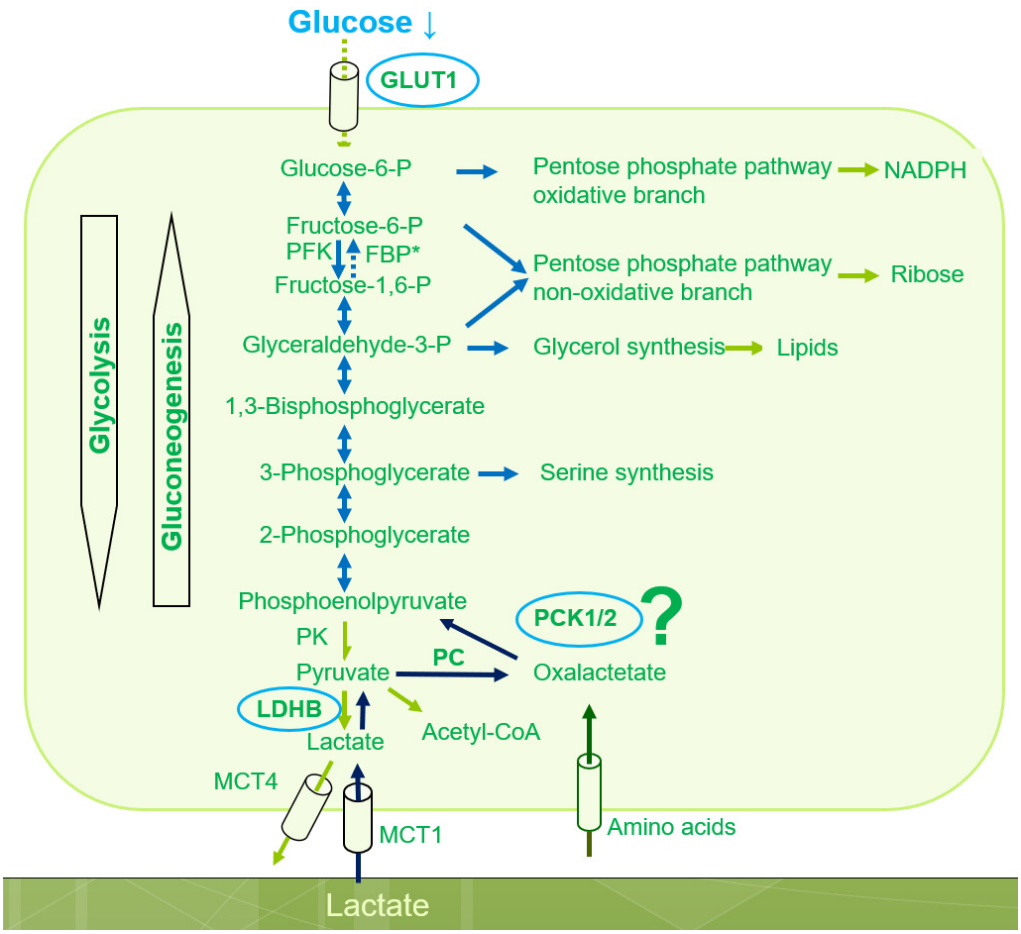

Figure 1. Glucose is taken up avidly by cancer cells, since glycolytic intermediates are branching into important biosynthetic pathways, *e.g.* for the generation of ribose, glycerol and serine. Gluconeogenesis, the reverse pathway of glycolysis, could replenish these intermediates. The key enzyme in this pathway is PEPCK. Its role in cancer cells is still poorly understood.
Figure adapted with kind permission from Katharina Leithner.

In a study by Leithner and colleagues, it was found that PCK2 is expressed in lung cancer cells, as well as in human NSCLC samples. PCK2 was found to be essentially involved in the conversion of stable isotope-labeled lactate to PEP in the direction of gluconeogenesis (71). In the same study, PCK2 was shown to enhance lung cancer cell survival in case of glucose scarcity (71). In a study on MCF-7 breast cancer cells, PCK2 was also found to act in a pro-proliferative and pro-survival manner (75). Furthermore, PCK2 silencing has been shown to result in an attenuation of lung cancer cell proliferation upon glucose starvation (71), and to effectively counteract the growth of lung cancer cell xenografts *in vivo* (72). In colon cancer cells, as well as in tumor-repopulating melanoma cells, PCK1, the cytoplasmic isoform of PEPCK, was found to act pro-tumorigenic *in vitro* and *in vivo* (74, 81). Interestingly, in the tumor-repopulating melanoma cell model, PCK2 overexpression reduced metastasis

formation, suggesting a pivotal role of PCK2 in primary tumors versus epithelial-mesenchymal transition and metastasis (82). In cancers originating from tissues where in the healthy state, high basal rates of gluconeogenesis are found, such as in liver- or renal cell cancer, a down-regulation of PCK1, or a reduction of the gluconeogenesis gene fructose-1,6-bisphosphatase 1 can be observed (83, 84). Downstream pathways fueled by PEPCK are used by tumor cells to overcome low-nutrient stress. There are four major PEPCK downstream pathways known today: first, the synthesis of serine and its consecutive conversion to glycine; second, the synthesis of glycerol phosphate; third, the synthesis of ribose phosphate, and fourth, glycogen synthesis (76). As it was recently discovered, in lung cancer cells undergoing low-glucose stress, glutamine is converted to serine and glycine via PCK2 at higher rates (72). PCK1 was found to mediate ribose phosphate synthesis from glutamine in colon carcinoma cells in moderately low glucose medium (2.5 mM), whereas under high glucose conditions (25 mM), conversion of glutamine to ribose was less prevalent (74).

Of note, lung cancer tissue is not the only one showing abbreviated gluconeogenesis, as mediated by PCK1 and/or PCK2. In malignant cells of various origins, PCK1 or PCK2 have been found to mediate the abbreviated version of gluconeogenesis, and particularly so under conditions of very low glucose supply, thus facilitating anabolic processes (71, 72, 74, 75). In downstream metabolic pathways, PCK1 or PCK2 are essentially involved in the *de novo* generation of the phospholipid glycerol backbone, as mentioned above (73), or else, of serine (72), and ribose (74, 85). The biosynthesis of glycolytic intermediate substrates is, to a large extent, realized by PCK1 and PCK2, both enzymes enabling malignant cells to proliferate despite glucose scarcity. In addition, a direct anchor point of PCK2 in mediating TCA cycle function has been demonstrated, since PCK2 catabolizes OAA synthesized by glutamine (86). Therefore, PEPCK mediates cancer cell metabolic adaptation, being a crucial prerequisite for glucose-independent tumor growth and proliferation (71, 72). When PEPCK is directly targeted and silenced, this was shown to reduce cancer growth in mouse models *in vivo* (72-74, 87-89). On the contrary, it was seen in liver- and kidney cancer cells that growth inhibition was a result of PEPCK activity (76, 90). Here, one could argue that in healthy liver and kidney tissue, PEPCK activity and gluconeogenesis is generally high, which might contribute to the growth inhibitory effect described here, which contradicts the observations in other cancer specimens.

When NSCLC cell lines are deprived of glucose, PCK2 expression was found to be upregulated, according to previous data (71, 72). Activating transcription factor 4 (ATF4), as well as endoplasmic reticulum (ER) stress (75), and the MYC protooncogene, BHLH transcription factor (MYC) superfamily member MondoA were also shown to cause an upregulation of PCK2 (91). Still, the precise interplay of catalysts for up- or downregulating PEPCK2 expression in the multilayered and complex cancer microenvironment *in vivo* is poorly

understood. Another important finding regarding PEPCK function in NSCLC is, that its activity is obviously lower in normal lung tissue, and higher in cancer specimens (71). Notably, PCK2 is the predominant PEPCK isoform in NSCLC, because it was shown that PCK1 levels are usually quite low in general (71, 72). A detailed analysis of PCK1/2 expression in a larger cohort of NSCLC, however, is still missing in current literature.

11.2.5 Glyceroneogenesis

A study specifically on glyceroneogenesis was performed by Leithner *et al.* in 2018, since until then little was known about glyceroneogenesis in cancer cells. However, it has been shown previously that both PCK1 and PCK2 play a role in the mediation of glyceroneogenesis (78, 92). Glyceroneogenesis usually occurs during lipolysis, allowing for the reesterification of excess fatty acids (78). The backbone of triglycerides is formed by glycerol phosphate, and glycerol phosphate is also essential for the biosynthesis of glycerophospholipids which are a key component of biomembranes (93). Glycerol phosphate, derived from glucose, is used for the synthesis of glycerophospholipids (94, 95). It was shown by Leithner and colleagues, that cancer cells expressing PCK2 use noncarbohydrate sources, for example glutamine or lactate, for the synthesis of the glycerophospholipid backbone, especially upon glucose-restriction (73). In this study, PCK2 expression was found to peak in A549 lung cancer cells cultured in a low-glucose (0.2 mM) and serum-free medium. In the 10 mM glucose- or serum-containing media, PCK2 expression was still present, albeit much weaker. A panel of different NSCLC cell lines was tested under glucose- as well as serum-starvation, and the PCK2 protein was upregulated in most of the cell lines investigated. It was hypothesized in this study that glyceroneogenesis is still ongoing in glucose-starved lung cancer cells featuring PCK2 upregulation, and that noncarbohydrate precursors are used as substrate. To prove this assumption, ^{13}C-labeled glutamine was used in A549 and H23 lung cancer cells cultured under serum-free and low-glucose conditions. Mass spectrometry was used to calculate the abundance of glycerophospholipid-bound glycerol. Indeed, the ^{13}C-labeled carbons were transferred to the glycerol backbone of glycerophospholipids in a time-dependent fashion. Glyceroneogenesis occurred at high rates at very low glucose levels (0.2 mM) and was practically absent at 10 mM glucose (73). Hence, the relatively low expression of PCK2 under high glucose conditions might explain why alternative carbon sources, other than glucose, contribute little to gluconeogenesis and glyceroneogenesis when glucose availability is sufficient. Notably, according to Leithner *et al.*, the first step of glyceroneogenesis, namely the conversion of glutamine to glutamate, was seen irrespective of glucose levels (73). In the same study it was also tested whether lactate is used as an alternative energy source for glyceroneogenesis. Therefore, uniformly labeled ^{13}C-lactate was used as a tracer, and it was

shown that under low-glucose and serum starvation, lactate was indeed used for glycerol-backbone biosynthesis. Still, glutamine appears to be the predominant precursor used for glyceroneogenesis. In summary, it was shown that in lung cancer cells upon glucose- and serum starvation, a vast proportion of glycerophospholipids contained carbons which had derived either from glutamine or lactate (73). It is thus very probable, that PCK2 upregulation under low glucose conditions endorses the use of non-glucose sources for the generation of glycerol, and glycerophospholipids, respectively. PCK2 renders cancer cells more flexible under metabolic stress and allows them to circumvent classical pathways in order to keep up growth and proliferation.

11.2.6 The Warburg effect

For decades it has been known that glucose is a central nutrient for cancer cells which allows them to obtain energy in the form of ATP by oxidation (for review see (96)). In mammals, the end product of glycolysis can be lactate or, in case of complete oxidation of glucose via mitochondrial respiration, CO_2. Since tumor cells use up glucose at much higher rates than the corresponding healthy cells, more lactate is produced, even when enough oxygen is available and in the presence of a functioning mitochondrial metabolism (96, 97). This phenomenon was first described by Otto Warburg, albeit that he did not fully resolve, why this unusual glucose metabolism is specific for cancer cells and what benefits it may provide. In the 1920ies, Otto Warburg and his colleagues observed that tumor cells, in contrast to the surrounding healthy tissue, used up glucose at enormous amounts. When Warburg found that glucose was fermented to lactate even under aerobic conditions, he termed this effect “aerobic glycolysis” (97, 98). The differences in glucose metabolism upon hypoxia and normoxia, in differentiated versus proliferative and tumorous tissues, respectively, are illustrated in Figure 2.
Interestingly, it was also observed that respiration alone allowed for the maintenance of tumor cell viability. Therefore, Warburg concluded that in order to kill cancer cells, both glucose and oxygen had to be eliminated (99). Building on Otto Warburg’s data, the English biochemist Herbert Crabtree subsequently studied the diversities in glucose and energy metabolism in cancer specimens. He found that the extend of mitochondrial respiration in tumors varied considerably, with many tumors indeed showing a substantial amount of respiration (100). Thus, it was concluded that tumor cells conduct aerobic glycolysis – but that there is also a considerable variability in glucose metabolism, either due to environmental or genetic factors. It was not until the 1970ies when the term “Warburg effect” was given to the phenomenon of aerobic glycolysis in cancer cells by Efraim Racker, whose research also focused on respiration and metabolism in cancer.

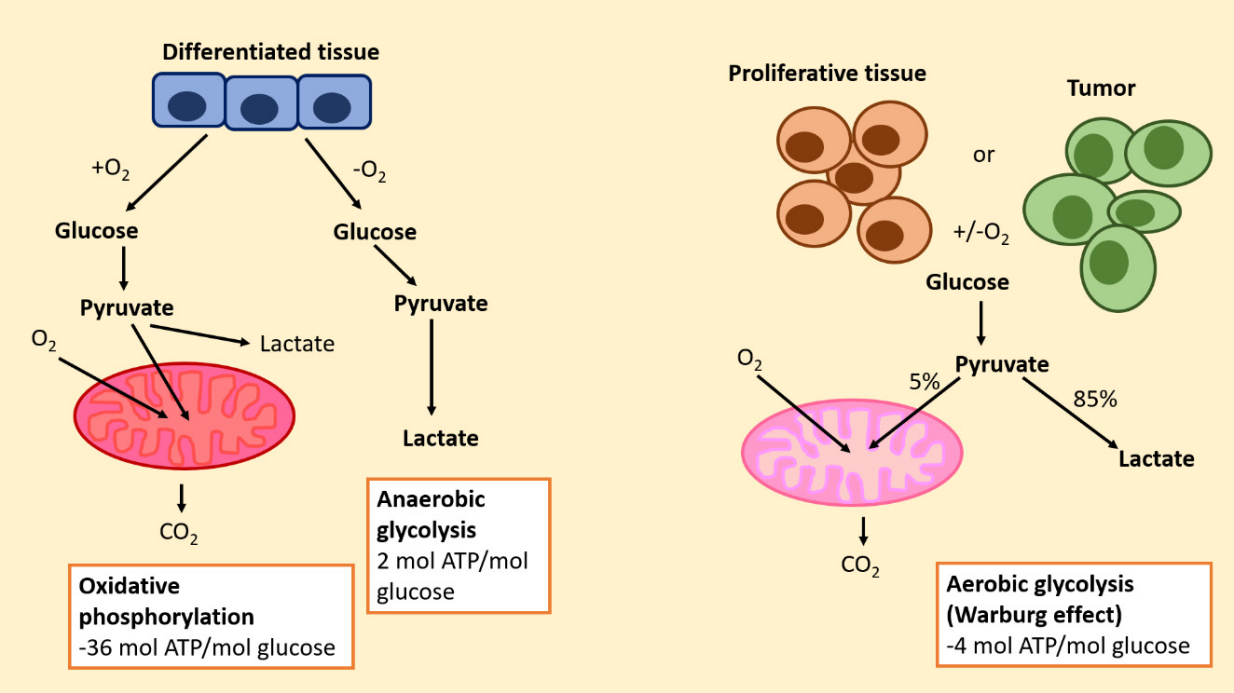

Figure 2. The difference between glucose metabolism in differentiated versus proliferative and malignant tissues, in the presence and absence of oxygen, respectively (37).

Racker hypothesized, that the cause of the Warburg effect might be an imbalance in intracellular pH, or disturbances in ATPase activity (101). Later, he observed together with his colleagues Flier and Birnbaum, that aerobic glycolysis can indeed be controlled by growth factor signaling. At about the same time, the discovery of oncogenes led to the conclusion that dysfunctional growth factor signaling is one of the root causes in tumor development (102-105). First, it was yet unclear whether the Warburg effect was only a bystander event in tumorigenesis, but more recently it was revealed that the Warburg effect actually is essential for tumor growth (106, 107). This “Warburg” phenotype of cancer cells, characterized by enhanced aerobic glycolysis, is mediated, at least in part, by an aberrant expression of members of the glucose transporter family (GLUTs) (108).

11.2.7 Members of the glucose transporter family in cancer

Generally, the predominantly glycolytic phenotype of tumor cells is linked to an upregulation of glucose transporters. The family of glucose transporters can be subdivided into three subfamilies (109), described by the gene symbol SLC2A, and the protein symbol GLUT. The nomenclature of GLUTs is heterogeneous and somewhat confusing, since over the past decades, several new members of the GLUT family have been described by independent research groups. What all GLUTs have in common are 12 membrane-spanning helices and

several conserved gene sequence motifs (110). Joost and colleagues analyzed sequence similarities of GLUTs in-depth, and came up with the corresponding sub-division of GLUTs into three categories: Class I comprises the most extensively studied GLUT family members, *i.e.* GLUT1 to GLUT4. The distribution to certain tissues is exclusive for each of them. GLUT1 is found in red blood cells and brain microvessels. GLUT2 is widely distributed in the mammalian liver and pancreas, GLUT3 is found in cells stemming from the neural crest, while GLUT4 is found predominantly in muscle tissue and in adipocytes (109). GLUT5, meanwhile represents a fructose-specific member of the GLUT family. Three closely related proteins, namely GLUT7, GLUT9 and GLUT11, constitute class II of GLUTs (111). Class III GLUT family members can be distinguished from other GLUTs by the absence of a glycosylation site in the first extracellular linker domain, and by the presence of a glycosylation site in loop 9 (109).

GLUT2 has a relatively low affinity for glucose and a high affinity for glucosamine (112, 113). Moreover, GLUT2 has been demonstrated to act as a galactose-, mannose- and fructose-transporter as well (114). GLUT2 is expressed in the basolateral membrane of intestinal and kidney epithelial cells (115), and mainly acts as a transepithelial transporter of glucose into the bloodstream. In hepatocytes, GLUT2 functions as both a promoter of glucose uptake from the blood, as well as glucose release (116). In the plasma membrane of beta-pancreatic cells, GLUT2 is highly expressed, functioning as a glucose-sensor and facilitating glucose-stimulated insulin secretion (117). GLUT2 has been reported to be overexpressed in liver cancer (118). Furthermore, GLUT2 expression is found in colon- and gastric malignant tumors, suggesting its potential role in these cancers (117). The GLUT3 transporter takes up glucose with high affinity (114), while also transporting galactose, mannose, maltose, xylose and docosahexaenoic acid (DHA) (119). GLUT3 mRNA is ubiquitously expressed in all types of human tissues, with a preponderance in the brain and testis (120). In neural cells, GLUT3 plays an important role, delivering glucose from the interneuronal space into the neurons (117). In NSCLC, GLUT3 is overexpressed when compared to healthy lung tissue, according to previous research (121). In breast cancer, on the contrary, GLUT3 expression tends to be particularly low or absent (122), while one study reported a higher GLUT3 expression in poorly versus well-differentiated breast tumors (123). In gliomas, GLUT3 is abundantly expressed, featuring higher levels than GLUT1 (124). It is generally assumed that GLUT3 acts pro-carcinogenic, facilitating glucose uptake in cancer specimens with high glucose turnover. GLUT4 transports glucose as well as DHA and glucosamine. Its gene sequence is also highly conserved in mammals, with 91-96% similarity of sequence between humans, bovines, rats and mice (117). GLUT4 is stored in tricellular tubulovesicular compartments and post-prandially stimulated by insulin, then translocated to the plasma membrane and plays a critical role in glucose homeostasis of the whole body (117). GLUT4 mRNA and protein are expressed in malignancies of the colon, breast, thyroid, pancreas and gastrointestinal tract, and in multiple

myeloma (125). Given the fact that these tissues are typically not considered insulin-sensitive, it is likely that GLUT4 expression increases during the process of carcinogenesis – a topic which has not been studied in-depth yet (117). Members of classes II and III of the GLUT family members will not be discussed in detail here.

The SLC2A1 gene encodes the GLUT1 protein. It is expressed at relatively high levels in most healthy tissues. Currently, most studies about GLUT family members have focused specifically on GLUT1, making it the most widely investigated member of membrane transport proteins as well. GLUT1 represents a highly conserved isoform, with a gene sequence that is 74-98% identical when comparing in fish, chicken, human, cow, rat and mouse (119). For glucose, GLUT1 shows the highest affinity, but it can ship galactose, mannose, glucosamine and DHA as well (112). Due to the high expression of GLUT1 in erythrocytes, this glucose transporter was previously termed the “erythrocyte-type glucose transporter”, making up 3-5% of the overall membrane protein content in erythrocytes. Of note, GLUT1 is abundantly and ubiquitously expressed in all types of non-malignant tissues under normal conditions (119). Given the fact that GLUT1 is abundantly distributed in healthy tissues in general, it must be emphasized that particularly high expression levels are observed in endothelial and epithelial cells forming blood-tissue barriers in the brain, eye, peripheral nerve, placenta and lactating mammary gland (126, 127). GLUT1 expression is very weak or below detection level in most epithelial healthy tissue specimens, as well as in benign tumors of epithelial origin (126, 128). Tumors frequently overexpress GLUT1, which allows them to perform glycolysis at high rates (128). Abundant GLUT1 expression results in increased glucose uptake into the cytoplasm of tumor cells (108, 128). The prognostic value of GLUT1 expression has been studied in a variety of cancer specimens (108). Interestingly, the existing literature on how GLUT1 influences tumorigenesis is somewhat contradictory: Some studies show GLUT1 overexpression to be associated with a worse prognosis, while other data show no significant association at all (128). In a large meta-analysis by Yu and colleagues from 2017, a total of 232 patients were included for an in-depth evaluation of the prognostic value of GLUT1 in pancreatic cancer. According to this review article, in pancreatic cancer GLUT1 overexpression was associated with a shorter overall survival (126, 129, 130). For the evaluation of lung cancer, three studies (518 patients) were included, and the pooled results suggested an overexpression of GLUT1 also to result in a significantly worse outcome, with a notable effect on overall survival (131-133). High GLUT1 expression has been linked to an adverse outcome (134), and chemotherapy resistance in lung cancer as well (135). Furthermore, an association of GLUT1 overexpression with an adverse outcome in colorectal cancer and oral squamous cell carcinoma has been reported (136, 137). No effect on prognosis of GLUT1 expression was observed in laryngeal cancer, ampulla of Vater carcinoma, extrahepatic bile duct-, cervical- and ovarian cancer (126). In the case of breast cancer, Kang and colleagues (138) found no significant association between

GLUT1 expression and overall survival, while Jang *et al*. (139) did identify a poor prognostic impact of high GLUT1 expression in breast cancer. Summing up the above-mentioned data, it seems that in most cancer specimens, high GLUT1 expression means an advantage for cancer cells, allowing them to consume higher amounts of glucose for optimum growth and proliferation which consecutively worsens prognosis. In an interesting study by Goodwin *et al*., aberrant expression of GLUT1 in squamous cell NSCLC (SCC) was investigated in-depth (140). Notably, GLUT1 expression was demonstrated to be abundant in SCC, but practically absent in lung adenocarcinoma (AC). Not surprisingly, elevated GLUT1 expression correlated with higher 18F-fluorodeoxyglucose (FDG) uptake and enhanced cellular glucose metabolism in general (140). Pharmacologic inhibition of glycolytic flux via a non-metabolizable glucose analogue, and a GLUT-specific inhibitor (WZB117), was shown to selectively suppress tumor growth in SCC, according to this study. Goodwin and colleagues sought to uncover the unique gene expression profile of lung SCC by conducting a search using The Cancer Genome Atlas (TCGA) database. They found a pooled analysis of mRNA-sequencing gene expression profiles from 501 SCC and 517 AC patient samples, outlining a subset of differentially expressed genes (141). Among them was also GLUT1, counting among the most significantly overexpressed genes in SCC (more than 10-fold median increased expression in comparison to AC) (140). Notably, when analyzing also mRNA expression of other members of the glucose transporter family, it became evident that GLUT1 is, by far, the most considerably expressed glucose transporter in human SCC patients. Interestingly, when comparing the proliferation marker Ki67 and proliferating cell nuclear antigen (PCNA) in the TCGA cohort, no correlation to GLUT1 expression was outlined. This finding suggests high GLUT1 expression not to reflect high cellular proliferation but rather to be phenotypically associated with SCC (140). Similar to our study, Goodwin *et al*. determined GLUT1 protein expression in tissue microarrays (TMAs) of SCC and non-squamous lung carcinoma samples. GLUT1 expression was predominantly confined to the SCC histology (79%), and very weak or absent in the non-squamous subtype (21%). By means of a patient-derived xenograft model, GLUT1 expression in human NSCLC was further validated. Across an array of SCC and AC xenograft samples, high levels of relatively homogeneous GLUT1 expression were seen in the SCC specimens, while expression levels in AC were generally weak and strictly confined to necrotic tumor areas in AC. This suggests an induction of GLUT1 upregulation by hypoxia (140). In the same study, lung SCC specific GLUT1 expression was tested in an animal model as well. For this purpose, the KrasG12D; Lkb1-null (KL) NSCLC mouse model was used. KL mice develop the whole spectrum of NSCLC subtypes, about 45% AC and 25% SCC. In the evaluated tumors of KL mice, GLUT1 was exclusively expressed in SCC, and was not detectable in AC. In accordance, a strong correlation between GLUT1 levels and the squamous marker tumor protein P63 (TP63) was observed (140).

Given the circumstance that in lung AC, GLUT1 does not play a key role like in the squamous cell histology, it is suggested that cellular glucose metabolism differs substantially between AC and SCC lung cancer.

11.2.8 Lactate dehydrogenase B

Another key enzyme in gluconeogenesis, or glucose metabolism in general, is lactate dehydrogenase B (LDHB). Lactate dehydrogenase is part of a family of NAD^+-dependent enzymes (142). Active LDH is a homo- or heterotetramer molecule, constituted by two different subunits: M and H. The letters M and H stem from the original first description of the subunits in the muscle (M) and heart (H). In humans, two genes with an exclusive structure encode these polypeptide subunits: LDHA (M) and LDHB (H) (143, 144). Oxidative phosphorylation is fueled by several precursor substrates, depending on their availability, in tumor cells that grow in highly vascularized areas (145). On the contrary, tumor cells that grow in a hypoxic environment with insufficient vasculature, become increasingly addicted to anaerobic glycolysis (as described above). LDHA is strongly linked to the Warburg effect, mediating the conversion of pyruvate to lactate, while LDHB is responsible for the reverse reaction. Tumor cells that are able to fuel oxidative phosphorylation by using a variety of substrates, are more often multidrug resistant and the patients' survival times decrease (146). The association of LDHB with tumor metabolism and its influence on prognosis is rather intricate (147). According to previous literature, LDHB upregulation is only found in certain cancer genotypes, depending on the rate of aerobic glycolysis (148). LDHB is silenced by promoter methylation in various cancer specimens, whilst in other tumor types it is overexpressed or amplified (147). For example, in basal-like and triple-negative breast cancer cell lines, a specific upregulation of LDHB has been described, as compared to luminal breast tumors (148). LDHB is upregulated in lung cancer cell lines characterized by rat sarcoma, G-protein (RAS) pathway activation and is a prerequisite for the *in vivo* KRAS-mutant lung tumor growth. Also, in other lung cancer subtypes, especially in MET proto-oncogene, receptor tyrosine kinase (c-MET) driven lung cancer specimens, and in EGFR driven tumors, high levels of LDHB have previously been described in cell culture experiments. While LDHB is upregulated in lung- or breast cancer (148, 149), other studies have demonstrated that LDHB expression is suppressed in pancreatic-, prostate- and gastric cancer (150-152). In liver cancer, loss of LDHB expression is associated with an adverse outcome (153). Thus, LDHB has been suggested as a future therapeutic target in lung cancer, particularly in LDHB high-expressing tumors. Considering that high LDHB expression is associated with a significantly shorter survival in lung AC patients, LDHB may also be used as a prognostic biomarker in lung cancer in the future (149). Interestingly, in hepatocellular carcinoma, LDHB expression levels, when compared to

adjacent healthy liver tissue, are significantly lower. In this case, low LDHB expression is associated with a worse prognosis (153). Similarly to GLUT1, this finding suggests an essential difference between cancers arising from tissues with a high gluconeogenic activity, compared to those where gluconeogenesis is performed at low rates in the healthy state: In the former, LDHB downregulation means a worse outcome, while in the latter cancer specimens, LDHB overexpression is associated with a poor prognosis. The general significance of LDHB on tumor cell death, including apoptosis, is poorly understood. One study showed LDHB inhibition in merkel cell polyomavirus skin cancer (MCPyV-MCC) cells to induce cleavage of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP), resulting in tumor cell apoptosis (154). LDHB knockdown also endorses taxol-induced cancer cell death, induced by cytochrome c release, as well as activation of caspase-3 and -7, and reduced expression of B-cell lymphoma 2 (Bcl-2). By reimplementation of LDHB, this pro-apoptotic effect can be reversed (155). On the contrary, some negative studies on this topic also exist, showing no apparent effect of LDHB on the cellular NADH/ nicotinamide adenine dinucleotide, oxidized form (NAD^+) ratio in these cells (150, 155). Autophagy is a self-degradative cellular event, which is crucial for nutrient rationing to maintain cellular homeostasis, particularly upon nutrient deprivation in a hostile microenvironment (156, 157). LDHB has previously been investigated with respect to autophagy. It was shown in experiments targeting LDHB, that this gluconeogenic enzyme controls lysosomal activity and basal autophagic flux of cancer cells (145). Silencing of LDHB-induced leupeptin-sensitive microtubule associated protein 1 light chain 3 (LC3-II) protein accumulation in SiHa human cervix adenocarcinoma cells resulted in a striking inhibition of autophagy pathways, as it was shown by accumulation of the autophagy substrate optineurin. Moreover, silencing LDHB had antiproliferative effects in cancer cells (142). In similar experiments on HeLa cervical cancer cells, LDHB silencing also led to a decrease in autophagy. In summary, LDHB impacts the autophagy flux of oxidative cancer cells, and silencing of LDHB results in the inhibition of basal autophagy, cancer cell proliferation and finally, apoptosis (145). The post-translational mechanism, whereby LDHB is regulated during the process of autophagy in cancer cells, is essentially regulated by sirtuin 5 (SIRT5), which is a binding partner for LDHB. SIRT5 deacetylates LDHB at lysine-329, thus endorsing the enzymatic activity of LDHB (158). Consecutively, deacetylated LDHB increases the autophagy of tumor cells, promotes lysosomal acidification and autolysosomal maturation, whereas silencing of SIRT5 results in LDHB acetylation and inhibition of its proautophagic activity (158).

11.3 Aim and scope.

With our research work, we want to provide a detailed insight into the expression pattern of PCK2, PCK1, LDHB and GLUT1 in NSCLC. Moreover, we wanted to find out about a possible intratumoral special heterogeneity of expression. To the best of our knowledge, the precise interplay of gluconeogenesis and glycolysis in NSCLC has not been thoroughly investigated yet. No data has yet shown in detail, whether inter- or intra-individual disparities of glucose metabolism in NSCLC exist. In addition, histology-related differences in the utilization of gluconeogenesis in NSCLC have not been investigated until today either. Hence, our study focuses on revealing the sophisticated interplay of glycolytic and gluconeogenic mechanisms in NSCLC.

12. Material and Methods

12.1 Patients

207 men and 135 women suffering from lung AC, and 93 male and 15 female subjects with lung SCC were included into our analysis. All tumor samples were placed on TMAs, which contained a mixture of the AC and SCC tumor specimens. We gained three TMA cores from each subject's tumor. The vast majority of tumor samples was acquired from surgical resection performed at the University Hospital of Graz, Austria, while only a minority of tissue samples stemmed from surgical procedures performed at a private sanitarium. We display the detailed patients' clinical data and characteristics in Table 1. The metastasis patient subgroup consisted of primary NSCLC stage IV (metastatic) samples which were also located on TMAs, alongside 54 NSCLC metastasis specimens, corresponding to the primary tumors. Table 2 illustrates clinical and patients' data of metastatic patients. Besides, formalin-fixed, paraffin-embedded (FFPE) samples of NSCLC, stemming from 29 subjects, as well as the corresponding healthy lung tissue, was provided by the Biobank of the Medical University of Graz. We used the patients' medical charts to extract their clinical data. Self-evidently, we had the institutional ethics review board approve our study protocol.

CHARACTERISTICS	ADENOCARCINOMA	N (%)	SQUAMOUS CELL CARCINOMA	N (%)
All Cases		342		108
Gender				
	Male	207 (60.5)	Male	93 (86)
	Female	135 (39.5)	Female	15 (13.9)
Age (yr)				
	Median	64	Median	64
	Range	16-86	Range	37-84
Stage (TNM 6th)				
	IA	105 (30.7)	IA	33 (30.6)
	IB	74 (21.6)	IB	24 (22.2)
	IIA	35 (10.2)	IIA	8 (7.4)
	IIB	69 (20.2)	IIB	29 (26.9)
	IIIA	33 (9.6)	IIIA	6 (5.6)
	IIIB	15 (4.4)	IIIB	8 (7.4)
	IV	11 (3.2)	IV	0 (0)

Table 1. Patients' characteristics.

CHARACTERISTICS	PRIMARY LUNG TUMORS	N (%)
All Cases		42
Gender		
	Male	27 (64)
	Female	15 (36)
Age (yr)		
	Median	59
	Range	37-79
Histopathology		
	Adenocarcinoma	25 (59)
	Squamous cell carcinoma	4 (10)
	Large cell	9 (21)
	Mixed / Other	4 (10)
Tumor grade		
	1	5 (12)
	2	14 (33)
	3	20 (48)
	4	1 (2)
	unspecified	2 (5)
Tumor stage	IV	42 (100)

CHARACTERISTICS	METASTASES	N (%)
All Cases		54
Gender		
	Male	35 (65)
	Female	19 (35)
Age (yr)		
	Median	60
	Range	36-79
Histopathology (of primary tumor)		
	Adenocarcinoma	35 (65)
	Squamous cell carcinoma	9 (17)
	Large cell	6 (11)
	Mixed / Other	4 (7)
Tumor grade (of primary)		
	1	5 (9)
	2	20 (37)
	3	25 (46)
	4	1 (2)
	unspecified	3 (6)
Type of metastasis		
	Brain	48 (89)
	Lymph node	1 (2)
	Other	5 (9)

Table 2. Patients' characteristics (samples of primary NSCLC and NSCLC metastases located on tissue microarrays).

12.2 Immunohistochemistry

Immunohistochemistry (IHC) was utilized for the assessment of TMA samples, and complete tissue sections as well. The following antibodies were used to stain tissue samples, after careful deparaffinization and antigen recovery: First, a PCK1 mouse monoclonal antibody (H00005105-M01, Abnova, Taipei City, Taiwan) diluted 1:50; second, a PCK2 rabbit polyclonal antibody (ab137580, Abcam, Cambridge, UK) diluted 1:200; third, an LDHB rabbit monoclonal antibody (EP1565Y, Abcam) diluted 1:800, and fourth, a GLUT1 rabbit polyclonal antibody (ab15309, Abcam) diluted 1:100. Simultaneously, we used isotype antibodies, serving as negative controls. The method of stable short hairpin ribonucleic acid (shRNA) induced silencing in cells, embodied in paraffin-cytoblocks, was utilized to test PCK2 antibody specificity (Figure 3 A). As a positive control for the PCK1 antibody, we used human liver samples (Figure 3 B). A pulmonary pathologist (LB) analyzed the slides, and had been blinded regarding patient group allocation, as well as patients' outcome. The arbitrary score we have used to define IHC staining intensity was: negative (0), weak (1), moderate (2) or strong (3).

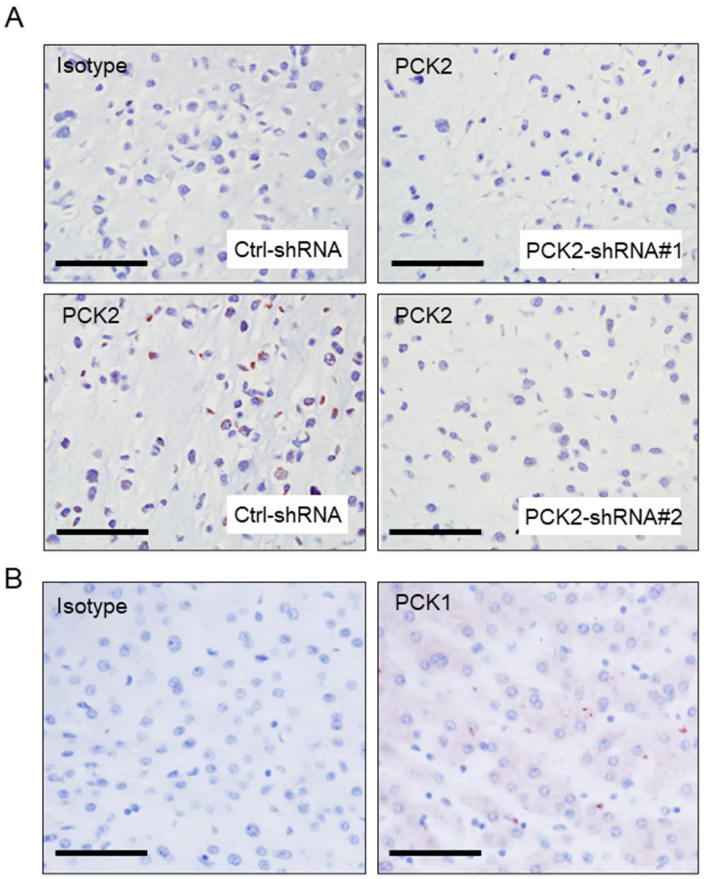

Figure 3. Control samples of positive and negative immunohistochemical staining for PCK1 and PCK2. A: Control- versus PCK2-shRNAs: Specificity of the staining is shown by silencing of PCK2 B: PCK1 staining versus the isotype control.

The percentage of positively stained cancer cells (range: 0-100%), defined IHC density. Consecutively, we calculated a score, the score being the product of staining intensity and density, ultimately adding up to a range of 0-300. In many cases, >1 core was obtained per patient. In this case, the arithmetic mean score of all cores from a single tumor was calculated. We defined a combined IHC score ≥1 as being the cutoff for “positive” versus “negative” IHC staining.

12.3 Survival analysis

The Social Insurance Agency of Austria provided the survival data of patients. Alternatively, we retrieved the date of death from a patient’s medical record, if available. The time period defined as “survival” was defined as the time between the pathological tumor diagnosis and the date of death, or last follow-up. We did not include patients who died within < 1 month post surgery into our analysis. Kaplan Meier curves were computed for PCK1, PCK2, LDHB and GLUT1, respectively, to evaluate overall survival. The cutoff for “present” or “absent” staining was an IHC combined score ≥1, as previously described. The multivariate analysis contained the following variables in addition: tumor grade, gender, patient’s age, and the tumor stage (6th TNM classification, valid at the time of diagnosis). In the TCGA cohort of lung AC specimens, we defined the median value of expression as the cutoff.

12.4 CellProfiler analysis

The Biobank of the Medical University of Graz provided us with FFPR samples of NSCLC, which were stained with the PCK2 antibody, using the same protocol as for the TMAs. Next, the CellProfiler software, version 3.1.8 was used for the evaluation of intratumoral heterogeneity (159, 160). Semi-automated analysis was performed to differentiate aberrations in the PCK2 staining pattern between the tumor margin and -core. We defined the thickness of the invasive margin of each tumor as being 1 mm, and we outlined the border of each tumor margin by histologic features. Certain parts of the tumor that were directly adjacent to the technical tumor margins (e.g. as a result from cuts), were manually eliminated from the CellProfiler analysis, because in these parts of the tumor it was not possible to accurately distinguish the margin from the tumor center. Consequentially, we did not include areas of the tumor harboring large blood vessels, bronchi and/or glandulae bronchiales, or histo-technical artefacts into the analysis. Exclusion of macrophages was realized by identification based on their high and intense IHC staining. Next, we utilized pre-defined intensity thresholds to outline

tumor portions of PCK2 (IHC)- and nuclear (hematoxylin/eosin, HE) staining. Consecutively, the overall staining intensity was quantified for the tumor margin and -center, respectively. We performed normalization of the tumor portion with PCK2 staining to the area of nuclei, by computing the ratio of area of PCK2 staining to area of HE staining. To directly compare the tumor margin to the tumor core, the ratio of area of PCK2 staining to area of HE staining was furthermore normalized to the tumor center. We were able to carry out semi-automated data analysis in 24/29 slides. Five sections needed to be eliminated from the analysis, because the tumor size was very small, or else, because identification of the tumor margin was not possible.

12.5 Cell lines

We obtained the human NSCLC cell line NCI-H23 (H23, ATCC number CRL-5800) from the American Type Culture Collection (ATCC, Manassas, VA). The human NSCLC cell line A549 (Cat. No. 300114), we acquired from Cell Lines Service (Eppelheim, Germany). The NCI-H460 cells (H460) were a kind gift from Martin P. Barr, Institute of Molecular Medicine, St. James's Hospital and Trinity College Dublin, Dublin, Ireland, to AH. We cultured H23 cells in RPMI 1640 medium, and then added 10% fetal calf serum (FCS, Biowest Nuaillé, France) and antibiotic agents for supplementation. A549 cells were put into DMEM-F12 medium (Gibco, Waltham, MA), and in addition, we added 10% FCS (Biowest), and antibiotics as well. Every three months, mycoplasma test was carried out. Cell line authentication was realized by Short Tandem Repeat (STR) analysis using the PowerPlex 16HS System (Promega, Madison, WI), for all above-mentioned cell lines.

12.6 Low glucose and hypoxia treatment

6-well plates at 150,000 cells per each well were used to plate the cells, and we let them attach over a time period of 24 hours. We washed them twice utilizing phosphate buffered saline, and after that, incubated them for 48 or 96 hours in a DMEM medium (Gibco), that was glucose-, glutamine- and serum-free. Next, the DMEM medium was enriched with varying glucose amounts, and with 2 mM glutamine. Cells were put both in ambient oxygen (21%), or in 1% oxygen, using the automated Xvivo System G300CL (BioSpherix, Lacona, NY). Media exchange by a fresh and pre-equilibrated media was done after 48 hours. We carefully prevented exposure of the low-oxygen cells to ambient oxygen through the whole course of the experiment.

12.7 Western blot

RIPA buffer (Sigma-Aldrich, St. Louis, MO), supplemented with protease inhibitors, was used for cell lysis on ice. Protein extraction was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Mini-PROTEAN® electrophoresis unit (BioRad, Hercules, CA). Next, proteins were transported to a polyvinylidene fluoride (PVDF) membrane (BioRad). A rabbit monoclonal antibody to PCK2 (ab187145, Abcam, diluted 1:2,000), a mouse monoclonal antibody to β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:3000) or a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody which is linked to peroxidase (Santa Cruz, 1:10,000), were applied to carry out immunodetection. Secondary, peroxidase-associated antibodies were utilized at a dilution of 1:3,000. By chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific), peroxidase activity was measured.

12.8 Quantitative real-time PCR

The peqGOLD Total RNA Kit (VWR, Vienna, Austria) was used to extract total RNA. Next, we applied the qScript complementary deoxyribonucleic acid (cDNA) synthesis kit (Quantabio, Beverly, MA) for reverse transcription of total RNA (1 µg) in a volume of 20 µl. We carried out real-time PCR (rtPCR) by means of the LightCycler 480 (Roche, Vienna, Austria) and the QuantiFast SYBR PCR kit (Qiagen, Hilden, Germany). We applied the following primers: 5´-CATCCGAAAGCTCCCCAAGTA-3´ (forward strand) and 5´-TGGAAATCAGCTGGGGACATC-3´ (reverse strand) for human PCK2; 5´-AGACGAGAGTTTCCTGGTCTCA -3´ (forward strand) and 5´-TTCCGGATCAGAGCCACAAC-3´ (reverse strand) for HK2, 5´-TGGCATCAACGCTGTCTTCT -3´ (forward strand) and 5´- AGCCAATGGTGGCATACACA -3´ (reverse strand) for HK2; 5´- CTACCACATCCAAGGAAGCA-3´ (forward strand) and 5´-TTTTTCGTCACTACCTCCCCG-3´ (reverse strand) for 18S ribosomal ribonucleic acid (rRNA). As a reference gene, 18S rRNA was used, because it was stably expressed. By substraction of the Cp number of the gene of interest from that of the 18S rRNA, ΔCp was computed. Next, we subtracted ΔΔCp-values of the reference group from ΔCp-values of the intervention group. Consecutively, we calculated the gene expression as $2^{\Delta\Delta Cp}$.

12.9 Antibody specificity control by shRNA mediated silencing

We transfected H23 cells with a subset of commercially available shRNA plasmids, that target PCK2, or with a non-silencing reference shRNA (Qiagen, Hilden, Germany), to achieve stable expression of PCK2 shRNA or non-silencing shRNA. Next, we performed a selection with 1 µg/ml puromycin (Sigma-Aldrich), following the manufacturer's instruction (73). Monoclonal subcultures, expressing two different PCK2 shRNA fabrics (#1 and #2), or else, reference shRNA, were engineered. We used puromycin for the cultivation of cells, aiming for the maintenance concentration of 0.5µg/ml. Puromycin was left out when performing the experiment. Western blot and quantitative PCR (qPCR) served as our tools to control, whether silencing had been effective (73). As the next step, we created cytoblocks of cells cultivated for 8 hours upon low glucose conditions (0.2 mM), that contained serum-free RPMI medium, utilizing human plasma. Calcium was added to induce clotting, and cytoblocks were consecutively fixed in 4% formaldehyde, embedded in paraffin, and stained with either the PCK2- or the isotype antibody. Figure 3 illustrates this experiment.

12.10 In silico gene expression analysis

Gene expression data of PCK1, PCK2, GLUT1 and LDHB and mutation data was retrieved from the TCGA database of AC samples using the UCSC Xena platform (https://xenabrowser.net/). TCGA gene expression data was generated by the TCGA Research Network (https://www.cancer.gov/tcga) or via cbioportal (https://www.cbioportal.org/) (162). Additionally, gene expression data from AC and paired non-involved lung tissue (GSE10072) (163) was analyzed using the GEO (Gene Expression Omnibus) platform (http://ncbi.nlm.nih.gov/geo).

12.11 Statistical analysis

We used the software package SPSS, version 25.0 (Chicago, IL), for data calculation and compiling. Since in the pre-existing literature, no standard deviation of the expression of the markers analyzed had been described in NSCLC or NSCLC metastases until today, we did not conduct a sample size calculation in this explorative analysis. By means of the Mann Whitney-U test, Spearman correlation analysis, two-sided, unpaired Student's t-test or one-group Student's t-test, differences between groups were calculated. These statistical tests were used as applicable. We considered P-values smaller than 0.05 as being significant.

13. Results

13.1 Validation of antibodies

First, we set out to establish and validate immunohistochemistry for PCK1 and PCK2, LDHB and GLUT1. NSCLC and normal lung, as well as paraffin-embedded normal liver were used. In all cases, isotype antibodies used at the same concentration as the primary antibody served as negative controls. We found intense, mostly membranous GLUT1 staining in lung cancer cells, and, as expected in erythrocytes. No staining in the stroma or in normal lung was observed. LDHB staining was found to be cytoplasmic, and present, as expected, in cancer cells as well, no background staining ocurred. Specificity of the PCK2 antibody was validated using stable short hairpin ribonucleic acid (shRNA) mediated silencing in cells, by means of paraffin-cytoblocks (Figure 3 A). For the validation of the PCK1 antibody, we used two different antibodies. One of these turned out to be not suitable for immunohistochemistry (poor staining on human liver, and lots of background staining; data not shown). The PCK1 antibody we finally utilized (see methods section) showed strong staining of hepatocytes, and only minimal background staining.

13.2 Gluconeogenesis is more frequent in AC than in SCC

The IHC expression of the gluconeogenic enzymes PCK1 and PCK2, as well as those of the glucose transporter primarily involved in the glycolysis pathway (GLUT1), was evaluated in specimens of primary NSCLC tumors, and the expression pattern was compared between AC and SCC. The expression of LDHB, mediating the interconversion of pyruvate as well as lactate, thereby being a crucial contributor to both pathways, was simultaneously assessed. GLUT1 showed a distinct location at cell membranes, but PCK1, PCK2 and LDHB were located in the intracellular area. Figure 4A shows the details of this analysis.

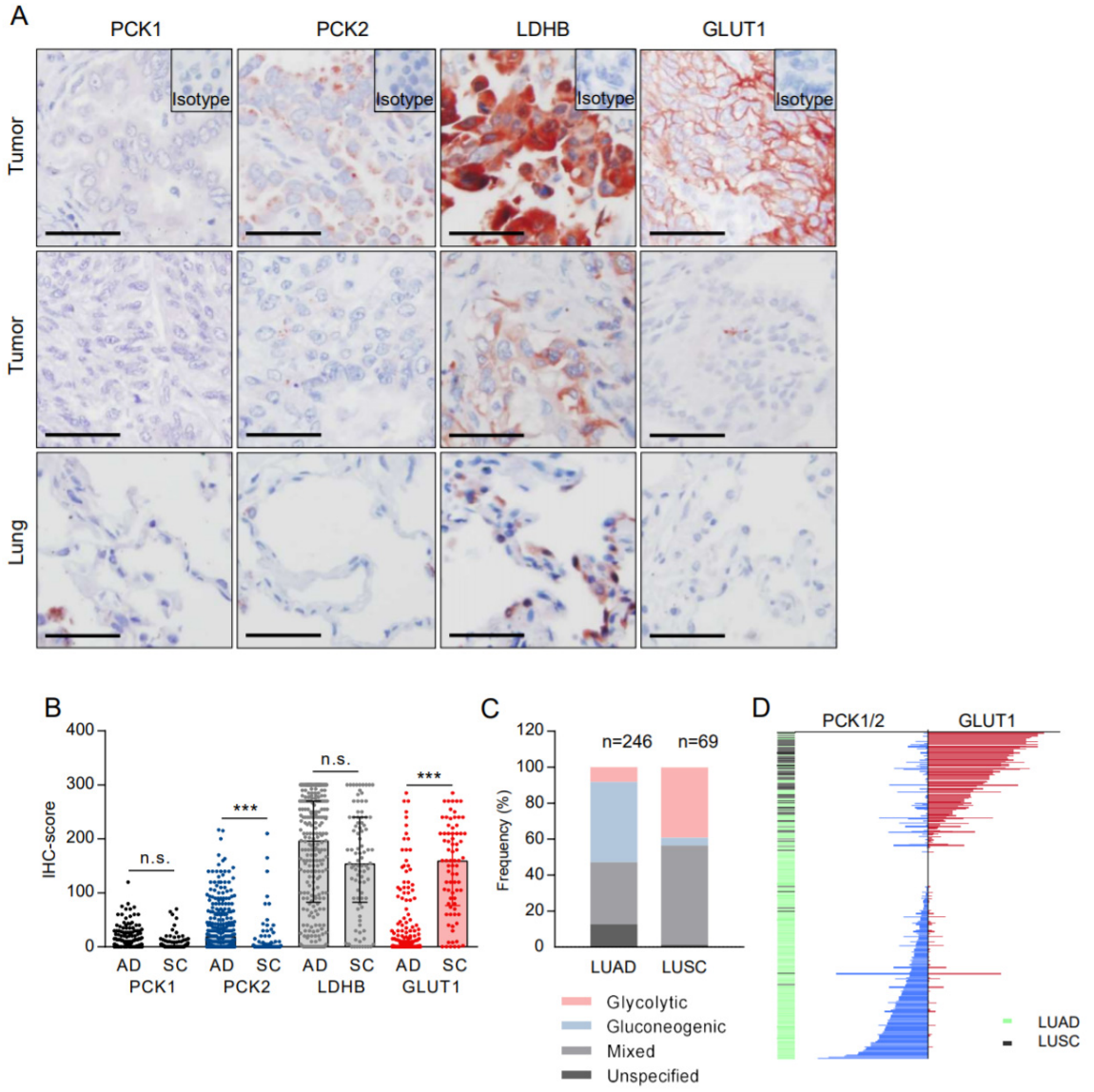

Figure 4. Markes of glycolysis and gluconeogenesis in NSCLC differ in relation to histological subtype and tumor size (1). (A) Strong or medium-intensity IHC staining (1st row), as opposed to a weak or negative staining (2nd row) for the enzymes PCK1, PCK2, LDHB and GLUT1 in tumors (1st and 2nd row), and healthy tissue (3rd row). Scale bar: 50 µm. (B). IHC scores of adenocarcinomas (AD) and squamous cell carcinomas (SC) are compared. The Mann-Whitney-U-test in AC and SCC patients was used for comparison of the groups, comprising samples with evaluable staining for PCK1 (n = 258/85), PCK2 (n = 264/94), LDHB (n = 257/92) and GLUT1 (n = 276/77). *** $P <$ 0.001. (C) Here, the frequency of the glycolytic (GLUT1 positive, PCK1/2 negative), gluconeogenic (GLUT1 negative, PCK1 or PCK2 positive) and mixed phenotype (GLUT1 positive and PCK1 or PCK2 positive) is illustrated. We defined samples which lacked immunopositivity for PCK1, PCK2 or GLUT1 whatsoever as “unspecified”. LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma. (D) A Waterfall plot visualizes the PCK1/2 IHC combined scores, and GLUT1 scores in a total of 317 NSCLC patients.

AC tumors featured significantly higher expression of PCK2 than SCC specimens. On the contrary, GLUT1 expression was more pronounced in the SCC- than in AC cohort (Figure 4 B). In AC, most of the samples (180; 69%) were positively stained for the gluconeogenic enzyme PCK2, whilst in SCC specimens only 38 (40%) of tumors were positive for PCK2 (Table 3). LDHB, which generally featured strong staining in most of NSCLC samples, was expressed to a similar extent in AC and in SCC. PCK1 staining, however tended to be weak in general (Figure 4 A and B, Table 3).

	AC	**SCC**
PCK1	n=258	n=85
positive	109 (42%)	43 (51%)
negative	149 (58%)	42 (49%)
PCK2	n=264	n=94
positive	180 (69%)	38 (40%)
negative	84 (31%)	56 (60%)
LDHB	n=257	n=92
positive	233 (91%)	84 (91%)
negative	24 (9%)	8 (9%)
GLUT1	n=276	n=77
positive	113 (41%)	72 (94%)
negative	163 (59%)	5 (6%)

Table 3. IHC staining results in primary NSCLC on tissue microarrays.

We additionally calculated Spearman correlations of the assessed markers, separately for both the AC- and the SCC cohort. PCK2 and GLUT1 correlated negatively (r = -0.2, $P < 0.001$) in AC, however not in SCC tumor samples. Conversely, in SCC PCK2 and LDHB (r = 0.33, $P < 0.001$), as well as PCK1 and PCK2 (r = 0.22, $P = 0.043$) showed a significant positive correlation. This indicates that in highly glycolytic tumors PCK2 expression tends to be low. Yet, when tumors were assigned to different metabolic phenotypes on the basis of PCK1, PCK2, and GLUT1 expression, a significant overlap was found (Figure 4 C): A “mixed” phenotype was defined as showing both, expression of GLUT1 as well as either of the two PEPCK isoforms. The mixed phenotype was present in 34.6% of AC and in 55.1% of SCC tumors. Purely glycolytic tumors (GLUT1 positive tumors lacking PCK1 or PCK2) were found in 8.1% and 39.1% in AC and SCC, respectively. Gluconeogenic phenotypes with positive PCK1 or PCK2 expression in the absence of GLUT1 expression were observed in 44.7% (AC) and 4.3% (SCC) of cases (Figure 4 C).

When we analyzed the correlation of glycolytic or gluconeogenic enzyme expression with tumor-related parameters, we found that in AC PCK2 expression decreased with incremental tumor size (r = -0.2, $P = 0.0013$). By contrast, GLUT1 showed a positive correlation with tumor size (r = 0.13, $P = 0.03$) as well as tumor grade (r = 0.38, $P < 0.001$). In SCC, PCK2 and tumor size were also negatively correlated (r = -0.24, $P = 0.02$)). PCK1 was negatively correlated with grade in SCC (r = -0.28, $P = 0.01$). Figure 5 A and Figure 6 show IHC scores in relation to the different T stages in AC and SCC, respectively.

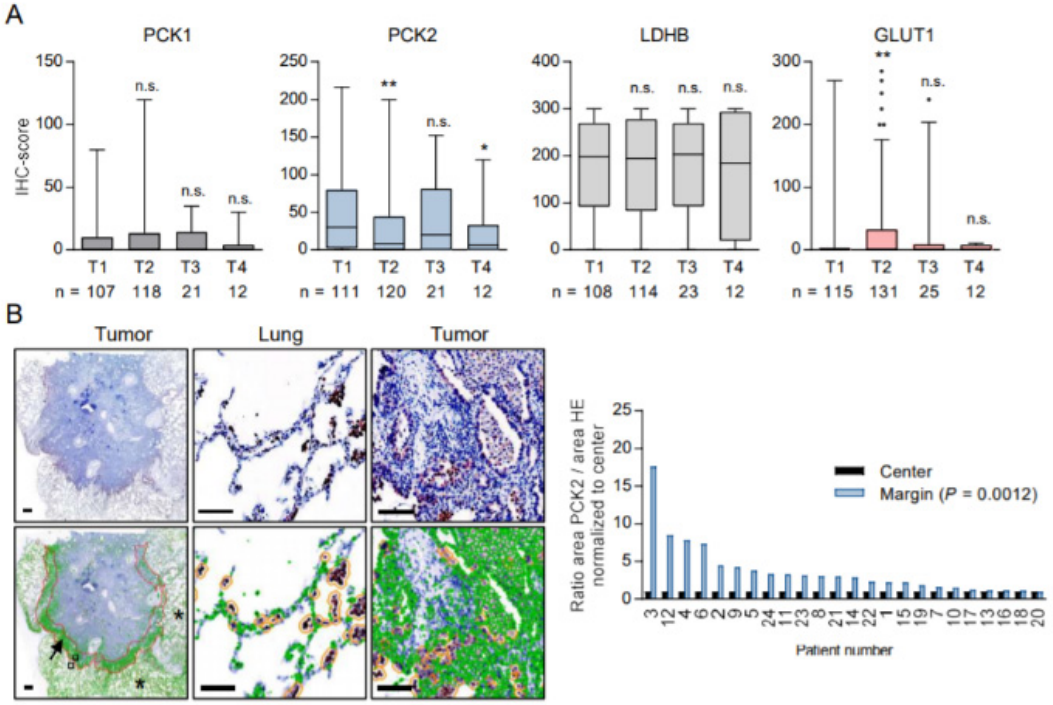

Figure 5. The intratumoral gluconeogenesis gene expression pattern is differential, and depends on tumor size as well (1). (A) The Mann-Whitney-T-test shows aberrant expression of glycolysis and gluconeogenesis genes, with respect to T stage (all T stages versus T1). * $P < 0.05$, ** $P < 0.01$. n.s. not significant; (B) PCK2 expression intensity is compared between the tumor margin (arrow) and the tumor core. Asterisks: indication of normal lung tissue. Scale bar = 100 µm. Automated exclusion of strongly stained macrophages (orange circles) was performed with the implementation of an appropriate cutoff value. Right side: Detailed results of the automated data analysis by the CellProfiler software are illustrated.

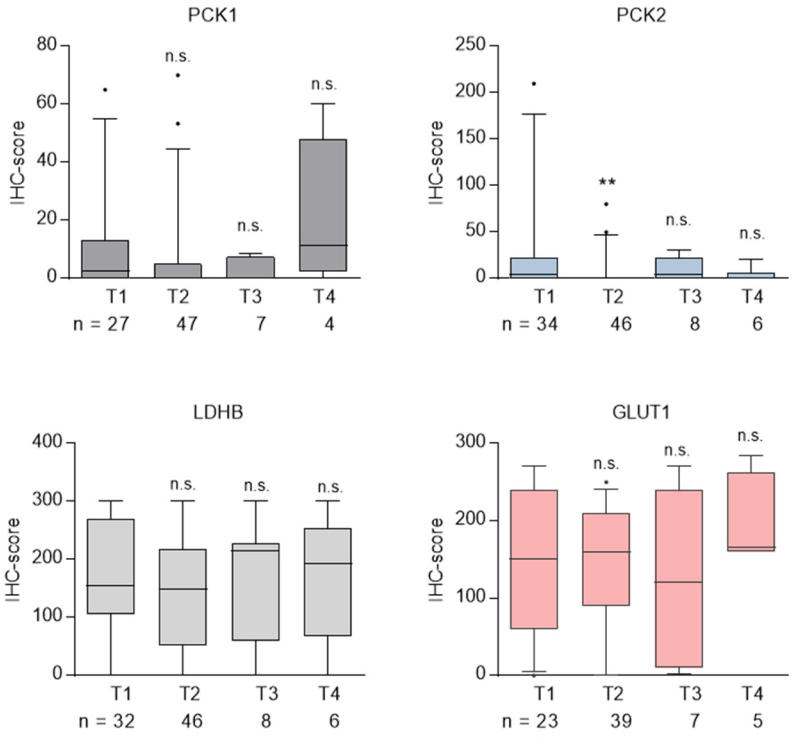

Figure 6. Gluconeogenesis and glycolysis markers are differentially expressed with respect to T-stage in SCC, as shown by IHC staining (1). * $P < 0.05$; ** $P < 0.01$; n.s., not significant.

When searching for a possible effect of the parameters age and gender on enzyme expression profiles, we found age at diagnosis showing a weak negative correlation with GLUT1 expression in AC (r = -0.138, *P* = 0.022). Interestingly, a weak positive correlation of age at diagnosis with PCK2 was outlined (r = 0.125, *P* = 0.042). Noteworthy, within the AC subtype, female gender was significantly associated with a higher PCK2 IHC score (the median score being 25, as compared to a median score of 10 in males, *P* = 0.017 on Mann Whitney U test), while the opposite relation was found for GLUT1 (*P* = 0.017).

13.3 PCK2 expression preferentially occurs at tumor margins

FFPE specimens of 24 whole NSCLC slides (AC: 10; SCC: 12; mixed/other histology: 7) were evaluated to find out, whether gluconeogenic and glycolytic cancer cells are located differently, with respect to the tumor margin, or -center. Hence, we perfomed a semi-automated data analysis, comparing the expression of PCK2 between the invasive tumor margin, as opposed to the tumor core. Intratumoral and alveolar macrophages featured a very strong staining in general, and had to be elimited from this analysis. We outlined a distinctly enhanced PCK2 staining in the tumor margin regions, as opposed to the tumor center (*P* = 0.0012; Figure 5 B). This finding was in line with our observations, made by visual inspection. Of note, we did not outline significant differences in GLUT1 or LDHB staining between the tumor core and -periphery, which was confirmed by the pathologist who carried out this analysis. PCK1 staining was generally weak in this relatively small cohort of patients, and a possible differential expression between the core-, as opposed to the margin regions could not be observed.

13.4 PCK2 and GLUT1 are differentially expressed in NSCLC and nonneoplastic lung tissue

In normal lung tissue, a weak to moderate staining for PCK2 was found in bronchial epithelial cells, but not in alveolar epithelial cells, although type II alveolar epithelial cells were stained positive on occasion (Figure 4 A). Alveolar macrophages generally featured a strong PCK2 staining and partially also PCK1 staining (Figure 4 A and Figure 5 B), while lymphocytes were basically negative. LDHB expression was abundantly present in alveolar and bronchial epithelial cells. By contrast, PCK1 staining was largely absent in the normal lung. In case of GLUT1, in healthy lung tissue positive staining was observed only in erythrocytes, while bronchial epithelial cells, healthy pneumocytes, endothelial cells, smooth muscle cells and macrophages were all negatively stained. A quantitative comparison of gluconeogenic and glycolytic enzyme expression in normal and cancerous lung tissue was precluded by the vast

expression of PCK2 in lung alveolar macrophages, which are present in highly variable amounts in lung specimens, and by the very heterogenous PCK2 expression in the different cell types (bronchial versus alveolar epithelial cells). Hence, we assessed mRNA abundance in the public AC dataset, GEO GSE10072 (161). We found a downregulation of PCK2 and an upregulation of LDHB and GLUT1 in the cancerous tissue when compared to normal lung tissue. Similar findings were obtained using the TCGA AC and TCGA SCC datasets (Figure 7).

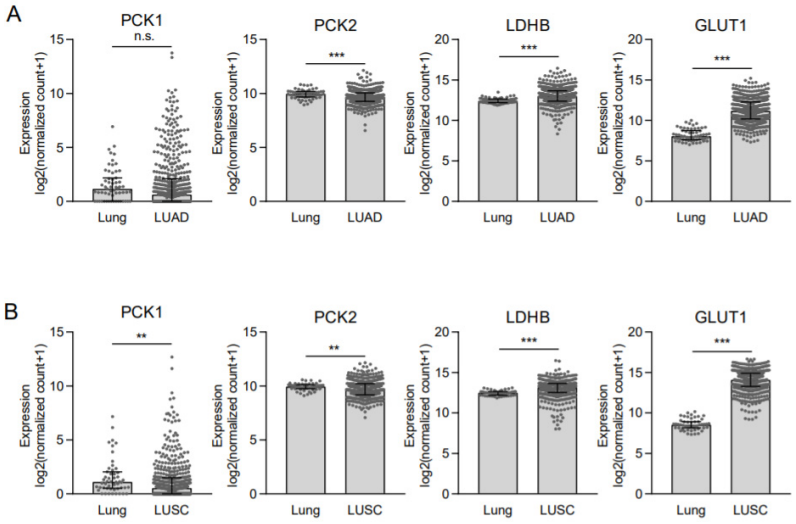

Figure 7. Gluconeogenesis and glycolysis enzyme expression in malignant tissue, as opposed to non-tumorous lung tissue from NSCLC TCGA datasets. A: For LDHB, expression levels were significantly higher in LUAD samples than in the normal lung. The same holds true for GLUT1, while for PCK1 no such association could be outlined. PCK2 was downregulated in LUAD tumor samples, as compared to normal lung. B: In the LUSC cohort, enzyme expression levels of PCK1, LDHB and GLUT1 were all significantly elevated as compared to healthy lung tissue. PCK2, however, was found to be downregulated in LUSC samples.

In SCC a lower PCK1 expression in tumor specimens, as compared to normal lung tissue was observed (Figure 7 B). While the observed reduced PCK2 expression in NSCLC compared to normal lung is in contrast to the findings on mRNA levels and PEPCK activity in a smaller, previously published cohort (71), the observed up-regulation of glycolytic markers in NSCLC is in line with published data (149).

13.5 The gluconeogenic phenotype and negative expression of GLUT1 are associated with a favorable outcome in AC

Since the above-mentioned data show a considerable inter-patient variability of gluconeogenic and glycolytic gene expression in NSCLC, our next step was to analyze their association with outcome. Overall survival, as shown by means of Kaplan Meier graphs (Figure 8), was significantly longer in AC patients whose tumors featured positive PCK2 staining. Conversely, in AC patients whose tumors were positive for GLUT1, the overall survival was significantly shorter (Figure 8).

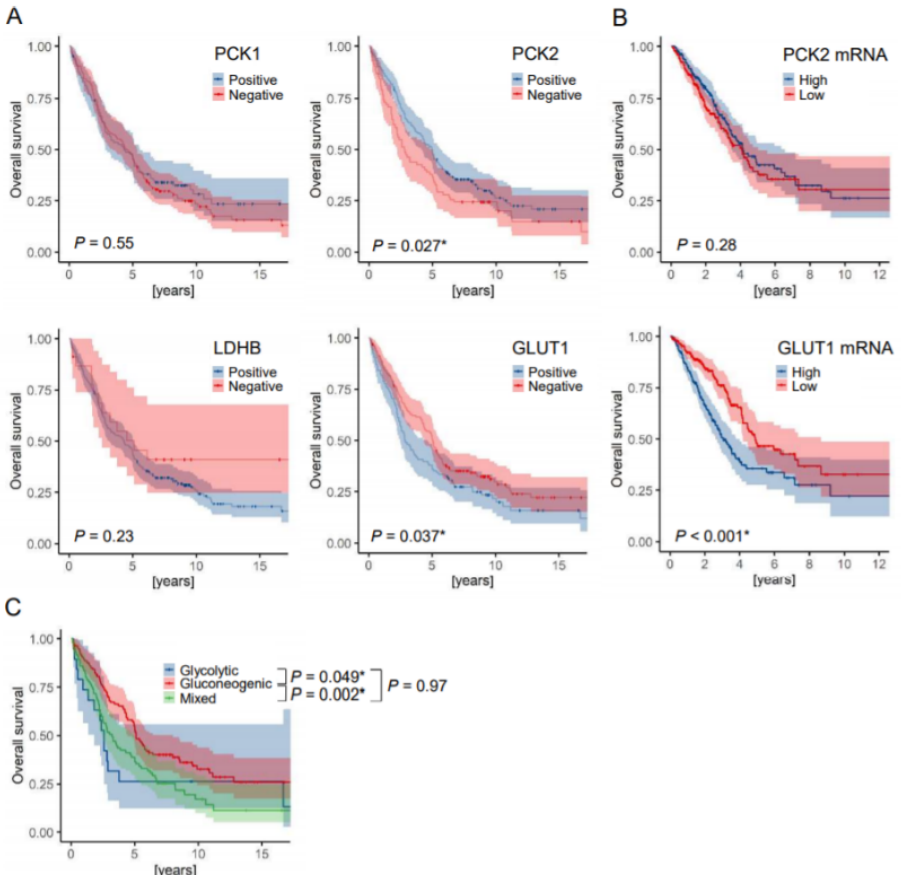

Figure 8. Overall survival and its association with gluconeogenic enzyme expression in lung AC (1). (A) The Kaplan Meier curves with a +/- 95% confidence interval for PCK1, PCK2, LDHB and GLUT1 positive and negative AC are illustrated here (cutoff = 1). (B) Here, the Kaplan Meier estimators for openly accessible TCGA AC data for PCK2 and GLUT1 mRNA, respectively, is shown (n=491; data was retrieved via the University of California, Santa Cruz (UCSC) Xena platform, https://xenabrowser.net/). We used median values as a cutoff. (C) The overall survival in AC patients, with respect to the metabolic phenotype, is pictured here. (A-C) The Logrank test was utilized for the calculation of differences in survival.

Similarly to our data, we found a significant association of GLUT1 expression with a poor outcome overall in the data drawn from the TCGA AC cohort (n=491). Yet, the association was not significant in the case of PCK2. When we analyzed the patient's survival times with respect to the different metabolic phenotypes, we found that in AC, glycolytic as well as mixed tumors were linked to a significantly shorter overall survival, as compared to gluconeogenic cancers (Figure 8 C). However, when comparing glycolytic and gluconeogenic AC specimens, we did not find any significant differences (Figure 8 C). Survival times were not, to any degree, influenced by any marker we analyzed, nor by the metabolic phenotype, in SCC samples (data not pictured). Still, it has to be kept in mind that the sample size was much smaller in the SCC cohort (see Table 1). PCK2 expression was linked to a significantly more favorable survival, according to the multivariate analysis as well, which comprised age, gender, as well as tumor stage and -grade (Table 4).

IHC SCORE	RELATIVE RISK	95% CI	*P*
G	1.27	1.07-1.52	0.008*
T	1.25	1.05-1.49	0.011*
N	1.47	1.22-1.77	< 0.001*
M	1.4	0.67-2.91	0.37
Gender (f. v.s. m.)	0.58	0.44-0.77	< 0.001*
Age	1.02	1.002-1.03	0.021*
PCK1 (score)	1.002	0.99-1.01	0.58
PCK2 (score)	0.995	0.992-0.999	0.006*
LDHB (score)	1.001	0.9992-1.002	0.42
GLUT1 (score)	0.9996	0.997-1.003	0.78

G, tumor grade; *significant at $P<0.05$

Table 4. Multivariate survival analysis in lung AC.

13.6 Gluconeogenesis is enhanced in NSCLC metastases as compared to primary tumors

Next, we wanted to explore, whether differences in glycolytic and gluconeogenic enzyme expression profiles between NSCLC primary tumors and metastases occur. To address this issue, we stained two additional TMAs representing 54 samples of AC and SCC metastases. Most metastatic samples stemmed from the brain (n=48) and to a lesser extent from other anatomic sites, including lymph node (n=1) or others (n=5), as well as non-corresponding NSCLC primary tumors from stage IV (metastatic) patients (n=41). The high rate of brain metastases is caused by the fact that NSCLC metastases are rarely treated by surgery, with

the exception of brain metastases. No significant differences in IHC expression between primaries and metastases were observed in the separate analysis of PCK1, PCK2, LDHB and GLUT1. However, the combined score of PCK1 and PCK2 was significantly higher in the metastasis cohort (P = 0.01, Figure 9 A). When the different phenotypes were determined based on expression levels of PCK1, PCK2, and GLUT1, an increase in the gluconeogenic and mixed phenotypes was found in the metastases (Figure 9 B).

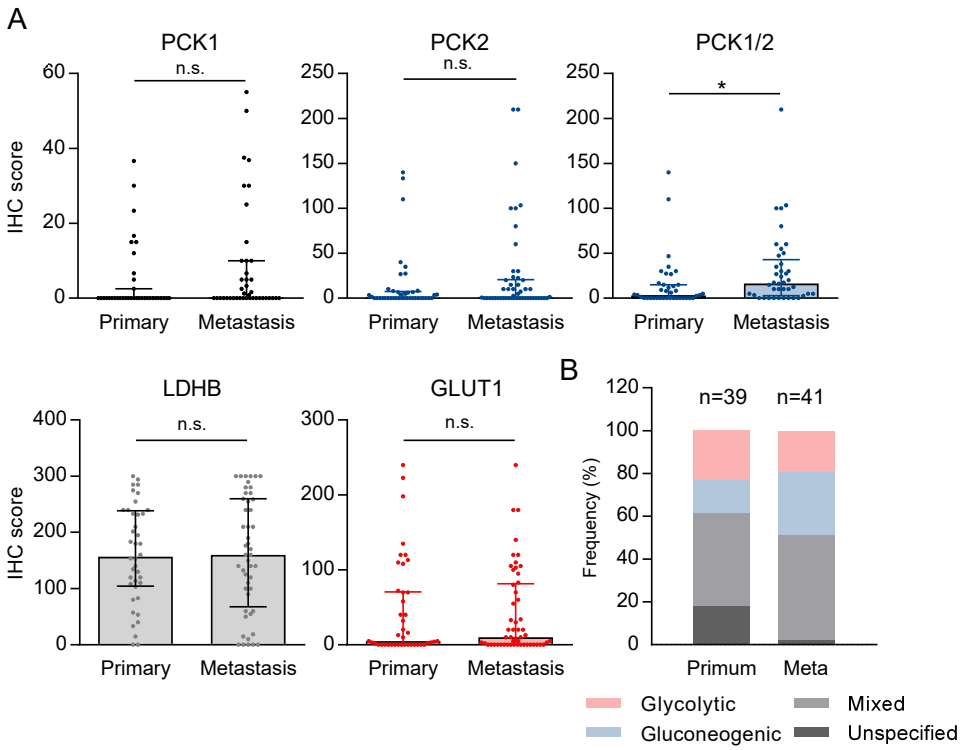

Figure 9. Primary tumors versus metastases, and expression of gluconeogenesis and glycolysis enzymes, respectively (1). (A) We evaluated in total 42 primary tumor specimens, stemming from metastasis patients, and 54 NSCLC metastasis samples. IHC expression did not significantly differ between the primary- and metastasis cohort, when we analyzed PCK1, PCK2, LDHB and GLUT1 separately. However, when combining the scores of PCK1 and PCK2 (meaning the sum of both scores), a significantly higher expression in the metastases was observed. We carried out a group comparison using the Mann-Whitney-U-test in primary tumors and metastases as well, in samples with evaluable PCK1 (n = 39/41), PCK2 (n = 41/50), LDHB (n = 40/48), GLUT1 staining (n = 42/53), or PCK1/2 combined scores (n = 39/41). * $P < 0.05$. (B) Here, the allocation of the glycolytic, mixed, or gluconeogenic phenotypes are shown. We termed those specimens, which were immunohistochemically negative for PCK1, PCK2 or GLUT1 “unspecified”.

13.7 PCK2 expression is decreased in KRAS mutated NSCLC, and GLUT1 is over-expressed in TP53 mutated tumors

Glycolysis is well known to be enhanced via growth-promoting signaling pathways in order to enable biomass production (162, 163). However, only little is known about the impact the activation of oncogenes or the inactivation of tumor suppressor genes exerts on the gluconeogenesis pathway. In the AC TCGA cohort, KRAS mutant tumors displayed a significantly reduced PCK2 expression, while GLUT1 expression was diminished in LKB1 (STK11) mutant tumors but enhanced in TP53 mutant tumors (Figure 10). These data suggest that the mutational background contributes to the determination of distinct metabolic phenotypes.

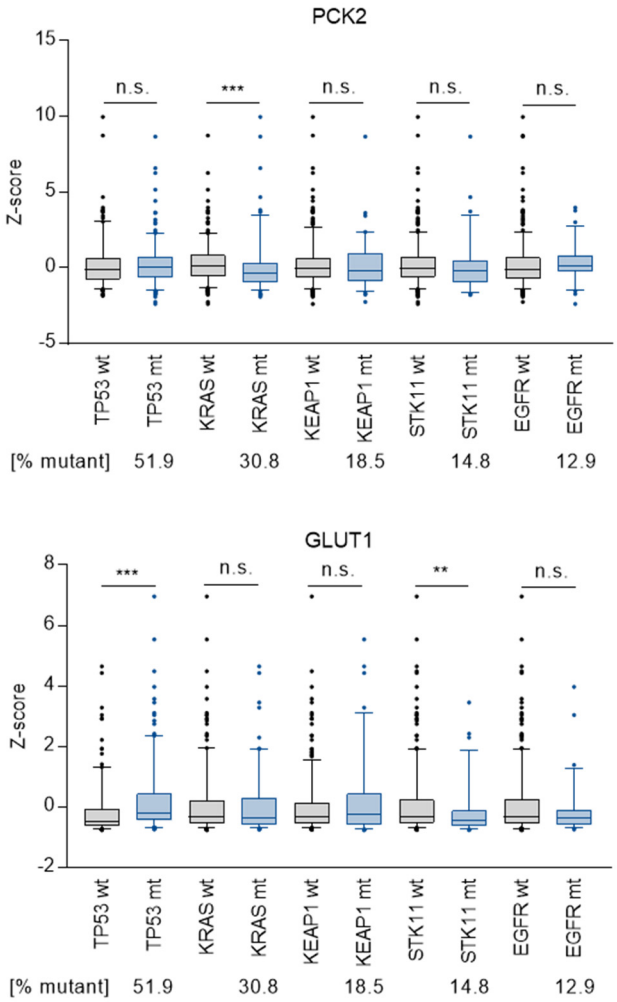

Figure 10. PCK2 and GLUT1 mRNA expression in lung AC featuring mutations of commonly mutated genes versus wild type samples. Data was inferred from the public TCGA database. KRAS mutant tumors featured a significantly lower PCK2 expression when compared with KRAS wild type specimens. GLUT1 expression was significantly elevated in TP53 mutant, as opposed to TP53 wild type samples.

13.8 Hypoxia differentially impacts gluconeogenesis and glycolysis

Tumor cell metabolic cascades are essentially regulated by the metabolic microenvironment, as well as nutrient- and oxygen supply, aside of cell-intrinsic contributing factors (163). Hypoxia is a powerful promoter of glycolysis, first and foremost via the up-regulation of glycolysis genes (163, 164). The impact of hypoxia in NSCLC tumor biology has been described in previous studies (165, 166). Besides, hypoxia reduced the expression of PCK2 in breast cancer cells as well (167), suggestive of an interaction of the gluconeogenesis pathway and hypoxia. Hence, it was our aim to investigate, how exactly hypoxia influences expression profiles of PCK2 and GLUT1 in NSCLC cell lines, and how expression changes with respect to varying glucose levels in the culture medium. Two lung cancer cell lines were cultured in this experiment, H460 and A549, in high glucose (10 mM) or low glucose (1 mM) media, for the evaluation of PCK2 and GLUT1 expression, respectively. We found increased expression of PCK2 upon “light” glucose starvation, as opposed to culture under high glucose conditions. PCK2 was strikingly downregulated by hypoxia in H460 cells, as compared to normoxic conditions, both in the high- and low glucose media (Figure 11 A).

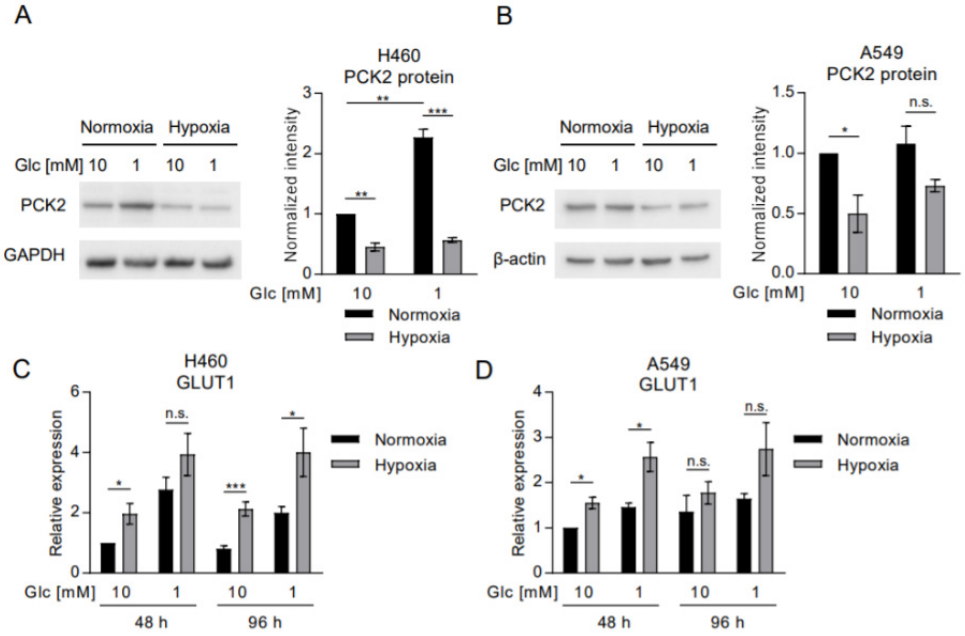

Figure 11. Hypoxia regulates the expression pattern of PCK2 and GLUT1 in lung cancer cell lines (1). In H460 (A) and A549 (B) lung cancer cell lines, expression profiles of the PCK2 protein are shown, either upon culture in a high glucose (10 mM) or low glucose (1 mM) medium, under normoxic and hypoxic (1% oxygen) conditions, respectively. Beta-actin and GAPDH served as loading controls. We normalized the integrated density to the first sample, as well as the loading control (C and D). Quantitative PCR was used for the evaluation of GLUT1 expression in H460 (C) and A549 (D) cells. The findings, displayed as mean +/- SEM, were obtained in four (A, B), six (C) or five (D)

independent experiments. We used the Student's t-test, or one-group Student's t-test, as appropriate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
Hypoxia experiments were performed by Katharina Leithner and by members of her research group.

In the A549 cells (Figure 11 B), analogical findings were observed. Noteworthy, mRNA expression of PCK2 was only insignificantly impacted by hypoxia (Figure 12), suggestive of a possible mechanism occurring in the post-translational phase. A similar post-translational mechanism had been described in the existing literature for PCK1 already (168), however, has not yet been investigated for the regulation of PCK2. On the contrary, GLUT1 mRNA levels, and hexokinase 2 (HK2) mRNA levels as well, both known as hypoxia-regulating glycolysis genes, were found to be strikingly elevated upon hypoxia, which is in line with data from the literature (163, 164). PCK1 mRNA was detected only at low levels in the two cell lines.

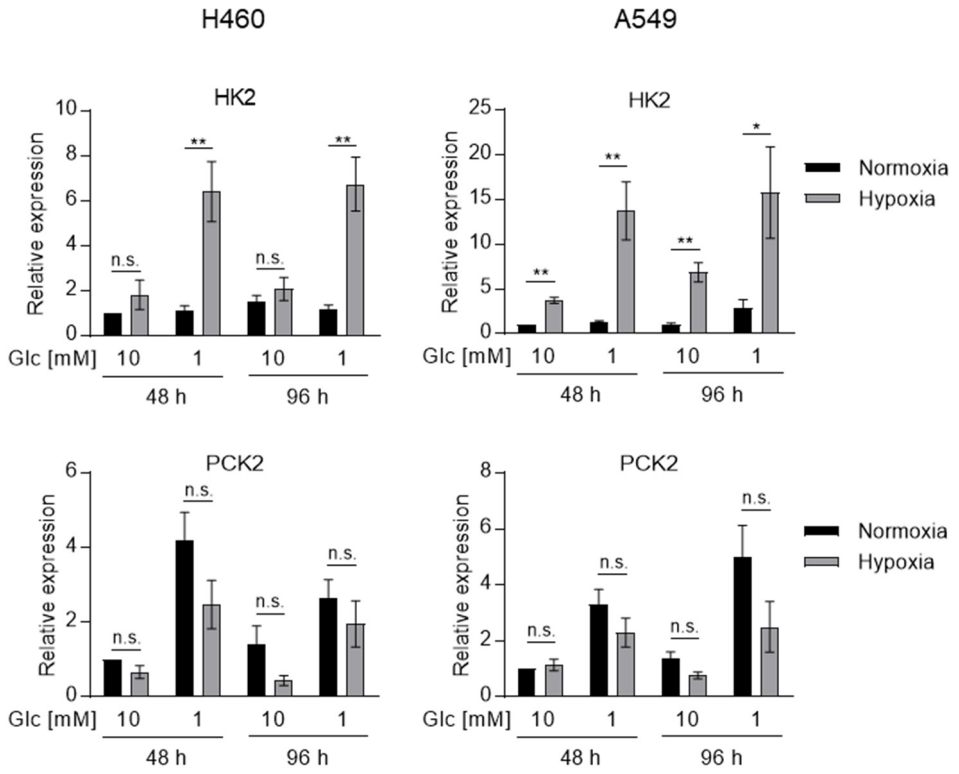

Figure 12. PCK2 and HK2 mRNA abundance in NSCLC cell lines is differential, depending on hypoxic versus normoxic conditions. For the H460 cells, HK2 expression was significantly elevated upon glucose starvation and hypoxia. A trend towards a higher HK2 expression under hypoxic conditions was also observed for the high-glucose medium, albeit insignificant. In the A549 cells, HK2 was also elevated upon hypoxia, which holds true for both low- and high-glucose conditions. The same experiments were carried out for PCK2, however, no significant observations were made.
Hypoxia experiments were performed by Katharina Leithner and by members of her research group.

14. Discussion

With this study, we aim to shed light on the intra- and intertumoral heterogeneity of glycolytic and gluconeogenic gene expression in primary NSCLC and NSCLC metastases. Our results suggest that most NSCLC samples show a mixed glycolytic and gluconeogenic phenotype and that the gluconeogenic phenotype is enhanced in metastases compared to primary tumors. Moreover, we found that oxygen availability contributes to the switch between these two metabolic states.

14.1 Hypoxia

In our study we found that chronic hypoxia reduces PCK2 expression while it enhances GLUT1 expression in NSCLC cells *in vitro*. Thus, low perfusion and the presence of hypoxia could partially serve as an explanation for the abundant GLUT1 expression in SCC compared to AC. In a high proportion of AC and SCC, both GLUT1 positive tumor cells and PCK1 or PCK2 expressing tumor cells co-exist. In some samples of AC and SCC, however, neither GLUT1, nor PCK1 or PCK2 expression was present. In these tumors, expression of either of the enzymes was probably below detection limits. We also cannot exclude the contribution of other GLUT isoforms to glucose transport.

In our study we analyzed the spatial distribution of both phenotypes in NSCLC by analyzing the abundance of gluconeogenesis and/or glycolysis markers in sections of complete tumors and performed an automated data analysis. Our findings revealed an unexpected enhancement of PCK2 on the tumor margins, compared to tumor centers, while for GLUT1 no preference could be found in our cohort. Since the tumor center has been shown to be more hypoxic than the tumor margin (169), central hypoxia might contribute to the decrease in PCK2 expression in this compartment, as suggested by our *in vitro* data.

As we have already written explicitly in the introduction part, hypoxia and glucose metabolism are branching into each other, so glucose metabolism upon hypoxia functions differentially, and *vice-versa,* oxygen uptake and distribution is different, depending on the nutrients available. Blood supply and angiogenesis is a third component that cannot be separated when discussing the cancer microenvironment.

Or findings show that, depending on perfusion, as well as nutrient- and oxygen availability, tumors either adopt a predominantly glycolytic, or gluconeogenic phenotype. Most probably, the most efficient way to ensure survival and continuous proliferation would be the flexibility of a given cancer to switch between those two phenotypes, depending on the respective conditions. Instead of going straight into apoptosis, especially aggressive tumors simply adapt their metabolism and cell division, so tumor growth and metastatic spread can continue.

In future, it will hopefully be possible to target the “metabolic flexibility” as such in cancer cells, by inhibiting the enzymes that primarily enable the switch from glycolysis to gluconeogenesis and *vice-versa.*

14.2 Implication of gluconeogenic enzyme expression on prognosis

A strong (*i.e.* positive) PCK2 expression evidently constitutes a favorable prognostic finding in AC, whereas a positive GLUT1 expression is rather linked to a worse outcome, as illustrated by our survival analysis. Two meta-analysis, with each of them including more than twenty studies about solid cancers, have been carried out, and showed that GLUT1 overexpression is significantly linked to an unfavorable overall survival in a variety of tumor specimens (134, 135, 137). The link between PCK2 expression and the patients’ outcome in different tumor entities, however, has only been investigated in few studies until today. In prostate cancer, for example, a high PCK2 expression is a predictor of a worse overall survival (86).
However, enzyme expression might not be a fixed, but rather a time-dependent event in NSCLC, meaning that upon glucose deprivation cancers can switch to a gluconeogenic phenotype, and back to the glycolytic one, as it fits the prevailing availability of glucose (and oxygen). Maybe it is not the expression of certain enzymes, captured as a snapshot, but the potential of flexibly reprogramming energy metabolism, after all.
More research is awaited with interest, with the aim of ultimately targeting unique properties of metabolic rewiring specific for cancers, that are not found in healthy cells, to minimize side effects.

14.3 Primary tumors versus metastasis

Interestingly, the combined PCK1 and PCK2 score was significantly elevated in NSCLC metastases compared to primary cancers, while glycolysis marker GLUT1 showed no differential expression in metastases versus primaries. As a limitation, primary samples from metastatic patients and metastases were derived from different patients and only a subset was corresponding. The low rate of surgeries of NSCLC metastases or biopsies taken from concurrent primaries and metastases precluded the immunohistochemical analysis of a larger number of corresponding samples. In previous studies an elevation of PCK1 or PCK2 was found in metastases of solid tumors compared to primary tumors (for review see (76)). Prostate cancer from metastatic patients showed higher PCK2 expression levels than prostate cancer tissue from non-metastatic patients (86). Accordingly, tumor-initiating cells (TIC) from prostate cancer showed higher PCK2 levels than the parental cells and PCK2 silencing reduced TIC

numbers by affecting levels of ROS and protein acetylation (86). Whether PCK1 and PCK2 play a mechanistic role in metastasis formation in NSCLC, however, remains to be elucidated in future studies.

14.4 The link between oxygen concentration and glucose consumption

According to previous research, a strong link between tumor glucose- / nutrient-metabolism and oxygen availability exists. A very recent study from 2020 reveals the relationship between tumor oxygenation and glucose uptake in pancreatic ductal adenocarcinoma by means of molecular imaging of the tumor microenvironment (170). The authors of this study utilized hyperpolarized magnetic resonance imaging (MRI) as well as electron paramagnetic resonance imaging approaches for the quantitative assessment of tumor glycolysis and oxygenation status, quantitatively. The assessment was performed on pancreatic ductal adenocarcinoma xenografts. Partial pressure of oxygen (pO2), FDG uptake and the lactate / pyruvate ratios for MiaPaCa-2, Hx766t and SU.86.86 xenografts were correlated. The three cell lines, although having a similar genetic background, differed strongly in terms of cancer microenvironment (170). It was found, that tumor oxygenation and lactate / pyruvate levels were inversely correlated, which has also been observed in previous studies (171). FDG uptake was higher when lactate and pyruvate were abundantly available, but inversely associated with pO2. The xenograft with the highest median pO2 value, namely SU.86.86, also showed the most striking overexpression of the angiogenesis biomarker CD31. In the Hs766t cells, showing the lowest pO2 values, expression of HIF1 was most abundant (170). The authors point out, that a considerable intratumoral heterogeneity of metabolic processes was observed, which is in line with our findings of different distribution of oxygen and, probably, glucose, in different tumor areas. Notably, there was a voxel-by-voxel-based positive correlation between pO2 levels and FDG-uptake, illustrating the inaccuracy of FDG-PET imaging (170). However, on larger distance scales, a negative correlation between pO2 and FDG-uptake was observed, and metabolic activity tended to be highest in the midst of the tumor where pO2 levels were lowest. Another interesting finding from this study was, when comparing the tumor margin versus tumor center, that good oxygenation and FDG-uptake negatively correlate in the tumor center, whereas at the margin region, FDG-uptake and pO2 levels showed a positive correlation (170). Using the lactate / pyruvate ratio, the authors of this study also attempted to investigate the relation of LDH activity and hypoxia, or differential LDH distribution in the tumor center versus the margin. However, lactate / pyruvate ratio, *i.e.* LDH activity, was uniformly distributed throughout the whole tumor, suggesting no influence of

hypoxia or normoxia whatsoever (170). Of note, we also observed a very nonspecific and uniform distribution of LDHB across most tumor samples, also suggesting a rather ubiquitous function of this enzyme rather than a specific role in tumor glucose metabolism.

Until today, no single and gold-standard technique for the imaging of hypoxic regions has yet been established (172). Although glucose usage is not a direct marker for hypoxia, both metabolic states share a pathway through HIF activation of the GLUT1 transporter (173). Thus, glucose uptake in the FDG-PET imaging has been proposed as a surrogate marker for hypoxia. Previous pre-clinical *in vivo* imaging studies have also demonstrated the correlation between hypoxia and glucose metabolism (174). Yamamoto and colleagues proposed, that tumor cells in the center are in a quiescent state, and oxidative phosphorylation serves as the main source of energy there, since the demand for metabolic building blocks produced by glycolysis is very low. Conversely, in the more metabolically active regions at the tumor margin, rapid growth stimulates glycolysis, supplying metabolic intermediates for the synthesis of proteins and nucleotides, leading to a positive correlation between FDG-uptake and pO2 (170). Another interesting report has examined the connection between oxygen-dependence and glucose consumption in solid cancers (175). For this purpose, fibrosarcoma (FSA) and rat mammary gland (R23230Ac) tumor xenografts growing in Fischer 344 rats were assessed using bioluminescence imaging, detection of Hoechst 33342 (a fluorescence chemical dye used in fluorescence microscopy for DNA staining; stimulated by ultraviolet light at a wave length of 340 nm, and emitting blue to cyan-dyed light) and immunohistochemical evaluation of the hypoxia marker EF5 [2-8-N-(2,2,3,3,3-pentafluoropropyl)acetamide] were performed in serial tumor samples (175). The researchers also tested for glucose- and lactate levels in the liver and blood. Moreover, cells were tested for glucose consumption and production of lactate *in vitro*. As expected, the hypoxia marker EF5 indicated maximum levels of hypoxia, and the most intense staining occurred in perinecrotic areas of the tumor. Glucose concentrations were highest in the liver, followed by blood and the tumor margin. In vital tumor regions located rather centrally, glucose levels were lower, reaching their lowest levels in near-necrotic areas. Between the mammary tumors and the FSA tumors, considerable differences were found: In R3230Ac tumors, glucose concentrations were consistently elevated in normoxic tumor regions as opposed to hypoxic ones, and maximum glucose levels in the cancer tissue came close to systemic blood levels. On the contrary, in FSA tumors glucose levels were nearby zero, irrespective of the presence or absence of oxygen (175). Lactate concentrations did not differ significantly between the two investigated tumor types. Upon hypoxia, both in R3230Ac tumors, and in FSA, lactate production markedly increased. The authors of this study conclude, that hypoxia triggers increased glycolysis, an observation which is termed after its discoverer, the Pasteur effect (176). However, keeping in mind the Warburg effect, one would probably

assume that also under normoxic conditions, glycolysis is performed at high rates in malignant tissues.

The authors of the above-mentioned analysis also state that aerobic glucose utilization ultimately results in low glucose levels in FSA, and a situation where the limited glucose supply narrows glucose uptake. We might add, that there must be an opportunity for cancer cells to circumvent the glycolysis pathway, going at least through the early steps of gluconeogenesis, and keeping up tumor metabolism, *i.e.* generation of intermediate substances needed for growth and proliferation.

In a study from 2004, the kinetics of pO2 fluctuations in FSA and in 9L rat brain tumors were investigated, upon conventional air and O2 breathing, respectively (177). The authors hypothesized that key factors relating to oxygen tension fluctuations probably vary not only between the two tumor types, but also in relation to the oxygen content of the inhaled gas. Investigation was performed in Fischer 344 rats. Oxygen microelectrodes were inserted into the tumors, which had been transplanted into the subcutis of the rats' hind legs. Consecutively, linear pO2 measurements were performed and linear pO2 measurements were recorded, simultaneously to the assessment of blood pressure measured via femoral artery access. Overall, 11 FSA and 12 9L tumors were investigated in this xenograft model (177). Rats were switched from air to 100% O2 breathing after 45 minutes. FSA tumors showed a higher median pO2 value (4 mmHg), as opposed to 9L tumors (1 mmHg). Pimonidazole staining (as commonly used for the staining of hypoxic regions) patterns in FSA and 9L tumors confirmed this finding. Considerable fluctuations in tumor pO2 over the measuring timer period was observed in both tumor specimens, yet, fluctuations were much greater in 9L tumors than in the FSA tumors. As expected by the researchers, O2 breathing significantly increased median pO2 values in FSA specimens (from 3 to 8 mmHg; $P < 0.005$) and also increased the frequency and magnitude of pO2 fluctuations. Interestingly, in 9L tumors, O2 breathing did not impact tumor pO2 at all (177). The authors conclude that both investigated tumor subtypes differ significantly with regards to spatial and time-dependent oxygenation conditions upon air- and O2 breathing, respectively. Fluctuations of pO2 are assumed to significantly impact response to radio- and probably chemotherapy, and could be a source of genetic instability, augmented angiogenesis and metastatic spread. These findings also highlight the considerable variability of oxygenation status across different tumor specimens, which might translate to considerable differences in tumor metabolism as well, since hypoxia and glucose metabolism in cancers are closely intertwined.

14.5 Glucose dependency in cancer

In recent years, more and more heterogeneities in cancer cell metabolic state, even within different parts of an individual tumor, have been identified (178). To the best of our knowledge, the spatial distribution of gluconeogenesis in solid tumors or its relation to glycolysis have not been studied so far. In this study we utilized gluconeogenesis and glycolysis enzyme expression profiling in a large cohort of NSCLC using immunohistochemistry, partially including also automated data analysis. This allowed metabolic characterization of tumor cells on a single cell level. PCK2 expression was found to be significantly higher in AC samples, as compared to SCC, according to our data. Conversely, for GLUT1, higher average IHC scores were found in SCC samples. The latter finding is in line with previously published data (134). When glucose dependency was assessed in a panel of SCC and AC cell lines, SCC cells displayed a highly glucose dependent metabolic phenotype whereas AC cell lines were rather glucose-independent and insensitive to silencing of GLUT1 (134). *In vivo* glucose uptake was found to be much stronger in SCC than in AC, as measured by ^{18}F-fluorodeoxyglucose uptake (179). Conversely, perfusion (as measured by perfusion MRI) has been shown to be higher in AC than in SCC (180).

The so-called glucose addiction of cancers is a crucial event which may help to find therapeutic strategies targeting cancer metabolism specifically in the near future.

It has previously been proposed that persistent activation of glycolysis creates a state of metabolic acidosis, toxic and potentially lethal for non-malignant cells (via the TP53-dependent apoptosis pathway, triggered by an increase in caspase activity) (181) – yet for cancer cells this metabolic state is not toxic, presumably because of mutations of the TP53 gene or other elements of the apoptosis pathway (182). Tumor cells – as opposed to healthy cells – show maximum growth rates in relatively acidic culture media with a pH of 6.8 (183). In this acidic environment, angiogenesis and the ability of invasion are at its peak. The dependence of cancer cells on glucose (or glycolysis) is also highlighted by the fact that glycolysis inhibitors such as oxymate, dichloroacetate (DCA) and pyruvate dehydrogenase kinase 1 (PDK1) small interfering ribonucleic acid (siRNA) induce apoptosis in cancers such as in multiple myeloma (184). That is, if tumors do not have the ability to circumvent glucose addiction, as we aimed to illustrate with our research.

Some glycolysis inhibitors, *e.g.* DCA combined with bortezomib (Velcade) in multiple myeloma, have already shown clinical effectiveness and synergistic cytotoxic effects (184). Several pathways are intertwined with the shift to enhanced glycolysis, and various oncogenes and transcription factors, *e.g.* C-MYC, RAS, TP53 and HIF1 upregulate elements of the glycolytic pathway (185). The hypoxic microenvironment associated with the preangiogenic phase of tumor development promotes stabilization of HIF1, which in turn endorses the expression of

GLUT1, LDHA, C-MYC and pyruvate dehydrogenase kinase 3 (PDK3), and all these molecules facilitate aerobic glycolysis as well (186). Another mechanism facilitating enhanced glycolytic activity in cancer is a switch to the embryonic isoform of pyruvate kinase, namely PKM2, because this isoform enhances anabolic processes dependent on glycolytic intermediates, facilitating tumor cell proliferation, whilst overexpression of HK2 has a similar effect as well (187). It is the synergism of genetic and epigenetic changes in cancer cells that contribute to the predominantly glycolytic phenotype. Moreover, the progressive development of resistance to chemotherapeutics is in part a consequence of the enhanced glucose catabolism, as it has previously been described in leukemia cells (188). Chemoresistance is also promoted by suppressing the activation of BAX and BAD, both pro-apoptotic signaling effectors that are regulated by glucose (189, 190). However, according to our observation it becomes evident that not only enhanced glycolysis is a prognostically bad event in cancer cells. Hence, if tumor cells become largely independent of glucose this promotes tumor progression and metastasis as well. We assume that it is maybe not so much the metabolism of a given tumor at a given timepoint that renders it so aggressive – rather, it is the general potential and possibility to switch between the glycolytic and gluconeogenic phenotype, adapting to nutrient availability, as a key event for rapid cancer growth and proliferation.

14.6 Gluconeogenesis in cancers – what do we know?

Multiple metabolic pathways including glycolysis, respiration and anabolic biosynthetic cascades are linked via the TCA cycle in cancer cells. Intermediates generated in the TCA cycle are distributed to various pathways, *e.g.* nucleic acid-, amino acid- or fatty acid synthesis. However, upon glucose deprivation, other nutrients that enter the TCA cycle have the potential to feed into these biosynthetic pathways. For the *de novo* synthesis of essential cellular building blocks such as nucleic acids and (glycero-) lipids, however, glycolytic intermediates are a prerequisite (61). These glycolytic intermediates can be obtained by shuttling non-carbohydrate precursors into the TCA cycle. This mechanism requires the action of the gluconeogenic enzymes PEPCK and partly also fructose-1,6-bisphosphatase (FBPase) (61).

As reviewed by Grasmann *et al*. (76), gluconeogenic enzymes are differentially expressed in cancers, mediating the abbreviated form of gluconeogenesis to produce biosynthetic intermediates. Apart from their function in abbreviated gluconeogenesis, gluconeogenic enzymes also act as regulators of glycolysis and the TCA cycle. In colon cancer, mutant KRAS has been linked to an increased PCK2 expression, and PCK2 was upregulated in colon carcinoma, as compared to healthy colon tissue (191). PCK2 was also upregulated in brain metastatic cells derived from breast cancer as compared to the parental breast cancer cells or

bone metastatic cells (192). The functional significance of PCK2 has not been analyzed in these studies, however. In primarily non-gluconeogenic organs, cancers tend to activate the gluconeogenic pathway, while for example liver cancer (the liver being highly gluconeogenic in the healthy state) poses an exception with regards to the regulation of gluconeogenesis (71, 153, 193). PCK2 activity was shown to be increased in human NSCLC, as it has previously been shown by Leithner *et al*. In the same study, PCK2 mRNA was slightly elevated in tumor compared to lung tissue, which is contrary to the findings obtained in in this study, However, the previous cohort was very small (n=19 patients), which might explain the conflicting results (71). Another interesting finding from the same study was, that lactate was converted to PEP in glucose-starved cancer cells, which confirmed PCK2 activity towards the pathway of gluconeogenesis. Furthermore, PCK2 silencing impaired survival of cancer cells upon glucose deprivation in two out of three investigated NSCLC cell lines (71). The inhibition of PCK1/2 also enhanced apoptosis in NSCLC cells growing as 3-dimensional spheroids, where known gradients of glucose and oxygen availability exist (71). Mendez-Lucas and colleagues discovered KRAS transformed NIH-3T3 fibroblasts featuring enhanced PCK2 mRNA when compared to the non-tumorigenic parental cell line (75). In breast cancer cells, silencing of PCK2 reduced glucose consumption, lactate production and proliferation. Upon PCK2 silencing, apoptosis induction via glutamine deprivation or ER stress increased considerably (75). According to a metabolomic and gene expression model of lung cancer cells, PCK2-mediated gluconeogenesis and serine synthesis was strikingly upregulated in a glucose-free medium, highlighting the function of PCK2 in the adaptation to glucose deprivation (72). When PCK2 was silenced, this put a halt on cell proliferation of different NSCLC cell lines upon glucose starvation. PCK2 silencing also reduced tumor progression in subcutaneous xenografts in a mouse model (72). *In vitro* analyses showed a reduced colony forming capacity of PCK2 silenced lung cancer cells upon glucose- and serum starvation (73).

PCK1 has been investigated in colon cancer, and it was found that PCK1 silencing markedly reduced proliferation and clonogenic growth of colon cancer cells, and reduced colon cancer xenograft growth as well (74). PCK1 was found to act pivotal, in the gluconeogenic, as well as in the reverse direction, depending on the glucose concentration in the medium (74). This further highlights the role of PCK1 in regulating cancer cell metabolic flexibility.

The role of FBPase, a PEPCK downstream enzyme of gluconeogenesis, appears to differ from the function of PEPCK in cancer cells. FBPase converts fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate in the second rate-limiting reaction of gluconeogenesis. FBPase has been shown to act as a tumor suppressor, enhancing oxidative phosphorylation and ROS levels, ROS being detrimental to cancer cells. Hence, loss of one of the two isoforms of FBPase, FBP1 or FBP2, leads to carcinogenesis, increased tumor growth and tumor

progression in multiple cancers, including breast-, pancreatic-, renal-, liver-, colon-, gastric-, lung-, cervical cancer and melanoma (90). FBP1 suppresses HIF1 alpha activity, binding to its inhibitory domain. HIF1 alpha regulates a vast variety of genes associated with metabolic reprogramming, augmenting the glycolytic phenotype and reducing oxidative phosphorylation upon hypoxic conditions (90). However, ectopic FBP1 leads to a considerable reduction of glucose uptake and glycolytic flux, downregulating GLUT1, HK2, phosphofructokinase 1 (PFK1) and LDHA in cancers (90). Overexpression of FBP1 represses PKM2 activity through dynamic structural switching from the active tetramer to the less-active dimer (187). Anabolic biosynthetis pathways branching from glycolysis – such as serine synthesis, glyceroneogenesis and the pentose phosphate pathway (PPP) also decline in case of FBP1 overexpression (194). Furthermore, FBP1 disrupts the adaptive response to hypoxia, enabled by pyruvate dehydrogenase kinase (PDK), which reduces the flow of pyruvate into the TCA cycle and thus decreasing oxidative phosphorylation. As a result, oxygen consumption is reduced (195). FBP1 further enhances oxidative phosphorylation by activation of the mitochondrial electron transporter complex I (194). Oxidative phosphorylation generates more ATP as compared to glycolysis, when oxygen availability is compromised, and electrons can escape from the electron transporter chain and be captured by O2 which causes high levels of ROS production and ATP depletion (194). Thus, FBP1 is a trigger for the metabolic switch from aerobic glycolysis to oxidative phosphorylation in cancers, leading to oxidative stress and cell death. Of note, however, FBPase it is not required for the biosynthesis of serine, glycine or glycerol from non-carbohydrate precursors.

Pharmacological inhibitors of PEPCK have been known since decades and are increasingly investigated as tumor-modulating agents. A specific PEPCK (PCK1 and PCK2) inhibitor, 3-mercaptopicolinic acid (MPA), enhanced glucose-starvation-induced apoptosis in lung cancer expressing high rates of PEPCK (71). Of note, a recently developed PEPCK inhibitor efficiently inhibited growth of different xenografts with no apparent toxicity in mice (88). So far, little is known about the interaction of PEPCK inhibition with other anticancer therapies. PEPCK expression has been suggested to predict susceptibility to chemo- and radiotherapy, since downregulation of PCK2 results in a low energy metabolism and decreased tumor cell proliferation in rectal cancer, causing a decreased response to anticancer drugs, especially 5-fluouracil (5-FU) (196).

While PEPCK inhibition might be favorable for growth of certain cancers, like lung, breast and prostate cancer (for review see (76, 197)), the opposite might be true in cancers arising in the liver or kidney. In liver cell cancer featuring low levels of PEPCK, administration of dexamethasone led to an inhibition of hepatocellular carcinoma (HCC) growth and angiogenesis, which was at least partly an effect of the upregulation of PEPCK transcription,

promoting higher rates of gluconeogenesis (193). Several studies noted a down-regulation of the gluconeogenesis enzymes FBP1 or PCK1 (and partly also PCK2) in cancers arising from the liver or kidney and a tumor-suppressive effect of gluconeogenesis (76, 88). Thus, there appears to be a striking difference in glucose metabolism, with regards to PEPCK, in cancers derived from tissues with high physiological gluconeogenic activity.

The utilization of gluconeogenesis by lymphocytes allowing metabolic adaptation in nutrient-low microenvironments has been recently investigated. By blockade of immune checkpoints, or else, the use of chimeric antigen receptor T cells, novel approaches of immunotherapy make T cells attack malignant cells. Interestingly, activated T cells themselves upregulate aerobic glycolysis and undergo a similar metabolic switch as cancer cells, resulting in a competition between T cells and cancer cells for energy substrates (198). In a tumor microenvironment poor in glucose, cancer cells and T cells compete for glucose, eventually leading to T cell exhaustion and an immune-suppressive condition in the cancer microenvironment. In an interesting study, it has been demonstrated, however, that by means of ectopic PCK1 it is possible to reprogram the metabolism of tumor-infiltrating T cells and restore their anticarcinogenic effect. PCK1 overexpression increased the generation of PEP, keeping up calcium and nuclear factor of activated T cells (NFAT) signaling, which resulted in higher tumoricidal properties of T cells and a prolonged survival in a mouse model of malignant melanoma (199). The authors of this study speculate that PCK1 probably allows T cells to consume also lactate and fatty acids as alternative energy sources, making them more independent of glucose (199). While this study suggests that T-cells function may be enhanced by forced expression of PEPCK, further studies are needed to clarify the role of PEPCK in immune cell function in malignancies. An interesting role of PEPCK in recycling lactate by macrophages has been proposed. Monocytes were found to take up lactate secreted from colorectal cancer cells, thereby generating glucose and promoting rapid tumor growth (200).

Summing up these data on gluconeogenesis in malignant disease, more research is clearly warranted. Tumor cells regulate the activity of gluconeogenic enzymes, that also have specific functions in healthy cells. Cancers are thereby enabled to continue growth and proliferation in their specific metabolic microenvironment. Gluconeogenic enzymes are powerful mediators of anabolic biosynthetic pathways and regulate a multitude of cellular functions. They might be future therapeutic targets in cancer therapy – always in consideration of their ubiquitous expression and function in various healthy tissues of the human body.

14.7 Ketosis and starvation as a treatment for cancer

Glucose is the predominant energy substrate for most cancers with glycolytic rates 8-200 times higher than normal tissues, accompanied by the production of 10% more ATP than non-malignant cells (201). High consumption of glucose is necessary for the production of biosynthetic intermediates (*e.g.* ribose for nucleotides, glycerol, pyruvate and citrate for lipids, amino acids and NADPH via the PPP) (202).

Most tissues in the human body, *e.g.* skeletal muscle, heart, and most other organs, are flexible when it comes to selecting the appropriate substrate for energy generation. During glucose scarcity (*e.g.* induced by ketogenic diets), glucose metabolism of healthy tissues decreases, and instead, free fatty acids (FFAs) are increasingly used to draw energy from. Ketone bodies are generated in the liver, which happens usually after a few days of depriving an individual of carbohydrates. If carbohydrate starvation is carried out over the time period of several weeks or even months, cells also cut their uptake of ketones, and additionally the permeability of the blood-brain-barrier for ketones is increased – these effects are termed “keto-adaptation” (203, 204). Some tissues, including the brain and central nervous system, however, do not fully undergo keto-adaptation and still rely on glucose as their main source of energy. The largest consumers of glucose are the brain and central nervous system. The brain does not metabolize FFAs. It will, however, readily use ketone bodies whose levels increase to 2.5-9.7 mmol/L in starved individuals, as compared to negligible levels of 0.01 mmol/L in non-starved subjects (205). It is assumed that the brain’s capability of using ketone bodies as an energy source was developed evolutionarily in order to ensure survival in times of glucose or carbohydrate scarcity. It has even been argued that if the human body were not able to adapt to ketosis, Homo sapiens could not have developed such a large and advanced brain (206).

The question arises, whether inhibition of glycolysis is a potential therapeutic option in cancers. Low carbohydrate diets, including also the so-called ketogenic diet are a means to limit the glucose supply in cancer cells (207). The obvious concern that arises, is not only the fact that starvation is highly unpleasant, but also leads to muscle wasting and weight loss, and both are serious adverse effects in probably already malnourished cancer patients. On the other hand, fasting has been shown to be relatively safe, if certain obvious contraindications such as cachexia are considered (208). In a previous report, the investigators sought to circumvent the adverse event of muscle- and weight loss by implementing a 4:1 ketogenic diet. The authors refer to the observation by the pediatrist Dr. Russel M. Wilder who found that intermittent starvation (*i.e.* caloric restriction) was indeed therapeutic for children suffering from epilepsy (but obviously no long-term solution). Wilder then invented the “ketogenic diet”, mimicking starvation by severe carbohydrate deprivation, but still providing adequate caloric intake (209).

The ketogenic diet is a high fat, low protein and very low carbohydrate diet (210). In the 4:1 ketogenic diet approach, carbohydrate intake must be limited to 20 g/day, and protein must be limited to an amount which allows normal (non-starvation) protein turnover, estimated at about 25 g/day in case of a 70 kg person. It has to be kept in mind, that any additional protein intake has the potential to be gluconeogenic and should thus be strictly avoided. The rest of calories, needed to ensure adequate intake, has to come entirely from fat (210). The ketogenic diet leads to a minimization of glucose uptake, increased cerebral ketone uptake and predominant use of FFAs in all organs apart from the brain, for energy production. Simone *et al*. even proposed that a ketogenic diet not only prevents muscle loss, but may even endorse muscle synthesis which could indeed be beneficial for cancer patients (211). Side effects of the ketogenic diet include constipation, salt loss, mild acidosis and increased risk of kidney stone formation, when applied long-term. The ketogenic diet has nevertheless be proven to be safe even in patients with advanced malignant disease (212). Some data suggest, that ketone bodies themselves are actually toxic to cancer (213, 214), as well as improving the immune system's capability to target cancer (215). This may be an additional benefit when implementing the ketogenic diet in cancer patients.

Moreover, it has been hypothesized that insulin is essential for the maintenance of tumor cell metabolism (216), and could impede ketogenesis. Notably, epidemiologic evidence exists about the therapeutic effect of metformin in cancer, a drug inducing mild hypoglycemia and usually administered for the treatment of diabetes mellitus (217).

Still, the approach of inducing hypoglycemia in cancer patients remains experimental, and optimum glucose levels for inducing tumor necrosis are not known. Moreover, even after ketoadaptation, too low serum glucose levels must be avoided for obvious reasons. Noteworthy, all systematic data which exists today on the topic of hypoglycemia as cancer treatment, stems from *in vitro* studies and cannot be directly translated to human beings. In a study on three cancer cell lines (218), between 55% and near 100% necrosis rates were observed after 48 hours of glucose starvation. However, the possibility exists that cancers might adapt to this state of constant glucose starvation, by shifting their metabolism towards gluconeogenesis and relying increasingly on ketone bodies and FFAs.

Our opinion on this approach of inducing ketosis in cancer patients is, that it is not well investigated yet, and poses considerable and potentially lethal risks for the patients. Furthermore, even if the patients' caloric requirements are met, catabolic processes, muscle tissue- and body weight loss might still occur, because of the low insulin levels.

From our data we conclude, that glycolysis and gluconeogenesis are both active in the majority of NSCLC, with the glycolytic marker GLUT1 being up-regulated in cancer versus normal tissue

and linked to a poor prognosis. The inhibition of glycolysis in tumors, together with concurrent blockade of adaptive mechanisms including gluconeogenesis, thus might be a potential therapeutic approach. The question whether ketogenic or other anti-glycolytic therapies might synergize with the inhibition of gluconeogenesis remains to be addressed in future studies.

14.8 Role of mitochondria in cancer metabolism

Eukaryotic cells acquired an intracellular symbiont several billion years ago, allowing for a more efficient cellular energy metabolism. It is assumed that these endosymbionts derived from a prokaryotic organism (219), and allowed for the evolution of multicellular organisms, and consecutively became an essential cell organelle, the mitochondria. Mitochondria can move within the cytoplasm, harbor an independent genome and protein translation system, and reproduce independently of the host cell (219). Essential mitochondrial genes are encoded in the nuclear genome, allowing for a distinct coordination of nuclear and mitochondrial gene expression. Mitochondria-derived metabolites are, for example, acetyl-CoA, alpha-ketoglutarate and NAD^+/NADH, all essential for cellular metabolism. On the other hand, nuclear encoded proteins and growth factor signaling strongly affects mitochondrial function (220, 221). Intuitively, one would assume that cancer cells make use of mitochondrial function to optimize their energy metabolism. However, early studies on this topic showed inconclusive data (222).

IDH2 is a mitochondrial enzyme catalyzing the oxidative decarboxylation of isocitrate to alpha-ketoglutarate. IDH2 mutations are known to occur in acute myeloid leukemia, gliomas, astrocytomas and chondromas. Mutation of IDH2 results in a neomorphic enzyme, catalyzing the conversion of alpha-ketoglutarate to D-2-hydroxyglutarate. Consecutively, an excess of D-2-hydroxyglutarate is produced, which acts as a competitive inhibitor of the ten eleven translocation (TET) family of enzymes that are essential for the demethylation of cytosine nucleotides in the DNA. Furthermore, the Jumonji-C domain histone Nε-lysine demethylases (KDMs) responsible for histone demethylation, are inhibited by D-2-hydroxyglutarate as well. Profound changes in the DNA and histone methylation pattern are the result from IDH2 mutation. Ultimately, this leads to carcinogenesis, as it has been proven in acute myeloic leukemia, where IDH2 mutations were found to lead to malignant transformation (223, 224). Most importantly, loss of this mutation in an established acute myeloic leukemia model resulted in severe growth attenuation, validating IDH2 as an oncogene and potential therapeutic target (224).

Of note, the majority of cancer specimens have been shown to harbor functional mitochondria (225). Yet, some tumors have the ability to circumvent the need of an intact TCA cycle via the

reductive carboxylation of alpha-ketoglutarate to isocitrate by the IDH1 and 2 enzymes (64). Mitochondria are not only responsible for the generation of ATP and biosynthetic precursor molecules, but also produce ROS. These mitochondria-derived ROS can be converted to H2O2 by superoxide dismutase (SOD2). H2O2 serves as a signaling molecule which is able to modulate the activity of ROS sensitive proteins like the FOS-JUN transcription factors. Perhaps the most relevant function of mitochondria in cancer is the initiation of the apoptotic response by release of cytochrome C into the cytoplasm (226, 227). Consecutively, caspases 3 and 7 are activated, and apoptosis is initiated. The anti-apoptotic BCL-2 family members control the apoptotic process in cancer cells – and the fact that several cancers overexpress BCL-2 for suppression of apoptosis, reveals another role of mitochondrial function in carcinogenesis (228, 229).

However, the role of mitochondria in cancer metabolism is clearly controversial. As already mentioned, latest data suggests that mitochondria function normally in cancers (225). Further research is warranted in this field.

14.9 Final remarks

One of the hallmarks of cancer is the reprogramming and rewiring of cell energy metabolism (230). The increment in glucose uptake and enhanced metabolizing of glycolytic intermediate substrates is observed in most cancer specimens. Thereby, anabolic processes of biosynthesis, and the restoration of reducing equivalents for growth as well as antioxidant defense, are achieved by the tumor cells. However, in the few clinical experiments conducted so far, attempting to target glycolysis as a therapeutic anticarcinogenic tool, only limited success was reported (231). Glycolytic intermediate substrates are supplied by the gluconeogenesis pathway, if glucose availability is very scarce, or in case of the inhibition of proximal glycolysis.

Our study shows that gluconeogenesis is frequently activated along with glycolysis in NSCLC, thus activation of gluconeogenesis and utilization of non-carbohydrate precursors for biosynthetic reactions may partially explain a lack of efficacy of glycolysis inhibitors. Targeting both, glycolysis and gluconeogenesis could potentially prove beneficial. Targeting glycolysis and gluconeogenesis would potentially allow to target different tumor niches and compartments simultaneously.

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