Towards personalised medicine for cancer

From initial therapy to follow-up

Molenaar, R.J.

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Chapter 3: In silico gene expression analysis reveals glycolysis and acetate anaplerosis in IDH1 wild-type glioma and lactate and glutamate anaplerosis in IDH1-mutated glioma

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Abstract
Hotspot mutations in isocitrate dehydrogenase 1 (IDH1) initiate lower-grade glioma and secondary glioblastoma and induce a neomorphic activity that converts α-ketoglutarate (αKG) to the oncometabolite D-2-hydroxyglutarate (D2HG). It causes metabolic rewiring that is not fully understood. We investigated the effects of IDH1 mutations on expression of genes that encode for metabolic enzymes by data mining The Cancer Genome Atlas. We analysed 112 IDH1 wild-type versus 399 IDH1-mutated lower-grade glioma and 157 IDH1 wild-type versus 9 IDH1-mutated glioblastoma samples in silico. In both glioma types, IDH1 wild-type was associated with high expression levels of genes encoding enzymes that are involved in glycolysis and acetate anaplerosis, whereas IDH1-mutated glioma overexpress genes encoding enzymes that are involved in the oxidative tricarboxylic acid (TCA) cycle. In vitro, we observed that IDH1-mutated cancer cells have a higher basal respiration compared to IDH1 wild-type cancer cells and inhibition of the IDH1 mutation shifts the metabolism by decreasing oxygen consumption and increasing glycolysis. Our findings indicate that IDH1 wild-type glioma have a typical Warburg phenotype whereas in IDH1-mutated glioma the TCA cycle, rather than glycolytic lactate production, is the predominant metabolic pathway. Our data further suggest that the TCA cycle in IDH1-mutated glioma is driven by lactate and glutamate anaplerosis to facilitate production of αKG, and ultimately D2HG. This metabolic rewiring may be a basis for novel therapies for IDH1-mutated and IDH1 wild-type glioma.

Introduction
Diffuse gliomas are infiltrative neoplasms that arise in the cerebral hemispheres of adults and are graded as World Health Organisation (WHO) II-IV based on histopathological characteristics. Grade II and III are lower-grade gliomas (LGG) of which a subset rapidly progress to WHO grade III glioma and eventually secondary glioblastoma (WHO grade IV), whereas others may remain relatively stable for longer periods of time. In contrast to this gradual progression of malignancy, the majority of glioblastomas arise de novo, and are then called primary glioblastoma. Complete surgical resection of diffuse glioma is impossible due to their highly invasive nature and without exception residual tumour is a source of recurrence and malignant progression. Median overall survival of LGG ranges widely from 3 to 15 years. Patients with de novo primary glioblastoma, the most common and aggressive primary brain tumour, have a median overall survival of 16 months. Despite all efforts to therapeutically target various pathways that are involved in glioma progression, significant improvement in survival has not been achieved since 2005, when temozolomide was added to irradiation as standard therapy.160

Hotspot mutations in the genes encoding for the metabolic enzyme IDH1 and, less frequently, IDH2 occur in 80% of WHO grade II-III and secondary WHO grade IV gliomas and are ancestral events in the formation of these neoplasms.15 In addition, IDH1/2 mutations occur in substantial percentages in various other tumour types, such as acute myeloid leukaemia (20–40%), chondrosarcoma (60%), intrahepatic cholangiocarcinoma (20%) and melanoma (12%).15 IDH1 and IDH2 are homodimeric enzymes that reversibly convert isocitrate to αKG with concomitant reduction of NADP+ to NADPH in the cytoplasm and mitochondria, respectively. The mutations lead to a neomorphic activity of the enzyme that converts αKG into the oncometabolite D2HG.13 D2HG competitively inhibits αKG-dependent enzymes, including histone demethylases35 and 5-methylcytosine hydroxylases30 that are
essential for epigenetic regulation of gene expression, including that of metabolic genes.\textsuperscript{161} \textit{IDH1/2} mutations change cellular metabolism via 4 mechanisms: 1) loss of wild-type \textit{IDH1/2} function that affects carbohydrate and NADPH/NADP+ metabolism; 2) accumulation of D2HG that further restricts the activity of various enzymes such as \(\alpha\)-ketoglutarate dehydrogenase (\(\alpha\)KGDH), succinate dehydrogenase (SDH) and complex IV of the mitochondrial electron transport chain, 3) epigenetic effects of D2HG on expression of genes involved in metabolism and 4) increased degradation of the hypoxia-response transcription factor HIF1\(\alpha\), a major inducer of expression of genes involved in glycolysis.\textsuperscript{31,43,143,162}

Reprogramming of cellular metabolism is one of the hallmarks of cancer.\textsuperscript{9} It is generally assumed that malignant cells use pyruvate for lactate production instead of fluxing it into acetyl-CoA in the TCA cycle, even in the presence of oxygen.\textsuperscript{163} This so-called Warburg effect is an accepted metabolic phenotype in cancer that allows rapid generation of biosynthetic intermediates at the expense of less efficient ATP production. As a result, cancer cells overexpress glucose transporters to compensate for their ATP demand via an abnormally high rate of glucose uptake. Presumably, the composition and availability of metabolites in the microenvironment, for example in the adult brain glucose, glutamate, glutamine and acetate, dictates which metabolites are the predominant carbon sources for cancer cells. These metabolites can all serve as precursors for macromolecules such as proteins, nucleotides and lipids.\textsuperscript{163} It has been reported that glioma cells have a higher rate of aerobic glycolysis than healthy brain tissue, and this is more pronounced in glioblastoma than in LGG.\textsuperscript{164,165} However, these observations may be biased because most analyses of glioblastoma tissue use resection material that likely contains angiogenic, hypoxic and necrotic regions. Metabolism in these areas may well differ from that in peripheral invasive regions that presumably do not experience hypoxia. Talasila \textit{et al.}\textsuperscript{166} reported recently that the centre the tumour centre of glioblastoma is particularly glycolytic, in line with the presence of perinecrotic hypoxia, and we previously observed similar metabolic phenotypes in preclinical orthopic xenograft models.\textsuperscript{167} However, how metabolic pathways in glioma are affected by the \textit{IDH1} mutation is not completely clear.

In order to evaluate the metabolic rewiring associated with \textit{IDH1} mutations, we performed an \textit{in silico} analysis of the transcripts that encode for metabolic enzymes and substrate transporters. These gene expression data were correlated with \textit{IDH1} mutational status in 677 LGG and glioblastoma samples derived from The Cancer Genome Atlas (TCGA). This study provides a comprehensive overview of the expression of key glycolytic, TCA cycle and glucose/lactate/glutamate/acetate anaplerosis enzymes in \textit{IDH1}-mutated glioma \textit{versus} \textit{IDH1} wild-type glioma.

\textbf{Materials and methods}

\textbf{Patient selection.} Gene expression analysis of LGG and glioblastoma data from TCGA\textsuperscript{168} was conducted on processed data of the UCSC Cancer Genome Browser portal\textsuperscript{169} and the cBioPortal for Cancer Genomics.\textsuperscript{170,171} The molecular data of 399 \textit{IDH1}-mutated \textit{versus} 112 \textit{IDH1} wild-type LGG and 157 \textit{IDH1} wild-type \textit{versus} 9 \textit{IDH1}-mutated glioblastoma samples were subsetted according to the \textit{IDH1} mutational status and analysed for expression and methylation of metabolic enzymes. Another 16 LGG and 198 glioblastoma tumours were excluded because of unknown \textit{IDH1} mutational status or lack of molecular data.

\textbf{In silico analysis.} Relative mRNA expression (RNAseq, IlluminaHiSeq) values were identified for relevant genes encoding for metabolic enzymes involved in glycolysis, the TCA cycle, glutamine-glutamate cycle and acetate and cydicate cycle (eTable 1). Annotations were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG). Mean normalised z-scores for mRNA levels were determined for \textit{IDH1}-mutated and \textit{IDH1} wild-type datasets. The expression of each gene in each sample was normalised separately. The returned value indicated the number of standard deviations away from the population mean. In the methylation data, the probes with the strongest negative correlation between the methylation signal and gene expression were included.
**Cell culture.** HCT116 *IDH1<sup>WT/R132H</sup> knock-in cells, generated by AAV-targeting technology GENESIS,<sup>172</sup> were kindly provided by Horizon Discovery. *IDH1<sup>WT/R132H</sup> and *IDH1<sup>WT/WT</sup> HCT116 cells were cultured in McCoy’s 5A medium (Gibco, Life Technologies, Thermo Fisher Scientific) in 5% CO<sub>2</sub> at 37°C. Media was supplemented with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific), 100 units/ml penicillin and 100 μg/ml streptomycin (both Gibco).

**Reagents.** AGI-5198 was purchased from MedChemExpress, oligomycin, carbonyl-cyanide-(trifluoromethoxy)phenylhydrazone (FCCP), antimycin A, rotenone, 2-deoxyglucose, glucose, *L*-glutamine and sodium pyruvate were purchased from Sigma-Aldrich.

**Measurement of oxygen consumption rate and extracellular acidification rates.** HCT116 cells, with and without pre-treatment of 1 μM AGI-5198, were grown in 5% CO<sub>2</sub> at 37°C and OCR and ECAR was measured using an XFe96 analyser (Seahorse Bioscience). HCT116 cells were plated in XF96 cell culture plates, 2.0*10<sup>4</sup> *IDH1<sup>WT/R132H</sup> HCT116 cells or 1.75*10<sup>4</sup> *IDH1<sup>WT/WT</sup> cells (to account for a more rapid proliferation of *IDH1<sup>WT/WT</sup> HCT116 cells relative to *IDH1<sup>WT/R132H</sup> HCT116 cells,<sup>48</sup> cells were seeded in each well of 96-well assay plates and incubated for 48 h prior to conducting the assay. For determination of OCR, medium was changed to DMEM supplemented with 10 mM glucose, 2 mM glutamine and 1.5 mM pyruvate. For the glycolytic flux, medium was changed to DMEM supplemented with 2 mM *L*-glutamine and the concentration of glucose added to initiate glycolysis was 25 mM. Four independent replicates were conducted with 10 technical replicates per cell line. Data were expressed as pmol of O<sub>2</sub> per minute and normalised by cell number measured by the CyQUANT Cell proliferation kit (Invitrogen™), which is based on a fluorochrome binding to nucleic acids. Fluorescence was measured in a microplate luminometer (ClarioStar BMG Labtech) with excitation wavelength at 485±10 nm and emission detection wavelength at 530±12.5 nm.

**Statistical analysis.** Statistical analysis included two-way Mann-Whitney U tests to determine significance of expression differences observed between *IDH1*-mutated versus *IDH1* wild-type samples and Kaplan–Meier estimates of survival with log-rank tests among strata. Relative expression data were visualised as box plots, depicting the 25<sup>th</sup>, 50<sup>th</sup> and 75<sup>th</sup> percentile, with 95% confidence intervals and significance levels are shown by (*; *P* < 0.05), (**; *P* < 0.01), (**; *P* < 0.001) and (****; *P* < 0.0001).

**Results**

We determined differences in expression levels of metabolic enzymes in *IDH1* wild-type versus *IDH1*-mutated glioma to increase our understanding of the metabolism of these two clinically very distinct types of gliomas. We included 399 *IDH1*-mutated and 112 *IDH1* wild-type LGG (WHO stage II: 49% and WHO stage III: 51%) and 157 *IDH1* wild-type and 9 *IDH1*-mutated WHO stage IV glioblastoma samples. The ratios of *IDH1*-mutated to *IDH1* wild-type tumours are in agreement with mutation frequencies from earlier reports of TCGA glioma data and other datasets.

**Expression of GLUT3 and glucose-metabolizing enzymes is low in *IDH1*-mutated compared to *IDH1* wild-type glioma.** To investigate the first rate-limiting step of cellular glucose metabolism, i.e. transport of glucose across the plasma membrane, we examined expression levels of *SLC2A1* and *SLC2A3*, the genes encoding for glucose transporters (GLUT1 and GLUT3). GLUT3 expression levels were low in *IDH1*-mutated glioma when compared with *IDH1* wild-type glioma, whereas GLUT1 expression did not differ between *IDH1*-mutated and *IDH1* wild-type glioma (Figure 1A). The rate-limiting, irreversible and glycolysis-related enzymes hexokinase (HK2 and HK3) and pyruvate kinase (PKM2), but not HK1 or the pyruvate kinase isoform PKLR, were also expressed at lower levels in *IDH1*-mutated glioma compared with *IDH1* wild-type glioma (Figure 1). High expression of HK2 was significant in *IDH1* wild-type LGG, whereas HK3 expression was significantly higher in *IDH1* wild-type glioblastoma. The rate-limiting enzyme of the pentose-phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD) was expressed at higher levels in *IDH1* wild-type glioma, but only
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in LGG and not glioblastoma. These results suggest that IDH1 wild-type glioma depend more on glycolysis than IDH1-mutated glioma.

Figure 1. mRNA expression levels of enzymes involved in glucose metabolism in IDH1 wild-type versus IDH1-mutated glioma.

IDH1 wild-type glioma show higher expression levels of genes that are typically upregulated in glycolytic cancer cells. (A) Analysis of gene expression levels of metabolic enzymes involved in transport and metabolism of glucose in IDH1 wild-type (n = 399) and IDH1-mutated (n = 112) LGG and IDH1 wild-type (n = 157) and IDH1-mutated (n = 9) glioblastoma samples obtained from the TCGA database. Relative mRNA expression levels are shown for IDH1 wild-type (blue) and IDH1-mutated (red). (B) LDHA and (C) LDHB expression and methylation according to IDH1 mutational status (blue: IDH1 wild-type, red: IDH1-mutated). Abbreviations: GLUT, glucose transporter; HK, hexokinase; G6PD, glucose-6-phosphate dehydrogenase; PK, pyruvate kinase; LDH, lactate dehydrogenase.
Increased TCA upstream of isocitrate, but decreased metabolism downstream of isocitrate in IDH1-mutated glioma as indicated by gene expression levels of enzymes involved in the TCA cycle in IDH1 wild-type LGG (n = 399) and IDH1-mutated (n = 112) LGG and IDH1 wild-type (n = 157) and IDH1-mutated (n = 9) glioblastoma. Relative mRNA expression levels are shown for IDH1 wild-type (blue) and IDH1-mutated (red). Abbreviations: PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; CS, citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase; αKGDH, α-ketoglutarate dehydrogenase; SDH, succinate dehydrogenase; FH, fumarate hydratase; MDH, malate dehydrogenase.

**IDH1 wild-type glioma express Warburg-effect genes, whereas IDH1-mutated glioma express TCA cycle genes.** The relative activities of lactate fermentation in the glycolysis and the TCA cycle are controlled by two sets of enzymes that determine the fate of pyruvate: pyruvate dehydrogenase (PDH) and the lactate dehydrogenases (LDHA and LDHB, where LDHA catalyses conversion of pyruvate into lactate and LDHB catalyses the reverse reaction). In IDH1 wild-type glioma, we observed a striking overexpression of LDHA relative to IDH1-mutated glioma (Figure 1A). Lower expression levels of LDHA in IDH1-mutated glioma were associated with hypermethylation of its promoter (Figure 1B). Conversely, IDH1-mutated glioma showed higher LDHB expression levels than IDH1 wild-type glioma. To maintain pH homeostasis, cancer cells up-regulate transporters such as the monocarboxylate transporters 1 and 4 (MCT1 and MCT4) for secretion of lactate. In IDH1 wild-type glioma, we observed higher expression of MCT1 and MCT4 as compared to IDH1-mutated glioma, suggesting increased transport of lactate in IDH1 wild-type glioma. These results indicate that IDH1 wild-type gliomas catabolise glucose in the glycolytic pathway with lactate as end product whereas in IDH1-mutated glioma lactate is converted into pyruvate.
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Conversion of pyruvate into acetyl-CoA for its entry into the TCA is accomplished by the pyruvate dehydrogenase (PDH) complex, the activity of which is controlled by pyruvate dehydrogenase kinase (PDK; isoforms PDK1, PDK2 and PDK3). PDK inactivates PDH via phosphorylation. PDH is a heterotrimeric enzyme complex composed of subunits PDHA and PDHB, forming the catalytic PDH component PDH E1, and PDHX, a non-catalytic regulatory subunit. In IDH1 wild-type glioma, we observed higher expression of regulatory genes PDK1 and PDK3 as compared to IDH1-mutated glioma. On the other hand, IDH1-mutated glioma showed higher expression levels of PDHA (in glioblastoma), PDHB (in LGG) and PDHX as compared to IDH1 wild-type glioma (Figure 2). Combined with the aforementioned glycolysis data these results suggest that in contrast to IDH1 wild-type glioma, IDH1-mutated glioma actively prevent the generation of lactate by silencing LDHA and maintaining high expression of LDHB, PDHA/B and PDHX. These conditions ensure that pyruvate is maximally used for TCA entry.

IDH1-mutated glioma have increased expression of TCA genes upstream of isocitrate and decreased expression of TCA genes downstream of isocitrate. Intermediate metabolites of glycolysis upstream of pyruvate can be shunted into macromolecular biosynthesis pathways without stalling glycolysis itself. In contrast, the TCA cycle must be continuously running and be completed to function optimally: generation of citrate by citrate synthase (CS) requires pyruvate-derived acetyl-CoA, the principal fuel of mitochondria, and the final TCA cycle metabolite (oxaloacetate). Mitochondrial products of a full TCA cycle are ATP and CO₂. The lack of glycolysis in IDH1-mutated gliomas suggests that these cancers apply alternative metabolic pathways that ensure sufficient energy and building blocks for macromolecular biosynthesis. To investigate this in detail, we examined expression levels of TCA-related enzymes in IDH1-mutated glioma and IDH1 wild-type glioma. CS and aconitase (ACO1/2), enzymes that function upstream of citrate oxidation, were expressed at higher levels in IDH1-mutated glioma than in IDH1 wild-type glioma (Figure 2). In contrast, expression levels of enzymes involved in downstream metabolism of isocitrate, including IDH1, IDH2 and IDH3A, αKGDH, succinate dehydrogenase (SDHB), fumarate hydratase (FH) and malate dehydrogenase (MDH2), were remarkably lower in IDH1-mutated glioma versus IDH1 wild-type glioma. These results suggest that IDH1-mutated glioma have an imbalanced TCA, with increased enzyme activity upstream of isocitrate, and decreased TCA cycle metabolism downstream of isocitrate. The imbalance resulting from low oxaloacetate production and high pyruvate influx causes disbalance that may be problematic for efficient citrate production. As a compensatory mechanism, oxaloacetate can also be produced directly from pyruvate by the activity of pyruvate carboxylase (PC). This reaction is counteracted by phosphoenolpyruvate carboxykinase 2 (PCK2) which catalyses the reaction of oxaloacetate to phosphoenolpyruvate. In IDH1-mutated glioma, PC levels were markedly higher than in IDH1 wild-type glioma, whereas PCK2 expression levels were significantly lower in IDH1-mutated glioma although differences in PCK2 levels between IDH1 wild-type glioblastoma and IDH1-mutated glioblastoma were less apparent (Figure 3). Collectively, these results indicate increased flux of pyruvate into the TCA cycle in IDH1-mutated glioma compared to IDH1 wild-type counterparts.

Higher lactate/glutamate anaplerosis in IDH1-mutated glioma and higher acetate anaplerosis in IDH1 wild-type glioma. We have previously postulated the hypothesis that glutamate could act as anaplerotic precursor for the TCA cycle in IDH1-mutated glioma. To test this assumption, we analysed the expression levels of mRNA of enzymes that are involved in the transport and conversion of these metabolites. Glutamine is a major source of TCA cycle metabolites via glutaminolysis, a process that converts glutamine to αKG via two subsequent deamination steps. To investigate its contribution to αKG replenishment in the TCA cycle, we determined mRNA expression levels of glutaminase (GLS and GLS2, converting glutamine to glutamate) and glutamate dehydrogenase (GLUD1 and GLUD2, converting glutamate to αKG). We also investigated mRNA expression levels of branched-chain amino acid transferase (BCAT1 and BCAT2) and glutamine synthetase (GS), which catalyse the reverse reaction of αKG into glutamate and of glutamate into glutamine, respectively.
In IDH1-mutated glioma, we observed strikingly higher expression levels of GLUD1/2 than in IDH1 wild-type glioma, whereas BCAT1/2 expression levels were much lower in IDH1-mutated gliomas than in IDH1 wild-type glioma (Figure 3), as has been reported previously. Low BCAT1 mRNA expression levels in IDH1-mutated glioma were associated with BCAT1 promoter hypermethylation (Figure 3B). We observed no significant differences between mRNA expression levels of GLS, GLS2 or GS between IDH1 wild-type glioma and IDH1-mutated glioma. These results suggest that IDH1-mutated gliomas predominantly utilise glutamate, rather than glutamine, for TCA cycle anaplerosis. Acetate can be utilised as an alternative substrate to generate acetyl-CoA and is transferred through the plasma membrane by MCT1 and MCT4. Acetyl-CoA synthetase (ACS1 and ACS2) converts acetate to acetyl-CoA, which can be catabolised in the TCA cycle. In IDH1 wild-type glioma, ACS2 showed an elevated mRNA expression compared to IDH1-mutated glioma (Figure 3), suggesting a higher contribution of acetate to the acetyl-CoA pool in IDH1 wild-type glioma. As indicated previously, higher contribution of acetate is in line with higher expression levels of MCT1 and MCT4 transporters in IDH1 wild-type glioma. Taken together, we show that relative to IDH1 wild-type glioma, IDH1-mutated glioma use lactate and glutamate (but lesser so glutamine) for the TCA cycle, by maintaining high expression of LDHB, PC and GLUD and silencing BCAT. In contrast, in IDH1 wild-type glioma glycolysis is more prominent and acetate is used to fuel the TCA cycle.

Figure 3. Contribution of glucose/glutamine/glutamate anaplerosis and acetate influx to TCA cycle metabolism in IDH1 wild-type versus IDH1-mutated glioma.

**Increased glucose/glutamate anaplerosis in IDH1-mutated glioma and high contribution of acetate in IDH1 wild-type glioma metabolism.** (A) Gene expression levels of metabolic enzymes involved in glucose anaplerosis, glutaminolysis and acetate metabolism in IDH1 wild-type (n = 399) LGG and IDH1-mutated (n = 112) LGG and IDH1 wild-type (n = 157) glioblastoma and IDH1-mutated (n = 9) glioblastoma. Relative mRNA expression levels are shown for IDH1 wild-type (blue) and IDH1-mutated (red). (B) BCAT1 expression and methylation according to IDH1 mutational status (blue: IDH1 wild-type, red: IDH1-mutated). Abbreviations: IDH1\textsuperscript{WTT}, IDH1-mutated; IDH2\textsuperscript{WTT}, IDH2-mutated; PC, pyruvate carboxylase; GLUD, glutamate dehydrogenase; GLS, glutaminase; BCAT, branched-chain amino acid transferase; GS, glutamine synthetase; ACS, acetyl-CoA synthetase.
Inhibition of the IDH1 mutation decreases oxygen consumption and increases lactate acidification. There is a scarcity of relevant models to study metabolic effects of IDH1/2 mutations in vitro. As an alternative we used the HCT116 IDH1<sup>WT/R132H</sup> knock-in cell line and its parental counterpart to investigate whether metabolic changes in IDH1<sup>R132H</sup> gliomas were reflected in this isogenic cell line pair too. We first measured oxygen consumption in HCT116 IDH1<sup>WT/R132H</sup> and HCT116 IDH1<sup>WT/WT</sup> cells in the Seahorse. The basal respiration was significantly higher in IDH1<sup>WT/R132H</sup> HCT116 cells than in IDH1<sup>WT/WT</sup> HCT116 cells, showing that also in this system IDH1-mutated cells have higher oxygen consumption and are more dependent on OXPHOS (Figure 4). To determine whether the IDH1-mutated is causally involved in the higher respiration rates, we tested the effects of the specific IDH1 R132H inhibitor AGI-5198. In line with expectations after 14 days of culture in the presence of the IDH1-mutant inhibitor AGI-5198 the respiration rate of HCT116 IDH1<sup>WT/R132H</sup> cells was significantly decreased and comparable to levels of HCT116 IDH1<sup>WT/WT</sup> cells (Figure 4B).

In order to investigate the glycolytic activity, we measured the extracellular acidification rate (ECAR) that determines the glycolytic phenotype of cells and is linearly related to lactate production. In HCT116 IDH1<sup>WT/R132H</sup> cells, we found a lower ECAR response to glucose compared to HCT116 IDH1<sup>WT/WT</sup> cells, indicating a lower glycolytic rate in IDH1-mutated cells (Figure 4). The glycolytic capacity was also significantly lower in HCT116 IDH1<sup>WT/R132H</sup> cells compared to HCT116 IDH1<sup>WT/WT</sup> cells (data not shown). After 14 days incubation in the presence of AGI-5198, we measured a slight increase of ECAR in HCT116 IDH1<sup>WT/R132H</sup> cells, indicating a shift of metabolism to glycolysis by inhibiting mutant IDH1 (Figure 4C). Taken together, we show that relative to IDH1 wild-type cells, IDH1-mutated cells are dependent on OXPHOS and inhibition of the IDH1 mutation decreases the oxygen consumption leading to a glycolytic phenotype.

Figure 4. The metabolic phenotype of IDH1-mutated and IDH1 wild-type cancer cells.

(A) Characterisation of metabolic phenotype; HCT116 IDH1<sup>WT/R132H</sup> cells (red) are dependent on OXPHOS, whereas HCT116 IDH1<sup>WT/WT</sup> cells (blue) cells are glycolytic. (B) The basal oxygen consumption rate (OCR) response of the HCT116 cell lines to 10 mM glucose, 2 mM glutamine and 1.5 mM pyruvate, with or without pretreatment with the IDH1MUT inhibitor AGI-5198 (1 μM, 14 days of incubation). (C) Extracellular acidification rate (ECAR) response of HCT116 cells to glucose (25 mM), with or pretreatment with AGI-5198 (1 μM, 14 days of incubation). All data were expressed as pmol of O<sub>2</sub> per minute and normalised by cell number measured by fluorochrome binding to nucleic acids. A representative experiment out of 4 is shown here, each data point represents mean ± SEM. Plots are visualised with 95% confidence intervals.
Discussion

The present study is the first to investigate the metabolic rewiring that is associated with IDH1 mutations in glioma in a comprehensive and integrated fashion. A summary of this metabolic rewiring is shown in Figure 5. We correlated the expression of key glycolytic, TCA cycle and glucose/lactate/glutamate/acetate anaplerosis enzymes with the IDH1 mutational status obtained from a large database containing data on both LGG and glioblastoma patients. We show that IDH1 wild-type glioma overexpress enzymes that play key roles in anaerobic glycolysis, i.e. genes that are typically upregulated in Warburg-like cancer cells. In contrast, expression of genes that encode for glycolytic metabolism are downregulated in IDH1-mutated glioma. Based on the axiom that gene expression levels are correlated with pathway activity, our data suggest that IDH1-mutated glioma predominantly catabolise glutamate, and to a lesser extent glucose, in the TCA cycle for αKG production. In support of this axiom, we confirmed these observations in functional studies by measuring lactate production and oxygen consumption in HCT116 IDH1WT/WT and HCT116 IDH1WT/R132H cells.

Glycolysis and lactate production are considered to be the major metabolic ways of processing of glucose in cancer cells as it generates both energy and carbon for macromolecular biosynthesis, required for sustained cell proliferation. In particular, GLUT3, HK2, PKM2 and LDHA play critical roles in initiating and maintaining the high glycolytic rates of rapidly proliferating cancer cells, and are associated with greater dependence on glycolysis than on OXPHOS. Our findings are in agreement with the existence of the Warburg effect in IDH1 wild-type glioma, whereas our data indicate that glycolysis and lactate production occur to a lesser extent in IDH1-mutated glioma. In line with this notion, silencing of HK2 increases oxygen consumption and decreases lactate production in glioblastoma cells. The elevated levels of LDHB in IDH1-mutated gliomas support the findings in previous studies that showed that IDH1-mutated glioma have reduced intracellular lactate levels compared to IDH1 wild-type glioma. Moreover, a recent study showed that the conversion rate of pyruvate into lactate was similar in IDH1-mutated glioma and normal brain, whereas IDH1 wild-type glioma showed a higher lactate production than normal brain. Our results also support previous reports that show that IDH1-mutated gliomas silence LDHA expression through hypermethylation of its promoter resulting in a low LDHA/LDHB ratio compared with IDH1 wild-type glioma or normal brain tissue. Reduced MCT expression in IDH1-mutated glioma is consistent with reduced glycolytic production of lactate as well as silencing of LDHA expression; cells that produce less lactate do not require an increase in its export and are less dependent on MCT expression.

Lactate production by IDH1 wild-type glioma cells has been suggested to acidify the extracellular space resulting in the death of surrounding normal brain cells and increasing the infiltrative potential of cancer cells. Our results suggest that IDH1-mutated glioma produce and secrete less lactate than IDH1 wild-type glioma which may correlate with a normalised tissue pH in IDH1-mutated glioma and the less aggressive behavior of IDH1-mutated glioma as compared to IDH1 wild-type glioma.

Compared with IDH1 wild-type glioma, IDH1-mutated glioma showed elevated expression levels of TCA cycle genes that function upstream of isocitrate whereas expression of TCA cycle genes downstream of isocitrate were decreased. In line with these observations, a recent study reported high expression of CS and ACO2 in IDH1-mutated glioma versus IDH1 wild-type glioma. We have shown previously that IDH1/2 enzymatic activity in IDH1-mutated glioblastoma is reduced compared to IDH1 wild-type glioblastoma. Consistent with these findings, a previous report demonstrated increased levels of isocitrate (2.7-fold) and D2HG (28-fold) in IDH1-mutated cancer cells versus IDH1 wild-type cancer cells. In addition to decreased SDH expression in IDH1-mutated glioma, a recent study demonstrated elevated succinate levels and protein succinylation in IDH1-mutated glioma samples. Taken together, the changes in gene expression levels in IDH1-
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Figure 5: Rewiring of metabolism by the IDH1 mutation.

IDH1<sup>WT</sup>/IDH1<sup>MUT</sup> and IDH1<sup>MUT</sup>/IDH1<sup>WT</sup> ratios indicate contribution of a particular pathway as calculated on the basis of gene expression levels in IDH1 wild-type glioma and IDH1-mutated glioma, respectively. IDH1 wild-type glioma catabolise glucose in the glycolysis with lactate as end product, whereas IDH1-mutated glioma prefer production of pyruvate from lactate and use the TCA cycle to generate isocitrate. Glucose, lactate and glutamate replenish the TCA cycle in IDH1-mutated glioma, to facilitate αKG production for consumption by mutant IDH1 to generate 2-HG, whereas acetate anaplerosis is important in IDH1 wild-type glioma.

Mutated suggest that mitochondrial isocitrate production is maximised, providing substrate for the cytosolic IDH1<sup>WT</sup>/IDH1<sup>R132H</sup> dimer complexes to generate αKG and D2HG, respectively.

Due to the lack of stable glioma cell cultures carrying an endogenous IDH1<sup>R132H</sup> allele, we used in vitro HCT116 colorectal carcinoma cells for functional studies. IDH1 mutations occur in 0.5% of colorectal carcinomas. Because IDH1<sup>R132H</sup> functions as a heterodimer with IDH1<sup>WT</sup>, 1:1 IDH1<sup>R132H</sup>:IDH1<sup>WT</sup> expression in IDH1<sup>WT</sup>/R132H HCT116 cells is more relevant than IDH1<sup>R132H</sup> overexpression models that are frequently used. Previous reports have shown that HCT116 IDH1<sup>mutated</sup> cells have reduced growth rates as compared to IDH1 wild-type cells under conditions of low oxygen tension suggesting that these are more dependent on OXPHOS.

Another major contributor to TCA metabolism in cancer cells is glutamine, which provides αKG via the glutaminolysis pathway. Moreover, 80% of D2HG generated by heterozygous IDH1<sup>WT</sup>/R132H HCT116 colorectal cancer cells is derived from glutamine, rendering this an important metabolite for D2HG generation. Whereas glutamine has been suggested to be an important carbon source in several types of cancers, experiments in xenograft models revealed that glioblastoma cells take up glutamine but do not directly oxidise it in the TCA cycle. Our analysis suggests an increased contribution of glutamate, rather than glutamine, to the TCA cycle in IDH1-mutated glioma, by maintaining high expression levels of GLUD1/2 and silencing or downregulation of BCAT expression. Our results support the findings of previous studies that demonstrated increased conversion of
glutamate to αKG by GLUD\textsuperscript{192} and reduced conversion of αKG to glutamate by BCAT1 in IDH1-mutated glioma, which is in line with promoter methylation of BCAT1 in IDH1-mutated glioma\textsuperscript{175,192}.

In line with our observations, a recent study demonstrated that IDH1 mutant overexpression in astrocytes results in increased PC expression and increased fractional flux through PC, suggesting that direct oxaloacetate production from pyruvate is critical for IDH1-mutated cancer cells to maintain TCA activity\textsuperscript{188}. Furthermore, metabolic flux studies in IDH1-mutated glioma models with [1–\textsuperscript{13}C]-glucose showed that 20% of 2HG was derived from glucose, suggesting that glucose is a less relevant donor of carbon for 2HG production than glutamate\textsuperscript{190}. On the other hand, we observed a high expression of PCK2 that shuttles oxaloacetate into glycolysis suggesting increased contribution from the TCA cycle to glycolysis in IDH1 wild-type glioma. In line with this observation, a study also reported high expression of PCK2 in IDH1 wild-type glioma versus IDH1-mutated glioma\textsuperscript{193}.

Finally, acetate metabolism can replenish the TCA cycle at the level of acetyl-CoA and this is an important anaplerosis pathway in the majority of glioblastoma\textsuperscript{191}. Our analyses suggest that this phenomenon is more important in IDH1 wild-type glioma than in IDH1-mutated glioma. Mashimo et al. did not stratify their glioblastoma cohort based on IDH1-mutated status but demonstrated higher expression of ACSS2 in glioblastoma than in grade II and III glioma and high ACSS2 expression in LGG patients with poor survival as opposed to low ACSS2 expression in LGG patients with prolonged survival. This resonates with the high frequency of IDH1 mutations in LGG cohorts and clarifies the observed association of ACSS2 expression and LGG patient survival, potentially reflecting the differences in ACSS2 expression and patient survival between IDH1-mutated glioma and IDH1 wild-type glioma.

Our transcriptome-wide approach study was performed on a large number of LGG and glioblastoma samples derived from TCGA. These data have been analysed previously in an effort to improve the pathological classification of glioma. It was proposed to categorise LGG in 3 molecular classes, based on IDH1, 1p/19q and TP53 status, instead of histology\textsuperscript{194}. In addition, IDH1 wild-type LGG appeared to be molecularly and clinically more similar to IDH1 wild-type glioblastoma than to IDH1\textsuperscript{MUT} LGG. Our studies support these conclusions in more detail, based on the profound similarities in metabolic transcript profiles both in IDH1 wild-type gliomas and in IDH1-mutated gliomas, irrespective of WHO grade. Of note, our data are confirmed by independent studies showing lower activity of HK, SDH and ACSS2 and higher activity of CS in LGG compared to glioblastoma\textsuperscript{184,191}. Considering the fact that the IDH1 mutation is more prevalent in LGG than in glioblastoma (80% versus 12%), this suggests that the IDH1 mutation is an important determinator in histological analyses and that metabolic analyses should be stratified by IDH1 mutational status and not histology.

These insights are relevant for rational development of metabolic anti-glioma therapies, because our data predict that inhibitors of glycolysis, such as HK2/3 and PKM2 may be the most promising for IDH1 wild-type glioma, whereas inhibitors of the TCA cycle and the glutamatolysis pathway such as metformin, chloroquine and epigallocatechin-3-gallate\textsuperscript{16} may possess a therapeutic index in IDH1-mutated glioma.