

Clinical-Kidney cancer

Transcriptomic and proteo-metabolic determinants of the grading system in clear cell renal cell carcinoma

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Received 28 December 2024; received in revised form 18 January 2025; accepted 23 February 2025

Abstract

Background: Pathological grade is a morphological parameter of clear cell–renal cell carcinoma (ccRCC) and an independent predictor of cancer-specific survival. The aim of this study was to identify grade-dependent metabolic signatures and corresponding gene and protein expression changes that connect variations in cancer metabolism with nuclear grade, especially in high-grade tumors.

Methods: Forty ccRCC samples were collected and stratified according to nuclear grade: 23 low-grade (LG = G1–G2) and 17 high-grade (HG = G3–G4) samples. In addition, 122 patients with sarcomatoid ccRCC (sRCC) were classified according to the abundance of sarcomatoid features as low sarcomatoid (LS; sarcomatoid component <20%; $n = 67$) or high sarcomatoid (HS; sarcomatoid component $\geq 20\%$; $n = 55$). Untargeted metabolomic analysis was performed. To study the relative changes in gene and protein expression in HG vs. LG ccRCC, data from 4 different datasets were downloaded and stratified according to nuclear grade. Immunohistochemistry and immunofluorescence were used to evaluate protein expression. Cancer-specific survival (CSS) and progression-free survival (PFS) were calculated using Kaplan–Meier analysis. Multivariate analysis was performed using a Cox regression model.

Results: The Warburg effect, in association with changes in Krebs cycle intermediates and related metabolites, was more prominent in HG ccRCC than in LG ccRCC. Additional alterations included metabolic reprogramming in the urea cycle and modulation of glutathione metabolism with the accumulation of reduced glutathione and carnitine derivatives in HG tumors, while the concentrations of long- and medium-chain fatty acids were greater in LG ccRCC. CSS and PFS were significantly decreased in patients with HS tumors. According to the multivariate analysis, the abundance of the sarcomatoid component was an adverse prognostic factor.

Conclusions: ccRCC is characterized by a particular grade-dependent metabolic reprogramming. Metabolic pathways and associated molecular alterations are grade-specific and could represent potential therapeutic targets, especially in HG tumors. sRCC subclassification based on the abundance of sarcomatoid components into HS vs. LS tumors have prognostic value. © 2025 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

Keywords: Renal cell carcinoma; Metabolomics; Metabolism; Grade; Sarcomatoid; Clear cell; Prognosis

1. Introduction

Renal cell carcinoma (RCC) represents approximately 3% of all human tumors. In 2025, the American Cancer Society estimated 80,980 new diagnoses of kidney cancer

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and 14,510 deaths from this tumor [1]. This tumor is extremely chemoresistant and partial or radical nephrectomy represents the standard therapeutic option for patients with RCC [2,3].

Clear cell RCC (ccRCC) represents the most common histological type, and according to the 2022 WHO/ISUP grading system, it can be classified into 4 grades of progressive undifferentiation.

This 4-tier grading system replaced the previous Fuhrman grade classification and represents an important prognostic parameter for risk stratification in patients with ccRCC. The assessment of tumor grade is fundamentally based on morphological parameters such as the degree of nucleolar prominence (for grades 1–3) and the presence of nuclear pleomorphism, anaplastic giant cells, rhabdoid morphology and sarcomatoid differentiation (for grade 4) [4]. The molecular determinants underlying tumor grade are still unknown, especially for high-grade tumors with sarcomatoid characteristics [5].

RCC is characterized by alterations in cellular metabolism that support uncontrolled cancer cell proliferation and tumor progression [6]. The use of high-throughput technologies to study the renal cancer transcriptome and metabolome showed that metabolic reprogramming is highly heterogeneous across different neoplastic histotypes. This heterogeneity is also present at the intratumoral level, resulting in a nonhomogeneous distribution of genetically distinct tumor cell subpopulations and determining resistance to anticancer therapies [7].

Previous studies investigated the role of different molecular alterations according to Fuhrman grade and demonstrated the existence of specific metabolic reprogramming involving glucose and lipid biosynthetic pathways [8,9].

In this study, we generated a large-scale metabolomic profile of human ccRCC according to the novel tumor grade classification system by combining metabolic data with transcriptomic and proteomic data. We analyzed the contribution of specific metabolic pathways to cancer cell survival and resistance to chemotherapy in primary renal cancer cells of different grades. Finally, we evaluated the molecular features and prognostic impact of high-grade tumors on the basis of the intratumoral abundance of sarcomatoid elements.

2. Materials and methods

2.1. Study population and tissue collection

Primary clear cell RCC ($n=40$) were collected from patients who underwent radical or partial nephrectomy (Supplementary Table 1). All specimens were immediately used to obtain primary cell cultures after frozen-section confirmation of the diagnosis. Two pathologists confirmed the presence of clear cell RCC in the neoplastic tissues and excluded tumor cells in the healthy specimens. Patients with diabetes mellitus and/or an estimated glomerular

filtration rate (MDRD equation) < 60 mL/min/1.73 m² were excluded from the study.

All patients were preoperatively staged by thoraco-abdominal computed tomography or magnetic resonance imaging. Tumor staging was reassigned according to the seventh edition of the AJCC-UICC TNM classification. Tumor grade on pathological tissues was attributed with hematoxylin–eosin staining in according to 2022 WHO/ISUP grading system. Written informed consent to take part was given by all participants. The protocol for the research project has been approved by the University of Bari IRB (n. 143/CE/2015) and conforms to the provisions of the 1995 Declaration of Helsinki.

2.2. Metabolite Analysis

Metabolic profiling of RCC samples was carried out at Metabolon Inc. All tissue samples were maintained at -80°C until processed. The sample preparation process was carried out using the Hamilton Company automated MicroLab STAR[®] system. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction, while allowing maximum recovery of small molecules. The resulting extract was divided into 2 fractions; 1 for analysis by LC and 1 for analysis by GC. Samples were placed briefly on a TurboVap[®] (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. For QA/QC purposes, a number of additional samples were included in each day's analyses. Furthermore, a selection of QC compounds was added to every sample, including those under test. These compounds were carefully chosen so as not to interfere with the measurement of the endogenous compounds.

2.3. Liquid chromatography/Mass spectrometry (LC/MS, LC/MS²)

The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into 2 aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in 2 independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient-eluted using water and methanol, both containing 0.1% Formic acid, while the basic extracts, also reconstituted using water/methanol, contained 6.5mM Ammonium Bicarbonate. The MS

analysis alternated between MS and data-dependent MS² scans using dynamic exclusion.

2.4. Gas chromatography/Mass spectrometry (GC/MS)

The samples destined for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramp is from 40° to 300°C in a 16 minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis. The information output from the raw data files was automatically extracted as discussed below.

2.5. Accurate mass determination and MS/MS fragmentation (LC/MS), (LC/MS/MS)

The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which had a linear ion-trap (LIT) front end and a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer backend. For ions with counts exceeding 2 million, an accurate mass measurement could be performed. Accurate mass measurements could be made on the parent ion as well as fragments. The typical mass error was less than 5 ppm. Characterizing ions with less than 2 million counts is a more laborious process. Fragmentation spectra (MS/MS) were typically generated in a data-dependent manner but if necessary, targeted MS/MS could be employed, as in the case of lower level signals.

2.6. Compound identification

Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. The identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. As of this writing, more than 1000 commercially available purified standard compounds had been acquired and registered into LIMS for distribution to both the LC and GC platforms for determination of their analytical characteristics. The combination of chromatographic properties and mass spectra indicated a match to the specific compound or an isobaric entity. Additional entities could be identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds may potentially be identified by future acquisition of a matching purified standard or by classical structural analysis.

2.7. Normalization

Data were collected over multiple platform run days and thus, “block normalized” by calculating the median values for each run-day block for each individual compound. This minimizes any inter-day instrument gain or drift, but does not interfere with intra-day sample variability. Missing values (if any) were assumed to be below the level of detection for that biochemical with the instrumentation used and were imputed with the observed minimum for that particular biochemical.

For studies spanning multiple days, a data normalization step was performed to correct variations resulting from inter-day instrument tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately. For studies that did not last more than 1 day, no normalization was necessary, other than for purposes of data visualization.

2.8. Bioinformatics and statistical analyses

Significance tests were performed with MedCalc 9.2.0.1 (MedCalc software, Mariakerke, Belgium) and “R” (<http://cran.r-project.org>). Global biochemical profiles were determined in human kidney tissue/tumor samples and compared across groups stratified by renal tissue pathology status. An estimate of the false discovery rate (q-value) was calculated to consider the multiple comparisons that normally occur in metabolomic-based studies. Comparisons of metabolites median values between different groups were evaluated by Mann–Whitney U test. In the cancer-specific survival (CSS) analysis, patients who died of RCC-unrelated causes or were lost to follow-up were censored. Progression-free survival (PFS) was calculated from the date of surgery to the date of disease recurrence. Estimates of CSS and PFS were calculated according to the Kaplan–Meier method and compared with the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards regression model to identify the most significant variables for predicting CSS and PFS. A backward selection procedure was performed with removal criterion $P > 0.10$ based on likelihood ratio tests. A P -value of < 0.05 was considered statistically significant.

2.9. Integration of metabolomic and transcriptomic/proteomic data

Clear cell-RCC transcriptome data derived from GSE40435, GSE150404, and TCGA datasets were used [10–12]. For proteomic analysis, Perroud dataset was used [13]. Data were divided into high-grade (HG, including G3 and G4) and low-grade tumors (LG, including G1 and G2).

MetaboAnalyst 6.0 [14] was used for the metabolite set enrichment and to integrate the data from the

transcriptomics, proteomics and metabolomics experiments. The main function of this module is to pinpoint the pathways involved in the underlying biological processes by combining the evidence based on alterations in both gene/protein expression and metabolite concentrations. In addition, a topology analysis was used to evaluate the relative importance of the genes/proteins/compounds based on their relative locations within a pathway. Over-representation analysis of the pathways was based on the hypergeometric test.

Gene set enrichment analysis (GSEA) was used to determine which pathways were statistically enriched across the renal cancer data sets [15]. The normalized enrichment score (NES) was used to evaluate the extent and direction of enrichment of each pathway.

2.10. MILLIPLEX® MAP human glycolysis pathway magnetic bead panel assays

The MILLIPLEX® MAP Human Glycolysis Pathway Magnetic Bead Panel (HGPMAG-27K, Millipore) was used for the quantification of the following proteins in tissue lysates: GPI/AMF (Glucose-6-phosphate isomerase/Auto-crine motility factor), ENO1 (Enolase 1), PKM2 (Pyruvate kinase isoform M2), LDHA (L-lactate dehydrogenase A chain), and LDHB (L-lactate dehydrogenase B chain).

For the immunoassay procedures, 25 µl of each dilute lysate sample in Assay Buffer (5 µg total protein/well) and HeLa cells lysate (positive control) were added into wells in duplicate, according to the manufacturer's instructions. To each well, 25 µl of the Mixed Beads were added and the plate was incubated for 2 hours at room temperature. Human glycolysis pathway detection biotinylated antibodies were added for 1 hour; each captured a specific bead. After that, the reaction mixture was incubated for 30 minutes with Streptavidin-PE conjugate to complete the reaction on the surface of each microsphere. Finally, the MILLIPLEX® MAP was analyzed by Luminex xMAP® technology. The immunoassay on the surface of each fluorescent-coded magnetic bead, MagPlex-C microsphere, was identified and quantified based on fluorescent signals. The median fluorescence intensity (MFI) was read with the Luminex 200TM instrument and measured with xPONENT® software.

2.11. Primary cell cultures from renal tissues

Tumor (ccRCC) tissue specimens derived from HG and LG ccRCC, were immediately placed in a Petri dish with phosphate-buffered saline (PBS) 1x and cut into small pieces of about 1 mm³. Each small piece of tissue was placed on the surface of the Petri dish with Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Life Technologies, Monza, Italy) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Milan, Italy) and 1% penicillin-streptomycin-L-glutamine (Sigma-Aldrich, Milan, Italy).

Cell proliferation was obtained around the kidney specimens as previously described [5]. Kidney epithelial tubular and neoplastic cells were isolated with EpCAM (CD326) Ab-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) under the effect of a magnetic field generated by the Mini MACS Separation Unit (Miltenyi Biotec), as previously described [16]. These cells were then characterized for EpCAM, and CA IX by immunocytochemistry. Primary ccRCC cells were used for proliferation studies at the second passage.

2.12. Cell viability assay

Cell viability after exposure to 5 mM of 2-Deoxy-D-glucose (2DG) or to 50 µM of Etomoxir and 10 µM cis-Diamminedichloroplatinum (II) (cisplatin) was evaluated using the trypan blue dye exclusion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the dye exclusion test and MTT assay, HG and LG tumor primary cells were seeded at a density of 1.5×10^5 and 1.5×10^4 cells in 6-well and 96-well plates, respectively (Sigma Aldrich, Milan, Italy) and incubated overnight at 37°C, 5% CO₂. In the first part of the experiment, the cells were exposed for 24 hour to 2DG or Etomoxir, or incubated in medium alone. After several washes to remove 2DG or Etomoxir, the cells were incubated for 72 hour. In the second part of the experiment, the cells were exposed to 2DG or Etomoxir for 24 hour and then were treated with cisplatin 10 µM for 1 hour and 2 hour. After several washes to remove cisplatin the cells were incubated for 72 hour. Each experimental condition was performed in triplicate.

2.13. Immunohistochemistry

Immunohistochemical evaluation of proteins expression was done on paraffin-embedded tissue sections derived from tumor samples. Sections (3 mm) of paraffin-embedded tissue were deparaffinized and rehydrated through xylenes and graded alcohol series. Slides were subjected to specific epitope demasking by microwave treatment at 700 W in citrate buffer (0.01 M, pH 6.0). After antigen retrieval, the tissue samples were incubated for 10 minutes with 3% H₂O₂ to block endogenous peroxidase activity. Sections were blocked with Protein Block Serum-Free (Dako) at room temperature for 10 minutes and then incubated with anti-NDUFA4L2 antibody (1:100 dilution, Proteintech, Chicago, IL, USA), anti-G6PDH antibody (1:250, Novus Biologicals, Littleton, CO, USA), anti-GPI antibody 1B7D7 (1:200, Novus Biologicals, Littleton, CO, USA), anti-GLUT1 antibody (1:200, Novus Biologicals, Littleton, CO, USA), anti-Ki67 antibody (1:100, Abcam, Cambridge, UK), anti-TKTL1 antibody (1:500, Novus Biologicals, Littleton, CO, USA), anti-PLIN2/ADFP antibody (1:200, Novus Biologicals, Littleton, CO, USA), anti-HIF1 alpha antibody (1:50, Abcam, Cambridge, UK), anti-HIF2 alpha/EPAS-1 antibody ep190b (1:150, Abcam, Cambridge, UK),

anti- c-MYC antibody (1:50, Abcam, Cambridge, UK) and anti-ACLY antibody 5F8D11 (1:300, Novus Biologicals, Littleton, CO, USA) at 4°C overnight. Binding of the secondary biotinylated antibody was detected using the Dako Real EnVision Detection System, Peroxidase/DAB kit (Dako, Agilent, Santa Clara, US), according to the manufacturer's instructions. Sections were counterstained with Mayer's hematoxylin (blue) and mounted with glycerol (Dako Cytomation). Negative controls were obtained by incubating serial sections with the blocking solution and then omitting the primary antibodies.

Sections of tissue were deparaffinized, rehydrated through xylenes and graded alcohol series and were stained with periodic acid-Schiff (PAS).

Digital images were obtained using the Aperio ScanScope C S2 device (Aperio Technologies, Vista, CA) and further analyses of the scanned images were performed with the ImageScope V12.1.0.5029 (Aperio). Specific staining was quantified by applying the Positive Pixel Count v9_v10.0.0.1805 algorithm (Aperio) and expressed as percentage of positive pixels in the analyzed area.

2.14. Immunofluorescence microscopy

Cryostat sections (5 mm) of RCC tissue samples included in OCT were rinsed with PBS for 15 minutes. Staining with BODIPY lipid probes (D-3922) (1:1000; Molecular Probes, Life Technologies, Monza, Italy) was determined by incubation for 30 minutes at room temperature. All preparations were counterstained with TO-PRO-3 (Molecular Probes).

Tumor renal cells (ccRCC cells) were seeded at a density of 2×10^5 on glass coverslips and left to adhere overnight at 37°C in 5% CO₂. These preparations were double-stained for NDUFA4L2 (Proteintech, Chicago, IL, USA) and MitoTracker™ Red CMXRos (Molecular Probes, Life Technologies, Monza, Italy). The expression and localization of proteins was evaluated by indirect immunofluorescence and confocal microscopy analysis. Mitochondrial mass was determined by incubating the live cells with 500 nM MitoTracker™ Red CMXRos in growth medium, for 20 minutes at 37°C. The cells were fixed using ice-cold 4% paraformaldehyde for 10 minutes at room temperature. Then the preparations were blocked with 1% BSA in PBS for 1 hour at room temperature and incubated overnight at 4°C with a primary antibody against NDUFA4L2 (1:25 in blocking) followed by incubation for 1 hour at 37°C with the secondary antibody goat anti-rabbit IgG FITC (1:200; Novus Biologicals). All preparations were counterstained with TO-PRO-3 (Molecular Probes). Negative controls were performed by omitting the primary antibodies. Specific fluorescence was acquired by a Leica TCS SP2 (Leica, Wetzlar, Germany) confocal laser-scanning microscope using an $\times 63$ objective lens.

3. Results

3.1. Clear cell RCC grade is associated with alterations in specific metabolic pathways

To obtain the first insight into the effects of metabolism-related gene expression on ccRCC grade differentiation, an *in silico* analysis was performed on data divided into high-grade (HG, including G3 and G4) and low-grade (LG, including G1 and G2) tumors using 3 public gene expression datasets (GSE40435, GSE150404, and TCGA) [10–12]. Next, we used GSEA to determine the enriched pathways in the HG vs. LG groups in each cohort [15]. GSEA of genes associated with grade differentiation revealed significant alterations in the following pathways: epithelial–mesenchymal transition and glycolysis for HG ccRCC and fatty acid metabolism for LG renal tumors (Fig. 1A).

3.2. Global metabolic profile distinguishes high-grade from low-grade ccRCC

Considering the leading role of metabolism-related genes in determining renal cancer grade, untargeted metabolomic analysis was performed on 40 ccRCC samples, including 17 HG and 23 LG ccRCC samples (Supplementary Table 1), using LC–MS and GC–MS platforms. The application of principal component analysis (PCA) to differentiate normal and pathological samples as a function of the global tissue metabolome showed that the 2 groups were metabolically distinguishable (Fig. 1B).

Next, to evaluate the impact of altered biochemical processes, we performed a metabolite set enrichment analysis using MetaboAnalyst 6.0 [14]. This functional approach showed that alterations in glycolysis, the pentose phosphate pathway (PPP), and aminosugar and glutathione metabolism had the greatest impact on HG cancer cell metabolism compared to LG ccRCC (Fig. 1C).

3.3. Integrated multiomics (metabolomics/proteomics/transcriptomics) analysis identifies grade-specific metabolic pathways

To compare the relative changes in gene and protein expression with metabolite accumulation in a grade-specific manner, we integrated the metabolomic data with gene expression data from TCGA [12] and proteomics analysis from the Perroud study [13].

The combined analysis of tumor metabolomics and proteomics revealed that biochemical pathways, including glucose, glutathione and tryptophan metabolism pathways, were significantly enriched in HG ccRCC patients ($P < 0.001$) (Fig. 1D).

Similarly, the integrated analysis of metabolomic and transcriptomic profiles using the GSE150404 dataset confirmed a significant enrichment ($P < 0.001$) in genes and

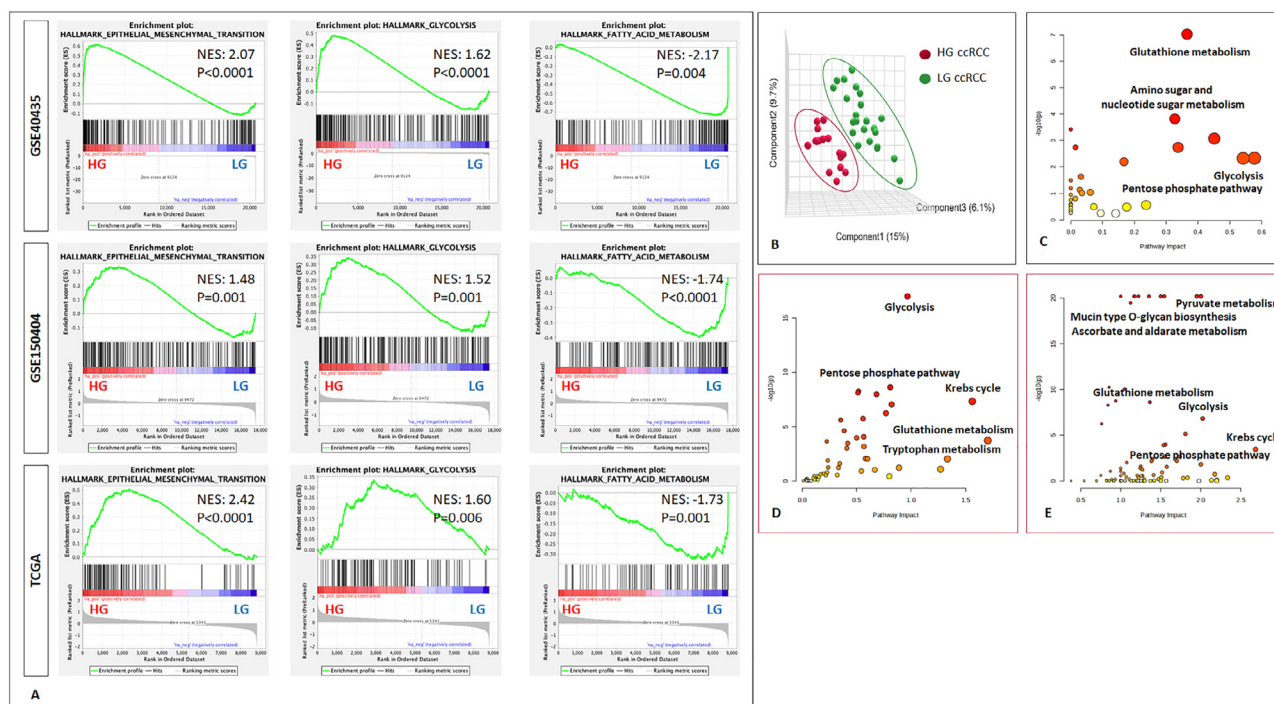


Figure 1. Gene set enrichment analysis (GSEA) of the GSE40435, GSE150404, and TCGA dataset (A). Principal component analysis (PCA) of the global tissue metabolome demonstrate that the 2 groups (HG vs LG ccRCC) are clearly distinguishable (B). Metabolic pathway enrichment analysis for the global metabolome (C), and for integrated analysis of the metabolome-proteome (D) and metabolome-transcriptome (E) in HG vs. LG ccRCC. The matched pathways are displayed as circles. The color and size of each circle are based on the *P*-value and pathway impact value, respectively. NES: normalized enrichment score.

metabolites involved in the Warburg effect and antioxidant metabolism pathways (Fig. 1E).

3.4. Metabolic perturbations in glucose metabolism and the pentose phosphate pathway (PPP) are grade dependent in ccRCC

The Warburg effect is one of the hallmarks of tumor cell metabolism. This metabolic alteration is characterized by a

shift toward glycolysis as primary energy source of cell, even when oxygen is available. Therefore, in this study we first evaluated whether a grade-dependent modulation of aerobic glycolysis was present in ccRCC tissues.

Increased glucose levels associated with elevations in upstream glycolytic intermediates (glucose 6-phosphate and fructose 6-phosphate) were found in HG tumors compared with LG ccRCC (Fig. 2). Consistent with these findings, glucose-6-phosphate dehydrogenase (G6PDH), in

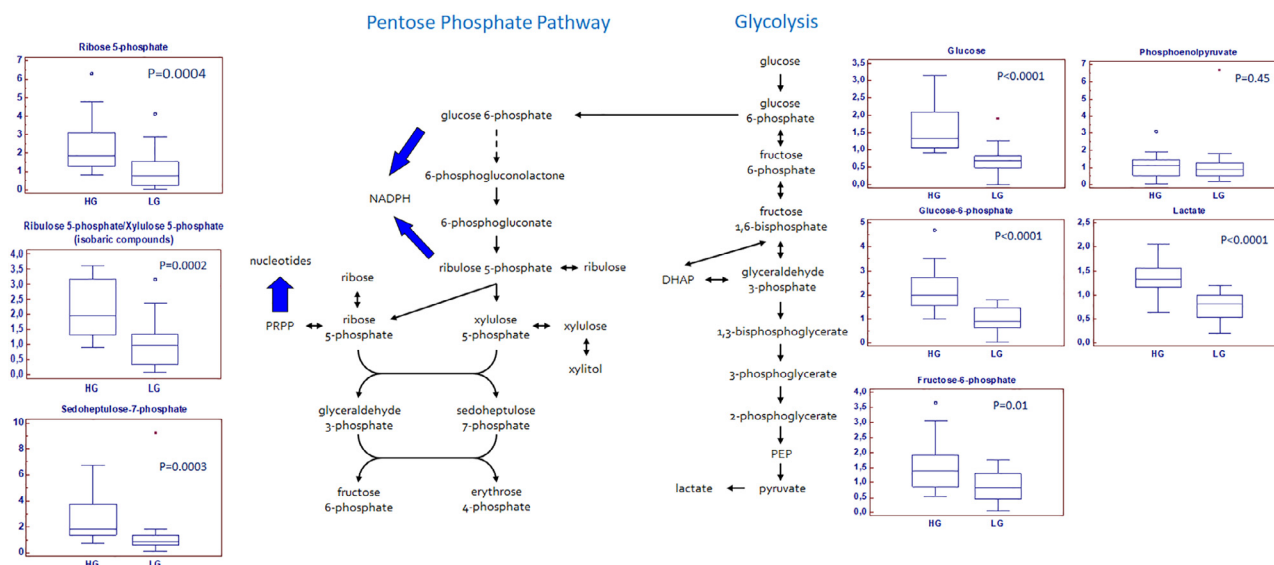


Figure 2. Schematic model summarizing the differences in glycolysis and the pentose phosphate pathway between HG and LG ccRCC. Y-axis: relative metabolite amount.

association with other glycolytic enzymes (including glucose-6-phosphate isomerase-GPI, enolase 1–ENO1, and pyruvate kinase isoform M2-PKM2; [Supplementary Fig. 1](#)), and the glucose transporter GLUT1 were overexpressed in HG tumors ([Fig. 3](#)). As a consequence of the Warburg effect, lactate accumulates in HG ccRCC, and in

support of this finding, we observed an increased LDH-A/LDH-B ratio that favors the expression of LDH isoenzyme 5 (LDH-A4), which is the isoform with the highest efficiency in lactate production ([Supplementary Fig. 1](#)).

Another interesting alteration involved the metabolism of aminosugars. In particular, we detected increased levels of

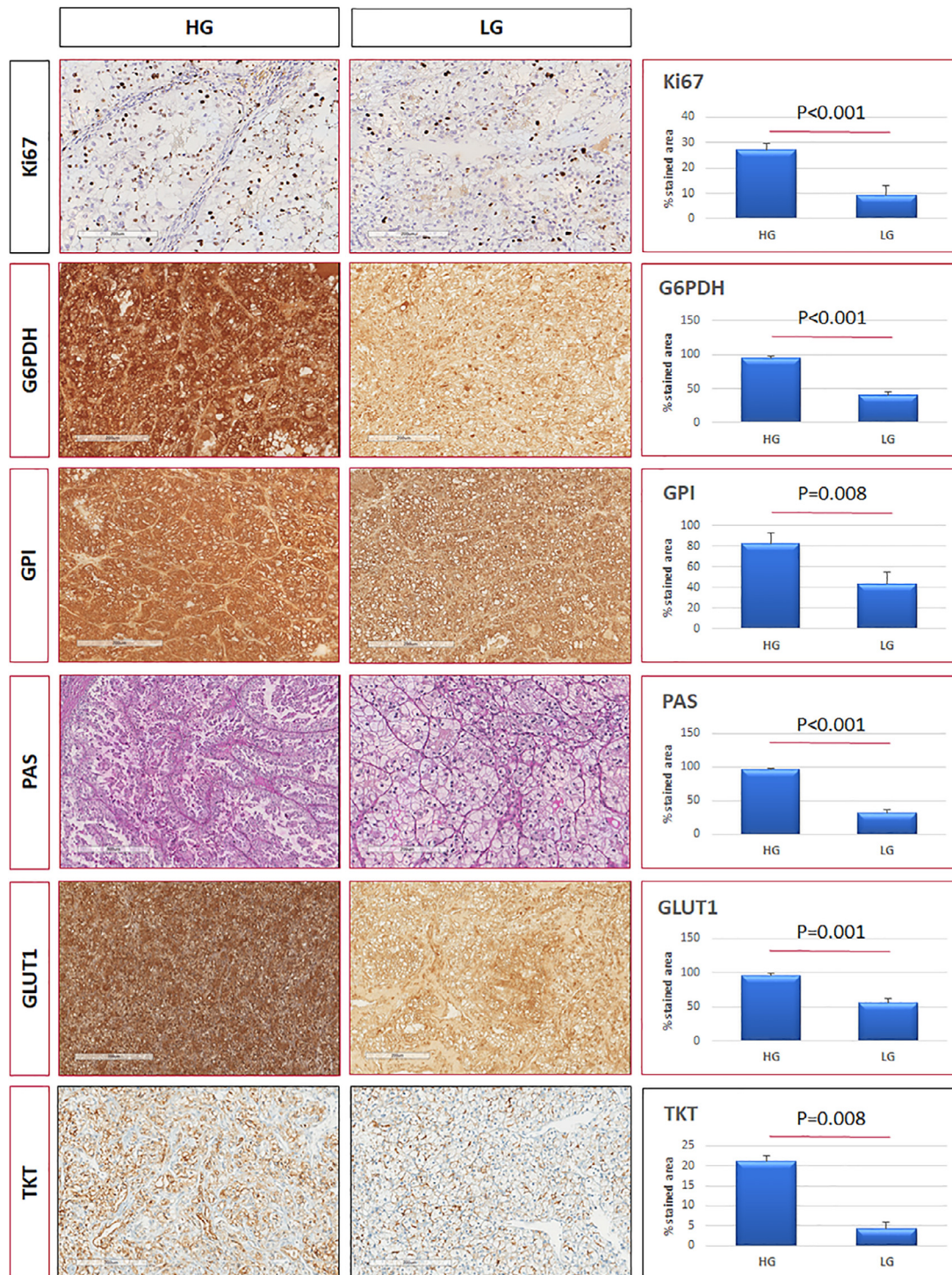


Figure 3. Immunohistochemical analysis of Ki67, glucose-6-phosphate dehydrogenase (G6PDH) glucose-6-phosphate isomerase (GPI), glucose transporter 1 (GLUT1), and transketolase (TKT) expression in HG vs. LG ccRCC. Periodic acid–Schiff (PAS) stain was used to detect polysaccharides.

amino sugar-related compounds (glucosamine, N-acetylglucosamine 6-phosphate, N-acetylneuraminic acid, and N-acetylmannosamine) in HG tumors compared with LG ccRCC, in accordance with the GSEA results (Supplementary Fig. 2).

The use of PAS staining for detecting carbohydrate macromolecules revealed intense positivity in HG- compared to LG-tumor (Fig. 3).

In ccRCC, metabolic flux through glycolysis is differentially partitioned, suggesting increased shunting of the metabolites of the upper part of glycolysis into the pentose phosphate pathway (PPP) [17].

In accordance with these findings, HG ccRCC was characterized by the accumulation of PPP intermediates (sedoheptulose 7-phosphate, ribose 5-phosphate, and ribulose 5-phosphate/xylulose 5-phosphate) (Fig. 2) and increased expression of the regulating enzymes G6PDH and transketolase (TKT) (Fig. 3).

The observed grade-dependent changes in PPP intermediates and enzymes supported the accelerated growth of HG tumors (as shown by Ki-67 labeling, Fig. 3), considering that the PPP supports the synthesis of nucleotides and NADPH for DNA replication, anabolic reactions and redox homeostasis.

3.5. Deregulation of the tricarboxylic acid (TCA) and urea cycles

High-grade ccRCC showed increased levels of metabolic intermediates in the upper part of the TCA cycle (citrate and succinate) and reduced tissue accumulation of fumarate and malate (Fig. 4A). These findings, in accordance with previous data showing impaired oxidative phosphorylation in ccRCC, suggest that HG renal cancer cells do not use the TCA cycle as their main energy source [5,10].

Compared with normal renal tissue, clear cell RCC is characterized by a reduced number of mitochondria, and this observation further supports the hypothesis that many biological processes involving these organelles are impaired, especially in HG tumors, as a consequence of increased NDUFA4L2 expression (Fig. 4B) [17]. Renal tubular epithelial cells typically express urea cycle enzymes, and 2 steps of this set of reactions take place in mitochondria. In addition, the TCA cycle and urea cycle are connected through the aspartate/argininosuccinate shunt.

Ochocki et al. [18] showed that ccRCC is characterized by alterations in ammonia metabolism and that multiple urea cycle enzymes, including arginase 2 (ARG2), argininosuccinate synthase (ASS1) and argininosuccinate lyase (ASL), were significantly downregulated in these tumors. We found a reduced accumulation of citrulline and urea in HG tumors compared to LG tumors (Fig. 4C). These alterations in ammonia metabolism, especially in HG ccRCC, aim to maintain elevated levels of pyridoxal-5'-phosphate for amino acid homeostasis and to avoid the toxic accumulation of polyamines (putrescine and spermidine) (Supplementary Fig. 3).

3.6. Antioxidants accumulate in high-grade tumors

In HG ccRCC, elevated levels of many antioxidants (alpha-, beta-, gamma-tocopherols, and ascorbate) were found (all $P < 0.0001$). In addition, increased biosynthesis of both the reduced and oxidized forms of glutathione was observed (Supplementary Fig. 4). These alterations in redox homeostasis could be explained by the greater proliferative rate of HG cancer cells, which need to increase their tolerance to oxidative stress. Tumors with greater malignant potential are characterized by increased production of reactive oxygen species (ROS) as a result of their intense metabolic activity. In this scenario, the accumulation of substances with antioxidant properties has the aim of counterbalancing the effects of oxidative stress and therefore reducing excessive genomic instability with consequent cell death.

3.7. The metabolism of fatty acids and acylcarnitines is differentially related to grade

RCC is characterized by a reprogramming of lipid metabolism in association with an altered expression of genes related to fatty acid metabolism [19,20]. In normal cells, before lipids can be used as fuels, triacylglycerol must be hydrolyzed to release free fatty acids (FAs). FAs are then linked to CoA to form acyl-CoA before they are beta-oxidized in the mitochondria. A special mechanism is required to transport long-chain FAs across the inner mitochondrial membrane. Long-chain FAs need to be conjugated to carnitine through a reaction catalyzed by carnitine palmitoyl transferase-1 (CPT1). We found an increased accumulation of FA-carnitine conjugates, especially in HG tumors. On the other hand, long-chain and medium-chain FA concentrations were increased in LG ccRCC patients (Fig. 5A). These observations were in accordance with the Bodipy dye results, which showed a grade-dependent increase in lipid deposition from HG to LG tumors (Fig. 5B). In addition, LG tumors showed significantly greater expression of ATP citrate lyase (ACLY) and perilipin2 (PLIN2) (Fig. 5B), 2 key proteins involved in FA biosynthesis and lipid droplet formation, respectively.

3.8. Specific treatments differentially affect renal cancer cell viability and cisplatin chemoresistance in a grade-specific manner

To study the contribution of specific metabolic pathways to renal cancer cell survival, HG- and LG- primary cancer cells were treated for 24 hours with 5 mM 2-DG and 50 μ M etomoxir.

2-DG is phosphorylated by hexokinase and generates a nonmetabolizable compound that inhibits both glycolysis and the PPP. Etomoxir is an inhibitor of CPT1 that catalyzes the rate-limiting step of FA β -oxidation.

MTT assays revealed that 2-DG treatment significantly affected the viability of normal tubular and HG ccRCC cells

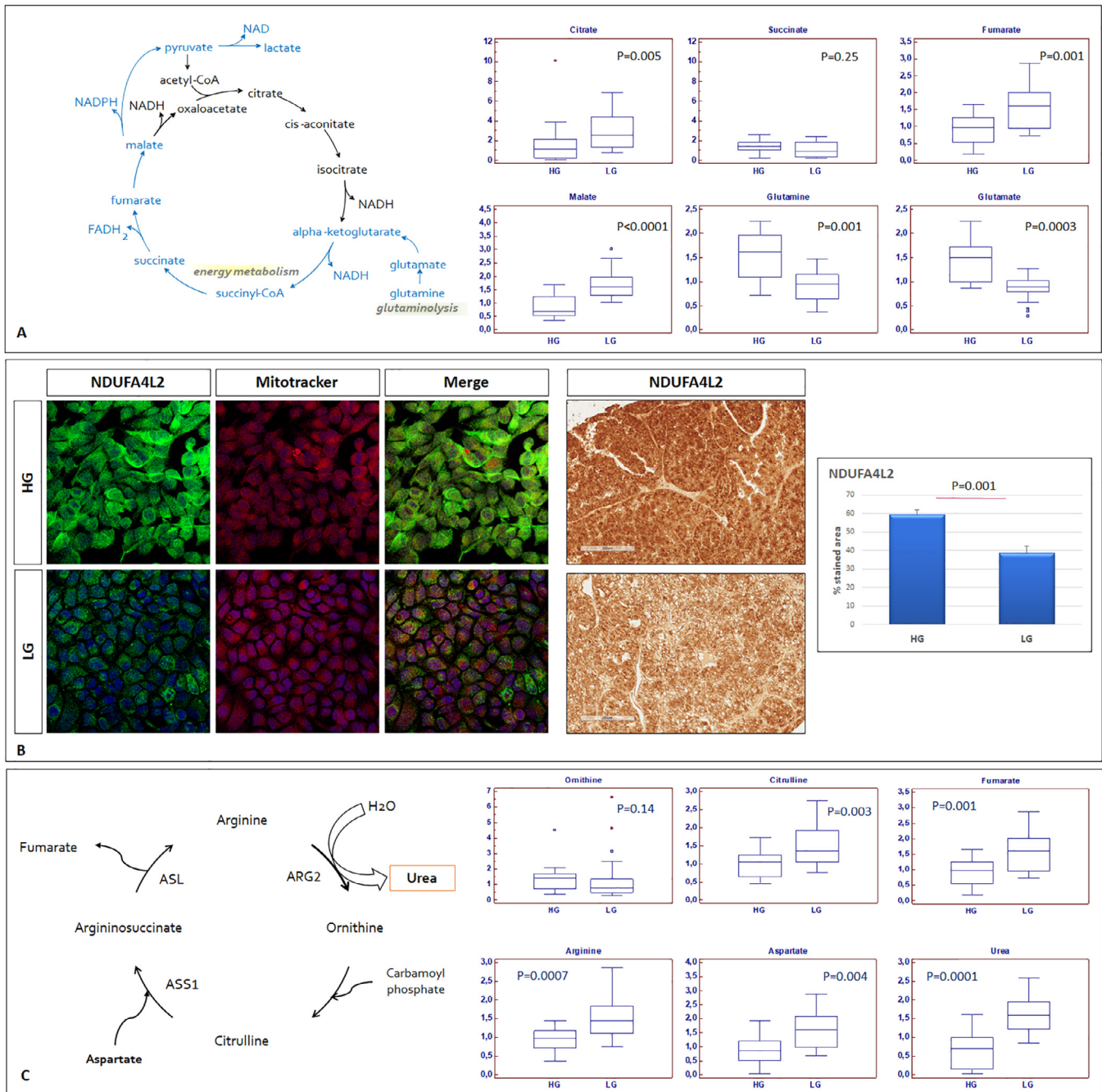


Figure 4. Schematic model summarizing the differences in tricarboxylic acid (TCA) cycle metabolites between HG and LG ccRCC (A). Immunofluorescence showing the differences in NDUFA4L2 and MitoTracker signal levels between HG and LG ccRCC (B). Differences in urea cycle metabolites between HG and LG ccRCC (C). Y-axis: relative metabolite amount.

but not of LG cancer cells, and these effects were inhibited by treatment with etomoxir (Fig. 6A).

Next, we evaluated the role of these specific metabolic pathways in regulating cancer cell resistance to cisplatin (CDDP)-induced cytotoxicity. HG- and LG-primary cancer cells were treated with CDDP in the absence or presence of their specific inhibitor. In particular, after cisplatin treatment, the death rate of HG primary cells treated with 2-DG was significantly greater than that of untreated cancer cells ($P < 0.001$; Fig. 6B). Similar results were observed in LG

cancer cells treated with etomoxir before cisplatin administration ($P < 0.001$; Figure 6B).

3.9. The presence of sarcomatoid features and their different abundances characterize a specific subset of ccRCC

To better characterize the biological and prognostic features of sRCC, we performed a metabolomic subanalysis in the HG group on the basis of the abundance of the

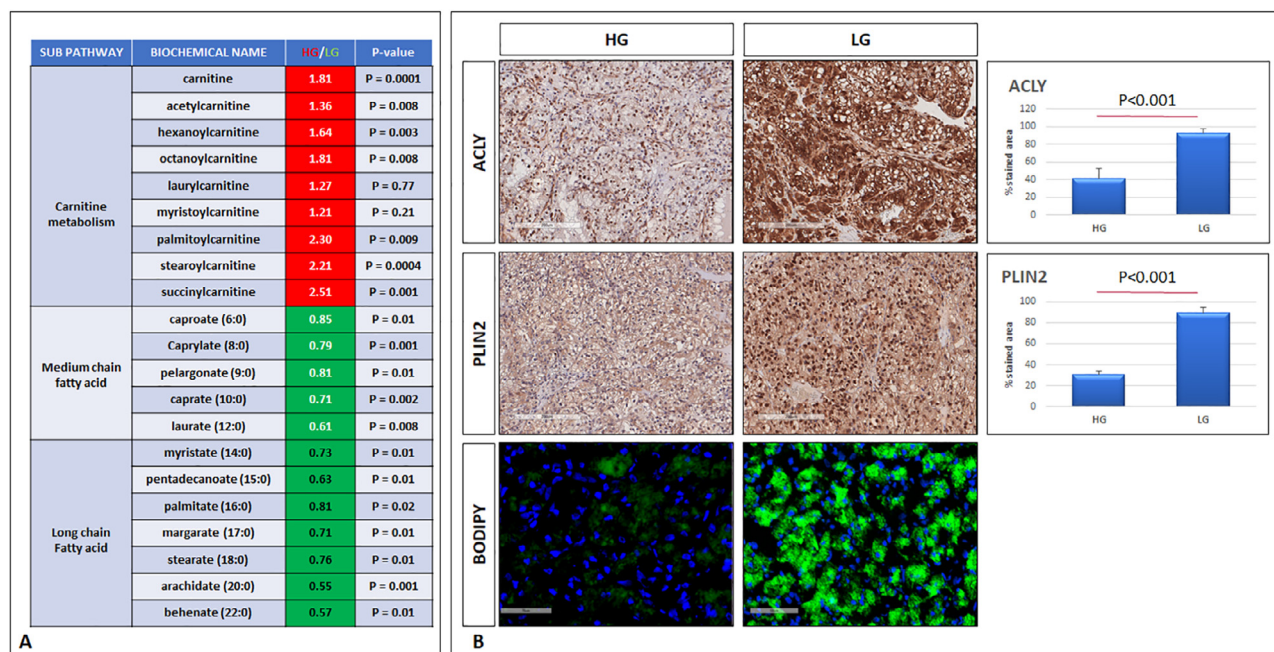


Figure 5. Alterations in fatty acid and acylcarnitine metabolism in HG vs. LG ccRCC (A). Immunohistochemical analysis of ATP citrate lyase (ACLY) and perilipin2 (PLIN2) expression is shown. Bodipy dye was used for lipid droplet visualization (B).

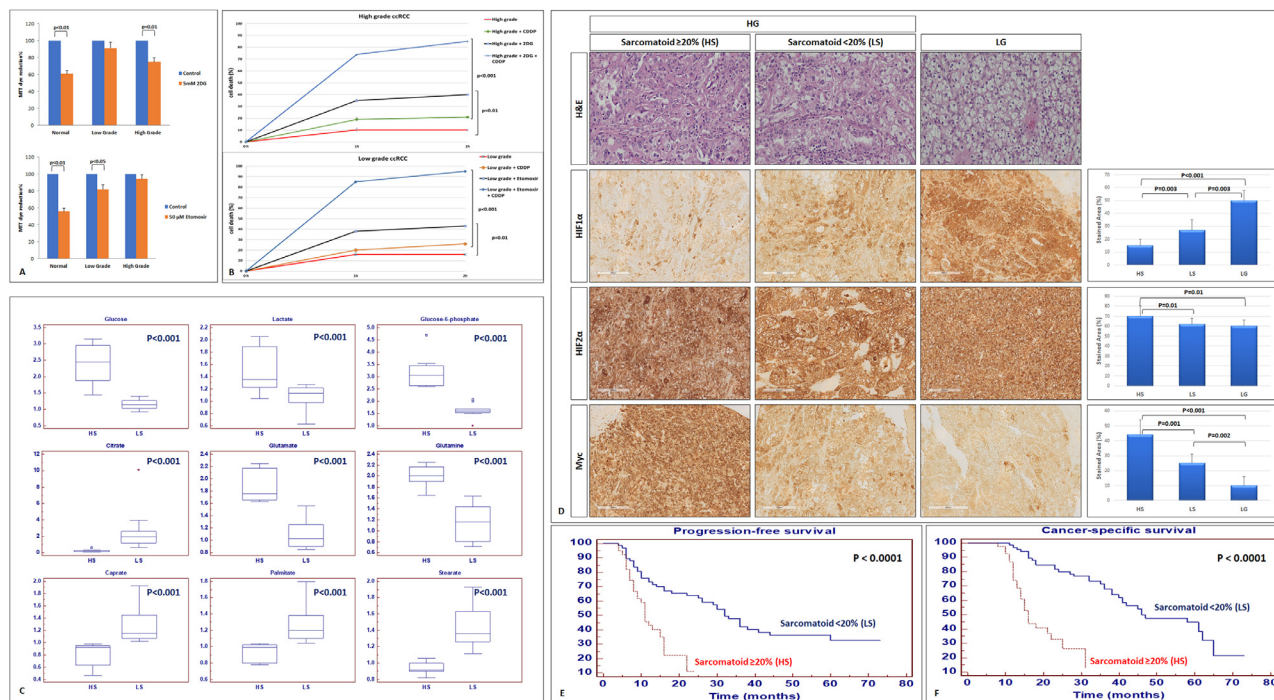


Figure 6. MTT assay showing that 2-DG treatment significantly affects the viability of normal tubular and HG ccRCC cells but not of LG-cancer cells, which are instead inhibited by treatment with Etomoxir (A). Grade-specific metabolic pathways regulate cancer cell resistance to cisplatin (CDDP)-induced cytotoxicity. After CDDP treatment, the death rate of HG primary cells treated with 2-DG is significantly greater than that of untreated cancer cells. Similar results are observed in LG-cancer cells treated with Etomoxir before CDDP administration (B). Metabolites accumulate differentially between high-sarcomatoid (HS; $\geq 20\%$ sarcomatoid component) and low-sarcomatoid (LS; $< 20\%$ sarcomatoid component) tumors (Y-axis: relative metabolite amount)(C). Immunohistochemical analysis of HIF-1 α , HIF-2 α , and Myc expression in HS vs. LS ccRCC (D). Kaplan-Meier progression-free survival (PFS)(E) and (cancer-specific survival (CSS) (F) curves, stratified by abundance of sarcomatoid component (HS vs. LS).

sarcomatoid component. In particular, HG ccRCC patients were subclassified into low-sarcomatoid (LS; <20% sarcomatoid component; $n=10$) and high-sarcomatoid (HS; $\geq 20\%$ sarcomatoid component; $n=7$) groups.

HS tumors exhibited increased accumulation of the glycolytic intermediates glutamate and glutamine, while LS tumors had increased levels of citrate and fatty acids, suggesting that metabolic reprogramming differs between these 2 subtypes of HG ccRCC (Fig. 6C). Previous studies have demonstrated that sRCC are characterized by differences in their transcriptomic profile compared to nonsarcomatoid RCC [21–24].

Therefore, we analyzed the expression of HIF-1 α , HIF-2 α , and Myc in LG, LS and HS tumors. We found a progressive decrease in the expression of HIF-1 α throughout ccRCC progression from LG to HG tumors with abundant sarcomatoid features. Conversely, Myc was highly expressed in HS tumors [25]. HIF-2 α was highly expressed in all subtypes and showed modest grade-dependent modulation, confirming the leading role of this transcription factor in ccRCC (Fig. 6D) [26].

Next, we evaluated the association between patient survival and the abundance of sarcomatoid components in a cohort of 122 patients with sRCC (Supplementary Table 2). Kaplan–Meier survival curves for CSS and PFS stratified by the abundance of sarcomatoid components are shown in Fig. 6E. CSS and PFS were significantly lower in patients with a sarcomatoid component $\geq 20\%$. Multivariate Cox regression analysis revealed that the abundance of the sarcomatoid component was an independent adverse prognostic factor for CSS and PFS (Supplementary Table 3).

4. Discussion

Determining tumor grade is a powerful and practical tool for understanding the biology of ccRCC, and furthermore, this morphological parameter has important prognostic implications.

For many years, the most used system for grading ccRCC has been a morphological system described by Fuhrman et al. [27], that evaluated both nuclear shape and size and nucleolar prominence. In the next years, the International Society of Urologic Pathologists (ISUP) proposed a novel, validated grading system [4] with a better prognostic performance compared to the previous system. This grading procedure is quite simple and reproducible in a standard pathology laboratory, however, the molecular components that underlie the determination of tumor grade are unknown.

Therefore, we used a multi-omics approach to integrate data derived from metabolomics, transcriptomics and proteomics studies with the aim of clarifying the molecular characteristics underlying the pathological grade.

We showed that the Warburg effect, in association with changes in Krebs cycle intermediates and related metabolites, was more prominent in HG ccRCC than in LG

ccRCC. These alterations described in glucose metabolic pathway are in accordance with previous studies that showed how the metabolic flux through glycolysis is differentially regulated, and mitochondrial bioenergetics and oxidative phosphorylation are impaired [17,28,29]. The impaired mitochondrial bioenergetics and increased glucose utilization through the pentose phosphate pathway especially in HG ccRCC indicate oncogenic signaling pathways promoting cancer through altered sugar metabolism. NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 (NDUFA4L2), a HIF-1 target gene highly expressed in ccRCC, plays a crucial role in bioenergetics, cell proliferation, migration, and angiogenesis [17]. Increased expression of NDUFA4L2 in HG ccRCC, is in accordance with the metabolic signature characterized by an anaerobic switch and impaired mitochondrial activity described in these tumors.

Additional alterations included metabolic reprogramming in the urea cycle, modulation of glutathione metabolism with accumulation of the reduced form of glutathione in HG tumors, and progressive increases in the levels of carnitine derivatives in a grade-dependent manner.

A previous study showed that ccRCC is characterized by alterations in ammonia metabolism, in association with reduced expression of multiple urea cycle enzymes [18]. This program of repression of urea cycle enzymes is intended to support biomass expansion through 2 mechanisms: maintaining elevated levels of pyridoxal-5'-phosphate for glycogen production and amino acids synthesis, and avoiding toxic accumulation of polyamines [30]. In this scenario, HG tumors were characterized by reduced production of citrulline and urea, and consequently by reduced accumulation of putrescine and spermidine.

Lipid metabolism has a fundamental role in RCC pathogenesis. De novo lipogenesis, elongation, and desaturation processes contribute to the production of lipids essential for tumor growth and progression. Dysregulation of enzymes like SCD1 and ELOVLs has been associated with enhanced cancer cell survival and migration, highlighting potential therapeutic targets [17,19,20].

In our study, lipid metabolism evaluation revealed increased accumulation of long- and medium-chain FAs in LG ccRCC, and these findings were confirmed by Bodipy staining of tissue samples. In contrast, FA-carnitine conjugates were increased in HG ccRCC, and these findings suggest that LG tumors preferentially synthesize and accumulate FAs compared to HG ccRCC that catabolize FAs for energetic purpose and synthesis of other molecules.

RCC is a typically chemo-resistant tumor. In the last years, a better understanding of the molecular basis of this cancer has led to identification of novel therapeutic targets, although the new molecules introduced into clinical practice yielded only partial responses, with no evidence of complete regression of metastases [31–35]. In consideration of the grade-specific metabolic reprogramming, we hypothesized that the inhibition of specific metabolic

pathways in HG and LG tumor-derived primary cancer cells could decrease cell viability and improve cisplatin susceptibility.

To explore this hypothesis HG and LG renal cancer cells were exposed to specific inhibitors of glucose and lipid metabolism. Our findings showed that HG- and LG-primary cancer cells treated with cisplatin in the presence of their specific inhibitor (2-DG for HG cancer cells, and Etomoxir for LG cancer cells), regain their chemosensitivity.

Sarcomatoid dedifferentiation represents one of the most aggressive features of ccRCC, and its molecular characteristics are poorly defined [36]. Bakouny et al. found that sRCC harbor distinctive genomic alterations (such as BAP1 mutations and CDKN2A deletions) that account for their aggressive biology [23]. In addition, sRCC is characterized by increased CD8+ T cell infiltration and PD-L1 expression, and by an immune-inflamed microenvironment, which makes it highly responsive to immune checkpoint inhibitors.

In this study, we found that sRCC is characterized by a metabolic profile distinct from that of HG tumors. This metabolic reprogramming is associated with progressively increased expression of Myc and HIF-2 α and reduced expression of HIF-1 α , probably with the aim of supporting the Warburg effect and simultaneously activating epithelial–mesenchymal transition. Finally, we evaluated the prognostic value of sarcomatoid component abundance in a cohort of patients with localized/locally advanced HG ccRCC. Patients with tumors characterized by abundant sarcomatoid features ($\geq 20\%$) had reduced PFS and CSS compared to those with low-sarcomatoid ccRCC, indicating the need to stratify patients with sRCC according to the extent of this pathological component to better define their prognostic risk.

The strength of our study is that it shows in ccRCC the existence of a grade-specific metabolic reprogramming, confirmed by the analysis and integration of data derived from different omics platforms. In addition, we demonstrated the prognostic role of the abundance of the sarcomatoid component in high grade tumors. The most important criticism is that the same tumor tissue was not used for the different omics platforms. Moreover, the prognostic value of sarcomatoid component abundance needs external validation, especially in the identification of an exact cut-off that allows the distinction between the different risk groups.

5. Conclusions

In this study, we aimed to determine the molecular characteristics of kidney tumors according to pathological grade. We found that ccRCC is characterized by particular grade-dependent metabolic alterations that could represent novel potential therapeutic targets. In addition, we showed that subclassification based on the abundance of intratumoral sarcomatoid component has a clinical significance. Grade 4 ccRCC subclassification into HS and LS groups

has a prognostic impact in terms of CSS and PFS, and it should be considered as a novel parameter for risk groups stratification in future clinical trials.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the University of Bari IRB (n. 143/CE/2015). Written informed consent to take part was given by all participants.

Consent for publication

Not applicable.

Availability of data and materials

Clear cell-RCC transcriptome data (GSE40435, GSE150404) were sourced from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>).

KIRC-TCGA expression data were retrieved from the TCGA database via UCSC Xena (<https://xenabrowser.net/>).

For proteomic analysis, Perroud dataset was used (DOI: 10.1074/mcp.M800252-MCP200).

Funding

No funding or other financial support was received.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Giuseppe Lucarelli: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Francesco Lasorsa:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Martina Milella:** Methodology, Investigation, Data curation. **Antonio d’Amati:** Methodology, Investigation, Data curation. **Giuseppe Ingravallo:** Methodology, Investigation. **Mariella Silecchia:** Methodology, Investigation. **Mariella Errede:** Methodology, Investigation. **Cristina Bianchi:** Methodology, Investigation. **Marco Spilotros:** Investigation, Data curation. **Michele Battaglia:** Supervision, Data curation. **Pasquale Ditunno:** Validation, Supervision. **Monica Rutigliano:** Validation, Methodology, Investigation, Data curation.

Acknowledgments

Not applicable.

Supplementary materials

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.urolonc.2025.02.016>.

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