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Evaluation of the metabolomic response of renal cell carcinoma cells to sunitinib and pazopanib by NMR metabolomics

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Abstract

Kidney cancer is the 3rd most prevalent urologic cancer worldwide, with an overwhelming majority being attributed to renal cell carcinoma (RCC). Due to the lack of symptoms until the disease has progressed, most cases are detected by chance during abdominal scans or by the presence of blood in the urine. Most kidney cancer treatment options rely on surgical techniques to remove a portion of the kidney, but this is no longer ideal when the disease has spread beyond the kidney cortex, resulting in a poor prognosis and outcome. Thus, to fully cure the patient, a systemic treatment approach that targets the metastatic cells that have spread rampantly throughout the body must be considered and implemented.

Novel therapies targeting specific genes and proteins involved in cancer cell growth and survival have emerged in recent years. Sunitinib and pazopanib, two tyrosine kinase inhibitors (TKIs), have been used as first-line therapy for advanced RCC. The present work aimed at providing new insights into the cytotoxicity and metabolic response of human metastatic RCC (Caki-1) cells exposed to these TKIs by combining traditional toxicological endpoints (MTT reduction and LDH leakage assays) with a metabolomics approach. Clinically relevant concentrations of sunitinib (2 μM) and pazopanib (50 μM) were used to further elucidate their metabolic impact. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to assess changes in the intracellular (endometabolome) and extracellular (exometabolome) metabolic profiles.

Both TKIs caused a concentration-dependent loss of cell viability, as measured by the MTT assay. Notably, no cellular membrane rupture was observed using the LDH assay up to a maximum concentration of 200 μM pazopanib. Metabolome analysis unveiled a higher impact of sunitinib on the endometabolome of Caki-1 cells (seven dysregulated metabolites), suggesting dysregulations on amino acid, glutathione and glycerophospholipid metabolisms. In contrast, pazopanib had a higher impact on the exometabolome of Caki-1 cells (seven dysregulated metabolites in culture medium), unveiling alterations on amino acid and energetic metabolisms. These findings demonstrate that sunitinib and pazopanib elicit distinct cytotoxic and metabolic responses. Characterization of the impact of these two targeted therapies in metastatic RCC can lead to a better understanding on their mechanism of action at the cellular level, therefore having the potential to improve existing therapeutic protocols in advanced stages of RCC, significantly improving clinical outcomes.

Keywords: renal cell carcinoma; tyrosine kinase inhibitors; cell viability assays; metabolomics; nuclear magnetic resonance spectroscopy

Resumo

O cancro renal é o 3º cancro urológico mais prevalente mundialmente, sendo uma grande maioria atribuída ao carcinoma das células renais (RCC). Devido à ausência de sintomas até que a doença se apresente em estado avançado, a maioria dos casos é detetada por acaso durante as tomografias abdominais ou pela presença de sangue na urina. A maioria das opções de tratamento para o cancro renal depende principalmente de abordagens cirúrgicas para remover parte do rim afetado, mas essa intervenção não é a ideal quando a doença já se espalhou para além do córtex renal, levando a prognósticos e resultados não favoráveis ao paciente. Assim, uma abordagem terapêutica sistémica deve ser considerada e aplicada a fim de atingir as células metastáticas espalhadas por todo o corpo.

Novas terapias direcionadas para genes e proteínas específicas envolvidas no crescimento e sobrevivência de células cancerígenas têm vindo a surgir nos últimos anos. O sunitinib e o pazopanib, dois inibidores da tirosina quinase (TKIs), têm sido usados como terapia de primeira linha para o RCC avançado. O presente trabalho teve como objetivo fornecer novos conhecimentos acerca da citotoxicidade e resposta metabólica das células metastáticas humanas de RCC (Caki-1) após exposição a esses TKIs, através da combinação de parâmetros toxicológicos tradicionais (ensaios de redução do MTT e do LDH) com uma abordagem metabolómica. Concentrações clinicamente relevantes de sunitinib (2 μM) e pazopanib (50 μM) foram usadas para elucidar melhor o impacto metabólico nas células. A espectroscopia de ressonância magnética nuclear de protões (RMN 1H) foi usada para avaliar as alterações nos perfis metabólicos intracelular (endometaboloma) e extracelular (exometaboloma).

Ambos os TKIs causaram uma perda de viabilidade celular dependente da concentração, conforme medido pelo ensaio MTT. De acordo com o de ensaio LDH, não foi observada alteração da viabilidade celular provocada pelo pazopanib até uma concentração de 200 μM. A análise do metaboloma revelou um maior impacto do sunitinib no endometaboloma das células Caki-1 (sete metabolitos desregulados), sugerindo alteração no metabolismo de aminoácidos, glutationa e glicerofosfolipídios. Em contrapartida, o pazopanib teve maior impacto no exometaboloma das células Caki-1 (sete metabolitos desregulados em meio de cultura), revelando alterações no metabolismo dos aminoácidos. Estes resultados demonstram que o sunitinib e o pazopanib provocam respostas citotóxicas e metabólicas distintas. A caracterização do impacto destas duas terapias direcionadas no RCC metastático pode levar a uma melhor compreensão do seu mecanismo de ação a nível celular, tendo assim potencial para melhorar os protocolos

terapêuticos existentes em estadios avançados do CCR, melhorando significativamente os resultados clínicos.

Palavras-chaves: carcinoma das células renais, inibidores da tirosina quinase; ensaios de viabilidade celular; metabolómica; espectroscopia de ressonância magnética nuclear

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Abbreviations and acronyms

AJCC - American Join Committee on Cancer
ANOVA - Analysis of Variance
BMI - Body Mass Index
CCLE - Cancer Cell Line Encyclopedia
ccRCC - Clear Cell Renal Cell Carcinoma
CE-MS - Capillary Electrophoresis-Mass Spectrometry
CE-TOFMS - Capillary Electrophoresis-Time of Flight Mass Spectrometry
chRCC - Chromophobe Renal Cell Carcinoma
CT- Computed Tomography
DFS - Disease-Free Survival
DMSO - Dimethyl Sulfoxide
ECACC - European Collection of Cell Cultures
ECM - Extracellular Matrix
FBS - Fetal Bovine Serum
FDA - Food and Drug Administration
FDR - False Discovery Rate
GC-MS - Gas Chromatography-Mass Spectrometry
HDAC - Histone-Deacetylase
HSQC - Heteronuclear Single Quantum Coherence
IFN-α – Interferon-α
IL-2 – Interleukin-2
IMCD - International Metastatic Renal Cell Carcinoma Database Consortium
KC - Kidney Cancer
LC-MS - Liquid Chromatography-Mass Spectrometry
LDH - Lactate Dehydrogenase
mRCC - Metastatic Renal Cell Carcinoma
MS - Mass Spectrometry
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMR - Nuclear Magnetic Resonance Spectroscopy
ODC - Optimal Multidrug Combination
OPLS-DA - Orthogonal Partial Least Square Discriminant Analysis
ORR - Overall Response Rate
OS - Overall Survival

PCA - Principal Component Analysis

PD-1 - Programmed Cell Death-1

PFS - Progression-Free Survival

PDGF - Platelet-derived Growth Factor

PLS-DA - Partial Least Square Discriminant Analysis

PPP - Pentose Phosphate Pathway

pRCC - Papillary Renal Cell Carcinoma

QC - Quality Control

RCC - Renal Cell Carcinoma

ROS - Reactive Oxygen Species

TCA - Tricarboxylic Acid Cycle

TKI - Tyrosine Kinase Inhibitor

TNM – Tumor, Nodes, Metastases

TOCSY - Total Correlation Spectroscopy

TSA - Trichostatin A

UPLC-MS - Ultra-performance Liquid Chromatography-Mass Spectrometry

VEGF - Vascular-Endothelial Growth Factor

VHL - Von Hippel Lindau Gene

1. Introduction

The kidneys are a pair of bean-shaped organs fundamental to the excretory functions. They are located in the abdomen, in its back portion. The main role that the kidneys hold within the human body is the purification and removal of water-soluble metabolites and by products through the urine. Second only to the liver, it has the highest rate of metabolization of compounds (Lara and Jonasch, 2015), primarily through the formation of glucuronide and glycine conjugates of several drugs and their metabolites (Vree et al., 1992). In addition, they also produce essential hormones, such as renin, a hormone that helps to control blood pressure and is a key player of the renin-angiotensin balance, and erythropoietin, the main hormone that regulates the development and differentiation of precursor cells into red blood cells and the global oxygenation of the body (Lara and Jonasch, 2015). The kidneys are also responsible for the reabsorption of several different essential minerals and amino acids through the proximal and distal tubules and to regulate the body's fluid balance (Figure 1). Moreover, they are responsible for preserving the acid-base homeostasis and the excretion of excess nitrogen from protein breakdown in the form of urea (van de Poll et al., 2004). Atop the kidneys are the adrenal glands that are responsible for the production of a plethora of different hormones.

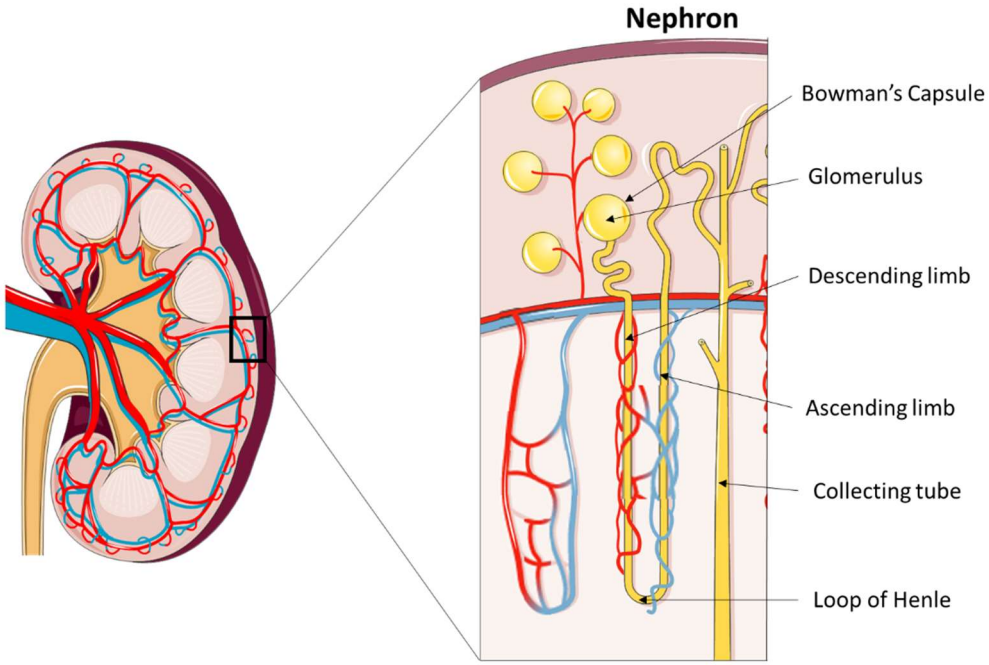


Figure 1 - Kidney and nephron anatomy.

Numerous pathologies can affect the kidneys, such as kidney stones, renal cell death due to toxic compounds (such as platinum compounds), among others. Notably, the kidney cells can also develop uncontrollable cell growth, subsequently leading to cancer (Webster et al., 2017).

Kidney cancer (KC), or renal cancer, is one of the main pathologies that can affect the kidneys and is characterized by an uncontrollable growth of cells eventually compromising the global function of the kidneys, rendering the organ without function. An overwhelming majority (85%) of the renal cancers are renal cell carcinomas (RCC), that stem from a primary growth in the proximal renal tubules. Rarer cases of KC can arise, such as transitional cell carcinoma or Wilms Tumors (Lerner et al., 2012). In the scope of this work, we will focus mostly on RCC.

Worldwide, KC represents about 2.2% of all new cancer diagnoses and 1.8% of all cases of mortality (Sung et al., 2021). The number of new cases for incidence and mortality are higher in men than in women (Figure 2) (World-Health-Organization-International-Agency-for-Research-on-Cancer, 2020). An early diagnosis is critical in the management of KC. In terms of survival rate, patients who present with localized KC have a 95% survival rate for 5 to 10 years, while patients who present with advanced disease have only an 18% chance for a 2-year survival (Linehan et al., 2009).

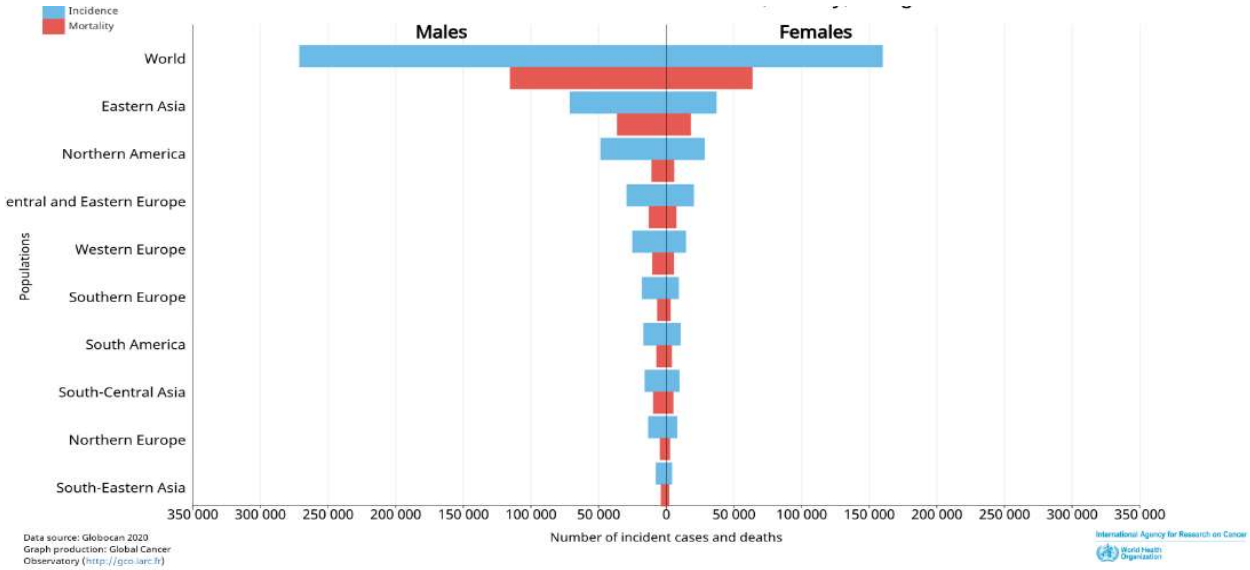


Figure 2 - Graphic representing the estimated number of new cases of kidney cancer in 2020 for incidence and mortality for both genders. Source: GLOBOCAN 2020.

Because KC arises from several different histological features and enzyme expression, genetic alterations and clinical processes, treatment options are determined based on the differences in these factors (Linehan et al., 2019). The first line treatment is always, whenever possible, surgical resection of the tumor (Lerner et al., 2012). However, this procedure is only feasible in localized tumors. At advanced stages, where the tumor has spread beyond the kidney's cortex, other forms of therapy should be considered. Herein, immunotherapy or targeted therapy have been emerging and cementing itself as potential treatment options (Hammers, 2016). Sunitinib is a multitargeted tyrosine kinase inhibitor that targets multiple different receptors, most notably platelet-derived growth factor (PDGF) receptors and vascular endothelial growth factor (VEGF) receptors. These two receptors are responsible for the neo-angiogenesis and tumor cell proliferation. It was approved as the first-line treatment for patients in advanced stage RCC (Tourneau et al., 2007). Multiple studies have validated sunitinib as a *de facto* first line treatment option for metastatic RCC, with improvements in both survival rate and progression-free survival (Moran et al., 2019a). Similarly, pazopanib is a PDGF and VEGF receptor inhibitor, but clinical trials have shown that it is more tolerable for administration without the need for alternating treatment schemes (Motzer et al., 2013)

Understanding the impact that both sunitinib and pazopanib currently have on their target cells and the cells surrounding the kidney is important for a better knowledge about its role in therapeutics and the overall impact on the quality of life of an individual. Metabolomics emerges as a promising area that can provide critical insights into some of these questions. This approach, in particular, can be employed to identify early biomarkers of certain cancers, as well as to improve the knowledge of disrupted metabolic pathways, and pathways involved in chemotherapy resistance (Chen et al., 2020). This stems mostly from the fact that cancer cells are highly metabolically active, leading to the production of by-products that can be clearly differentiated from healthy cells. Pharmacometabolomics, a branched field of metabolomics, has proven to be a powerful tool to deepen the knowledge on the mechanisms involved in drug mode of action and their particular toxicokinetic profile, in order to study adverse drug reactions either in single therapy or through combined therapy. This knowledge has the potential to improve existing therapeutic protocols in advanced RCC, leading to a better toxicity management and overall improvement in the quality of life for all patients.

1.1 Renal Cell Carcinoma: staging, histologic subtypes, and treatment

Renal cell carcinoma (RCC) is a disease characterized by uncontrollable growth of cells originating from the renal epithelium, with each type differing in location and primary cell growth. It can be further divided upon the current stage of the cell growth. Normally, two different classification types are used in order to adequately assess its stage and overall spread: the Robson grade and the tumor, nodes, and metastases (TNM) classification (Brierley et al., 2016). TNM is usually preferred over the Robson grade. Once the tumor has infiltrated deep into the bloodstream or the lymph system, and spread to other organs, it is deemed a metastatic growth (Delahunt et al., 2021). Typically, this occurs at later stages of the tumor, and is directly correlated with higher rates of mortality. The Robson grade typically relates the tumor location and where it has disseminated upon to assess a prognosis of the disease. It is graded from stage I to IV, being stage I a localized growth inside the kidney capsule, while stage IV implies glandular and tissue invasion (such as the adrenal glands, located above the kidneys) or the appearance of secondary metastatic growths in other organs (commonly the lung) (Kinouchi et al., 1999). In the TNM classification, that was proposed by the American Join Committee on Cancer (AJCC), the tumor grading is based on 3 main parameters:

a) T (Tumor) associated to a number, typically refers to the location and size of the tumor. This can range from T0 where there is no evidence of a primary tumor, to T4 where the tumor expands beyond the renal capsule;

b) N (Node) relates to the existence of tumor cells at the lymphatic glands, identifying which and how many of these glands have tumor cells. It can range from N0, where there is no presence of metastases on regional lymphatic nodes to N2 where there is the presence of tumor cells in more than one lymphatic gland;

c) M (Metastases) which evaluates the presence of metastases directly on organs. It can be classified as M0, where no metastasis are detected or M1 where there are distant metastases from where the tumor was originally developed (Telloni, 2017).

Similar to the Robson grade system, in the TNM classification, the patient is categorized on a certain risk group, and the prognosis and the type of treatment to follow is selected.

In order to fully characterize the disease, the tumor has to be assessed based upon its histological features and the main cell from it has developed. These means that there are several phenotypes of RCC, and subsequently different clinical courses, and responses to

therapy, which are caused by mutations in different genes (Linehan et al., 2019). So far, at least 17 different genes are known that can cause different variations of RCC. These gene mutations can affect the cells ability to respond to changes in oxygen, iron, nutrients, among others (Linehan et al., 2019). RCC has numerous risk factors that can exacerbate the risk of developing the disease. Some of these factors can be controlled, such as heavy smoking, obesity and acquired hypertension, while some can be intrinsic, such as genetic factors. As with other types of cancer, the existence of a family history of RCC can lead to a higher risk to develop RCC (Clague et al., 2009).

RCC can be divided into five diverse subtypes based on their histopathologic features, being the most commonly occurring, the clear cell renal cell carcinoma (ccRCC), followed by papillary renal cell carcinoma (pRCC), chromophobe renal cell carcinoma (chRCC), and rare types of RCC, such as collecting-duct carcinoma, also known as Bellini duct carcinoma, and renal oncocytomas. Each subtype can then present common mutation, namely morphologic alterations, such as sarcomatoid differentiation, which generally carries a poor prognosis (D'Avella et al., 2020). With a particular focus on ccRCC, an interesting discovery is the consistent inactivation of the Von Hippel-Lindau (VHL) tumor suppressor gene. Through the discovery of this suppression, therapeutic targets on the mTOR and VEGF pathways were developed, significantly improving therapeutic outcomes. It was further demonstrated that the reintroduction of a wild-type VHL in tumor cells that lack VHL expression cause significant lost metastatic potential *in vitro (*Cowey and Rathmell, 2009*).* Further studies identified several other mutations, such as PBRM1, BAP1, SETD2, and KDM6A. ccRCC is most closely associated with mutations, but it also has a high degree of hypermethylation.

From a clinical point of view, ccRCC is characterized by the lack of symptoms at early stages, evolving then to a multitude of different clinical manifestations. Typically, an initial diagnosis of the disease is given only in advanced stages of the disease, although most are found accidentally in routine exams. Image-based studies, such as ultrasounds or CT scans are the common first line exams, followed by biopsies in order to correctly diagnose the type of RCC. Macroscopically, ccRCC is characterized by a golden yellow color lesion, but this can vary depending on the stage of the tumor. It is defined by a high variety of the growth patterns, and also present a high degree of vascularization, due to the consistent upregulation of VEGF receptors (Petejova and Martinek, 2016). Furthermore, ccRCC is characterized by an innate resistance to chemotherapy and radiotherapy (Ata-ur-Rehman et al., 2015). Referring to pRCC, it usually originates mostly from two different familial syndromes, that increase the risk of papillary-type differentiation of RCC, being hereditary pRCC. This is an autosomal dominant syndrome that stems from a mutation of the MET

gene on chromosome 7, region q31 (Fernandes and Lopes, 2015). The other type of pRCC is commonly associated with Hereditary Leiomyomatosis, and commonly affects patients at earlier ages. This type originates from a mutation of the fumarate hydratase gene, resulting in a deficiency in the Krebs cycle. Histologically, it is characterized by multifocal growth, with frequent necrosis observed microscopically. Moreover, the cancer cells typically have a large nucleus with hemosiderin inclusions in the cytoplasm (Merino et al., 2007).

As for chRCC, it can be divided into two subtypes, the classic and oncocytic, as well as holding an hereditary disease, named Birt-Hugg-Dubé syndrome, caused by mutations in the folliculin gene on chromosome 17 region p11.2 (Schmidt et al., 2001).

RCC therapeutic outcome is primarily dependent upon the stage of the primary tumor. Early stage RCC has higher rates of survival than advanced stages of the disease. Localized RCC can be treated with partial or radical nephrectomy, ablation or active surveillance. Typically, surgery is always the first therapy option, as it completely removes the tumor cells, and tends to prevent a recurrence of the tumor growth. However, the tumor must be localized and of a certain size for surgery to be considered. Partial nephrectomy is employed mostly in very small RCC wherein just a small portion of the affected kidney is removed. This type of surgery allows the patient to maintain both kidneys function intact. In more advanced types of RCC, a full kidney removal is performed, leaving the patient with only one working kidney. Unfortunately, ~30% of patients with this localized disease can and will eventually develop metastases, which is associated with a higher mortality and will require therapy against the VEGF and mTOR pathways. However, treatment response is varied and most patients still progress into advanced stages of the disease (Hsieh et al., 2017). Currently, to adequately select the first-line of treatment, the most important factor is the tumor stage. The 5-year recurrence-free survival at stage I is >95% while at stage III this number drops to just below 40% (Janowitz et al., 2013). Several models are used to predict outcomes and classify the risk of disease progression in order to assess the state of RCC after surgical resection. It is based upon stage, tumor size, presence or absence of necrosis and the nuclear grade of the cells (Barata and Rini, 2017).

Before the recognition of biomarkers as tools that can be used in the diagnose or the emergence of targeted therapy, the standard therapy was an immunotherapy approach with interferon-α (IFN-α) and/or interleukin-2 (IL-2). However, the patients' responses revealed high toxicity and a low overall response rate (ORR) (Garcia and Rini, 2007). Other approaches were tested in various clinical trials, such as hormone therapy, chemotherapy, and radiotherapy, but all failed to demonstrate any clinical benefit. Nowadays, there are several clinical trials based on newly discovered targets, such as the use of girentuximab,

a monoclonal antibody against carbonic anhydrase IX (Belldegrun et al., 2013). Perhaps the most impactful studies with antiangiogenic therapies and mTOR inhibitors, consist on the use of bevacizumab (Escudier et al., 2007), sorafenib, sunitinib (Motzer et al., 2007) and pazopanib (Sternberg et al., 2010). The most used targeted therapies for each type of RCC mentioned above are described in **Table 1**. An important finding from these trials was that these targeted therapies were effective in prolonging disease-free survival (DFS) in cases of ccRCC. However, a fine balance between benefit and tolerability must be established, as in all trials several off-target effects were demonstrated, with a significant number of patients having to scale down the dosage in order to lessen these toxic effects. Nonetheless, these VEGF and PDGF receptor inhibitors demonstrated superiority when compared to previous standard-of-care, with still clear improvements in quality of life, progression-free survival (PFS) (improved from 5.5 to 11 months) and overall survival (improved from 23 to 26 months) (Motzer et al., 2013; Motzer et al., 2007). A common practice in order to benefit from these improvements without submitting the patient to severe off-target effects is the use of alternative delivery schedule, such as a 2-weeks on/1-week-off, or a full out treatment break (Ornstein et al., 2017). A different tentative approach, this one bringing back the old setting of immunotherapy is the use of programmed cell-death 1 inhibitors (PD-1), such as nivolumab and pembrolizumab (Barata and Rini, 2017). Lastly, other therapeutical approaches are currently under clinical trials, such as vaccines consisting of multiple tumor-associated peptides, or cell-based therapies focusing upon the activation of the CD-4 and CD-8 lymphocytes, recruiting them to target the cancer cells themselves, rather than the administration of any time of therapeutical agent (Amin et al., 2015; Walter et al., 2012).

With this in mind, the main caveat to treatment selection remains the need to fully characterize the disease, specifically histological characteristics, biomarker production, localization and hormone expression, to adequately select the treatment option that will yield the most efficient results from the start, without exposing the patient to severe off-target effects, or to a poor benefit-to-risk ratio.

Table 1 - Molecular targeted agents as treatment for the different types of RCC.

RCC types	Treatment	References
ccRCC	All IMDC risk patients: Pembrolizumab + Axitinib, Nivolumab + Cabozantinib OR Lenvatinib + Pembrolizumab (first line) IMDC intermediate- and poor-risk patients: ipilimumab + nivolumab (first line) Alternative: Sunitinib, Pazopanib or Cabozantinib	(Ljungberg et al., 2021)
pRCC	First line: Nivolumab + Ipilimumab OR Pembrolizumab OR Sunitinib/Sorafenib Second line: Axitinib Third line: Cabozantinib Situational: Everolimus + Bevacizumab	(Tachibana et al., 2021; Vera-Badillo et al., 2015)
chRCC	First line: Sunitinib OR Everolímus if non-sarcomatoid, Second line: Pazopanib OR Cabozantib Third line: Axitinib OR Atezolizumab + Bevacizumab	(Garje et al., 2021)
Bellini duct carcinoma	First line: Prefer chemotherapy Second line: Nivolumab OR Sunitinib	(Dason et al., 2013)

IMCD: International Metastatic Renal Cell Carcinoma Database Consortium (model for metastatic disease)

Both sunitinib and pazopanib, tyrosine kinase inhibitors (TKIs), were previously used as first-line treatments in metastatic ccRCC (Lalani et al., 2017; Ruiz-Morales et al., 2016; Wang et al., 2020). However, with the continuous development of more effective therapies in this area, the clinical practice guidelines for RCC were updated in the early 2020s (Aeppli et al., 2021; Powles et al., 2021). Recent studies that use TKIs alone (Choueiri et al., 2017) or in combination with immune checkpoint inhibitors (ICIs) (Rini et al., 2019) or combination between ICIs (Motzer et al., 2018) have shown better results comparing with sunitinib or pazopanib. In a recent study, lenvatinib, an antiangiogenic agent, and pembrolizumab, an anti-programmed cell death-1 (PD-1) monoclonal antibody, were tested in order to discover if they had the same efficacy when used in a combined regimen as compared when used as a standalone treatment option in ccRCC using sunitinib as the control (Motzer et al., 2021). The data showed a significant advantage for the combination lenvatinib plus pembrolizumab with a significantly longer progression-free survival and overall survival compared with sunitinib alone (Motzer et al., 2021). These findings led FDA to recommend lenvatinib plus pembrolizumab for first-line treatment of advanced ccRCC, regardless of International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk group (Powles et al., 2021). As lenvatinib plus pembrolizumab, others compounds/ combinations, such as ipilimumab plus nivolumab (Motzer et al., 2018), pembrolizumab plus axitinib (Rini

et al., 2019) and cabozantinib (Choueiri et al., 2018) have showed better results comparing with sunitinib. However, sunitinib and pazopanib are still alternatives to PD-1 inhibitor-based first-line combinations when immunotherapy is contraindicated or not available (Powles et al., 2021).

1.2 Metabolomics in cancer research

1.2.1. Definition and strategy

The area of metabolomics is the last in the cascade of “omics” sciences. This cascade also includes genomics, the study of the genes; transcriptomics, the study of transcriptomes and their function; and proteomics, the study of proteins (Tokarz et al., 2020). Metabolomics entails the comprehensive analysis of all metabolites involved in the metabolic network of living organisms and their response to pathophysiological or other stimuli (Nicholson et al., 1999). The metabolome comprises all small molecules with a molecular mass below 1200 Da, such as lipids, sugars and amino acids in a biological sample (Tokarz et al., 2020). Various biospecimens may be used in the metabolomics workflow, ranging from immortalized or primary cultured cells to biological fluids (e.g., saliva, urine, blood plasma/serum) and tissues (Graziani et al., 2018).

The first step of a metabolomics analysis consists in a rapid sample preparation, followed by the analysis of the sample by high-throughput analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS). Then, the complex raw data generated is gathered and analyzed by bioinformatic tools to achieve reliable biological interpretation (Chen et al., 2019).

The metabolomic approaches can be divided into two major categories: untargeted (global) or targeted metabolomics (**Figure 3**). These approaches differ in several factors, mainly in the objective and purpose of the experiment, but also in the complexity of the sample preparation, the number of metabolites detected, and the experimental accuracy and precision. The untargeted or global metabolomics, also known as metabolic profiling, aims to determine all measurable analytes simultaneously present in a sample (Tokarz et al., 2020; Wang et al., 2019a). It is used as a hypothesis-generating tool leading to the development of hypothesis that can be validated later by targeted approaches. The main advantage of the untargeted metabolomics approach is the collection of large amounts of raw data without the pre-existing knowledge of sample composition. In fact, this approach can potentially reveal altered metabolites and pathways previously unknown (Schrimpe-Rutledge et al., 2016). The difficulty to identify these unknown metabolites and the large amount of time to process all the raw data obtained are some of the disadvantages associated with this approach (Wang et al., 2019a). In addition, the untargeted approach is not focused on the absolute quantification of the metabolites, so it usually provides only

semi-quantitative data (Tokarz et al., 2020). On the other hand, the targeted metabolomics aims to determine the absolute concentration of a defined set of metabolites with similar chemical structure and/or a related biological function in order to test a specific hypothesis. In this approach, a standard calibration curve can be prepared for each metabolite for accurate quantification because all the metabolites in the study are already pre-selected based on the prior knowledge of the specific metabolic pathway included in the hypothesis (Tokarz et al., 2020). Compared with the untargeted approach, the targeted metabolomics is more precise and sensitive and has higher reproducibility, whilst presents lower false-positive results. One disadvantage of this approach is that the compound of interest for the study must be known *a priori* and it must be available in its purified form. In addition, the limited group of simultaneously quantified metabolites can lead to an increased risk of missing interesting biological processes (Tokarz et al., 2020; Turi et al., 2018). An intermediate option considering semi-targeted assays can also be adopted with the main goal to analyze large quantities of metabolites with a known chemical structure, defining approximate metabolite concentrations (Broadhurst et al., 2018).

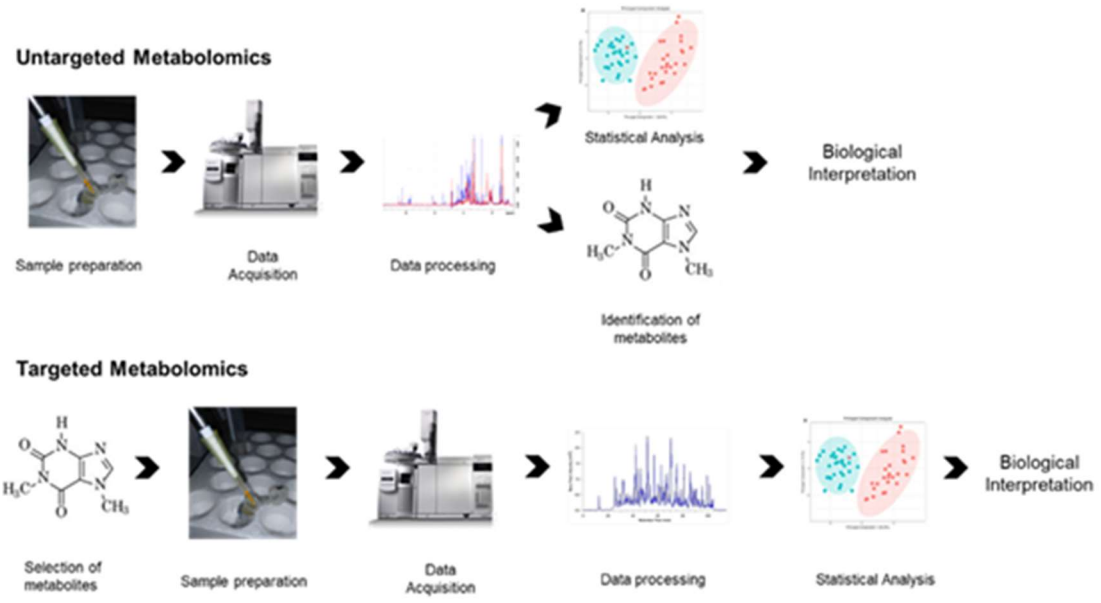


Figure 3 - Schematic representation of the untargeted and targeted metabolomics workflows

Metabolomics is gaining popularity in many areas of biomedical research, including oncology. Several studies in the literature show that certain metabolites can be candidate biomarkers for distinguishing tumoral from non-tumoral tissue (Henry and Hayes, 2012; Liu, 2019). Indeed, this approach can help to improve cancer diagnosis, monitoring and

treatment (Schmidt et al., 2021). Some clinical applications of metabolomics in cancer research include identification of cancer risk factors, discovery of biomarkers for cancer screening, diagnosis and monitoring, and basic overall cancer biology to understand the fundamentals of cell-cell communication and the tumor microenvironment (Schmidt et al., 2021).

Metabolites can become ideal biomarkers in cancer screening, diagnosis and monitoring because they can be easily measured from non-invasive samples, such as blood or urine (Vermeersch and Styczynski, 2013). In fact, this approach allows the identification of potential biomarkers by comparation of metabolite levels between healthy and unhealthy individuals. In recent years, several comprehensive reviews of metabolic biomarkers in the most diverse types of cancers have been published (Bamji-Stocke et al., 2018; De Matteis et al., 2021; Goh et al., 2020).

Cancer biology and therapeutics are two other clinical applications of metabolomics research that have recently received a lot of attention. The study of the cancer biology is very important because it enables the study of the dynamics in metabolic pathways, which can help to identify metabolites that are altered in specific cancers. Moreover, metabolomics can provide new insights to customize a specific treatment reaching to an individualizing therapy that will allow to achieve more adequate therapeutic outcomes than simply applying the standard therapy (Misra, 2020).

1.2.2. *In vitro* models for cancer metabolomic studies

As cancer is a widespread disease in the modern world, there is the innate necessity to study cancers adequately outside of *in vivo* systems in order to get a complete understanding of the disease. Through *in vitro* models we are able to rapidly test the interaction of certain drugs with the cancer microenvironment and assess its therapeutic potential without the need of performing experiments on animals. We are also able to gain critical insights on the behavior of a tumor, namely in invasion potency, extracellular matrix remodeling, and extravasation (Hulkower and Herber, 2011). Models can vary in their constitution and complexity, ranging from primary cell lines to 3D models of the tumor microenvironment (Katt et al., 2016). The primary goal of the study of the cancer microenvironment ultimately dictates the complexity of the model. If the goal is to simply study the efflux of cancer drugs by multidrug resistance proteins of the cancerous cell line, a simple monoculture (2D model) will suffice. If the goal is to verify the metastatic potential and the invasion properties of the cells, a co-culture of the cancer cells with an extracellular

matrix (ECM) or ECM-producing cells is performed, or a microenvironment with microvessels that allow blood flow (Katt et al., 2016). Having this in mind, the main component of any tumor model is the cancer cells. Commercially available cancer cell lines (**Table 2**) are widely used in research because they provide comparable results, are easy to manipulate and growth, and are a staple of molecular biology throughout the research world (Holliday and Speirs, 2011).

Table 2 - Examples of some widely used cancer cell lines with origin in different cell types. These data were obtained from the European Collection of Cell Cultures (ECACC) and Cancer Cell Line Encyclopedia (CCLE).

Cancer cell line	Specie		Morphology
Caco-2	*Homo sapiens*	Colorectal adenocarcinoma	Epithelial
Caki-1	*Homo sapiens*	Renal cell carcinoma	Epithelial
HeLa	*Homo sapiens*	Cervix adenocarcinoma	Epithelial
MCF-7	*Homo sapiens*	Breast adenocarcinoma	Epithelial
PC3	*Homo sapiens*	Prostate adenocarcinoma	Epithelial
HEP-G2	*Homo sapiens*	Hepatocellular carcinoma	Epithelial
HT-29	*Homo sapiens*	Colon adenocarcinoma	Epithelial

Thus, immortalized cancer cell lines have been the most extensively sought and used for metabolomics studies. Some advantages and disadvantages of this type of model are listed in **Table 3**. It allows for more controllable experimental designs and the elimination of some confounding variables such as age. The use of patient-derived cells is a way of adequately assessing the gene profile of the particular tumor and also *an in* vitro model of the tumoral cells behavior and microenvironment (Ferreira et al., 2013). However, the patient-derived sample typically contains a heterogeneity of cell types, resulting in cross-contamination of individual cultures (Aparicio et al., 2015). Despite the advantages, the simplicity of these models never allows for a correct understanding of the tumor microenvironment, such as their angiogenic potential, which in turn can lead to missed opportunities for novel therapeutic targets. In addition, cultured cells are usually placed in ideal nutrient and CO_2 levels, which does not occur in tumors, as both nutrient and O_2 deprivation (hypoxic core) are standard in many tumors (Chambers et al., 2014).

Table 3 - List of some advantages and disadvantages of the use of cancer cell lines as models in cancer research (Ferreira et al., 2013)

Advantages	Limitations
Easy to handle and manipulate	Cross contamination
High variety available	Genomic instability
Unlimited auto-replicate source	Loss of heterogeneity
Easy substitution	Contaminations (e.g., mycoplasma)
Immediate accessibility	Characteristics of the cells may change
Reproducibility of results	Different environment of the tumor
High homogeneity	

The use of multicellular tumor spheroids allows to overcome this limitation because they consist in aggregates of different cell types that frequently interact in the tumor microenvironment and are grown in a 3D matrix (Fennema et al., 2013). They adequately mimic the interaction between multiple cells of a tumor and their cell-cell interactions. Spheroids are characterized by the presence of a necrotic, hypoxic core, much like the tumor core *in vivo* and is characterized by expressing several biomarkers comparable with neo-angiogenesis. They also have their own extracellular matrix, drug transfer gradients and specific nutrient demands (Zang et al., 2021). When applied in the field of metabolomics, the 3D model allows for a more robust and characterized metabolic profile of the tumor because it represents a more realistic approximation to *in vivo* systems. The metabolomic profiles that is possible to obtain in different areas of a grown spheroid allows for information regarding the drug distribution within the spheroid (Pampaloni et al., 2007). In turn, establishment of a complex spheroid requires multiple days of cellular culture, potentially limiting rapid analysis if time is of the essence and requires multiple cell cultures to be combined at the same time, where the possibility of a problem or poor cellular interaction can make this option less appealing.

1.2.3. Analytical techniques for metabolomic studies

As most metabolomics research is performed in complex matrices, ranging from cells to tissues or biofluids, different techniques can be employed, each with their own advantages and limitations. The primary goal of a metabolomic assay is to obtain a more

comprehensive coverage of the metabolome; thus, different techniques can be used in different types of assays, mostly defined by the properties of the specific classes of metabolites to be analyzed (Segers et al., 2019). There are several techniques that allow a quantification of very small quantities of analytes, with reliable and reproductible results. Most commonly used techniques are liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy. The advantages and limitations of these analytical techniques are described in Table 4.

NMR is based on the absorption and release of energy of some atoms after the application of an external powerful magnetic field. With regards to metabolomics, it can quantify a wide range of metabolites, often reaching into the micromolar (μM) levels (Emwas et al., 2019). It enables the non-destructive analysis of the samples, can be readily automatized and allows the simultaneous analysis of multiple molecules present in a metabolome (Agin et al., 2016). This technique requires low sample quantities and minimal sample preparation before the analysis (Markley et al., 2017). NMR also provides reproducible results that offers insight into the molecular structure of a metabolite. As we are able to obtain large quantities of information from a single NMR analysis, it is expected that it significantly improves the understanding of biological processes and subsequently the biochemical signal cascades or pathways (Fan and Lane, 2011). Nonetheless, the low sensitivity makes this technique useless when analyzing low concentrations of various metabolites (Kohler et al., 2016). Moreover, multiple overlapping peaks from several molecules appear in the NMR spectrum which is a challenge in the data interpretation. Although 2D NMR methodologies can be performed for metabolite identification, the output of the method will be negatively affected by overlapping quantitative information (Segers et al., 2019).

Table 4 - Advantages and limitations of the most used analytical techniques in metabolomics (Emwas et al., 2019)

	Advantages	Limitations
NMR	• Fast analysis • Minimal sample preparation • Non-destructive • Able to quantify metabolite levels • Able to detect the metabolite structure and identify unknown compounds • High level of reproducibility • Suited for analysis of all kinds of samples from fluids to tissues	• Low sensitivity • Signal overlap • Need high resolution equipment for more complex samples • Inferior in targeted analysis studies
GC-MS	• High efficiency • High resolution • High precision • Low quantity of sample needed per analysis • High sensitivity • Superior in targeted analysis	• Long analysis • Limited to volatiles or compounds that can be derivatized • Unable to analyze non-volatile, polar or thermally labile compounds • Destructive analysis
LC-MS	• High sensitivity • Can analyze metabolites over a wide polarity range • Allow analysis of compounds with high molecular weight (e.g., proteins) • No need for derivatization step • Superior in targeted analysis	• Lower reproducibility • May require different ionization techniques to maximize metabolite detection • Demanding sample preparation • Destructive analysis

Mass spectrometry (MS) is a technique that consists upon the ionization of the molecules and subsequent mass analysis and ion detection, leading to a spectrum representing the signal intensity of detected ions as a function of the mass-to-charge (*m/z*) ratio. MS enables the analysis of metabolites present in low concentrations, potentially reaching the nanomolar (nM) range in certain cases (Zhang et al., 2018). MS also offers a higher resolution when compared with NMR. It is usually coupled with separation techniques, such as liquid chromatography (LC-MS) or gas chromatography (GC-MS). In

GC-MS, a derivatization step may be needed because this technique is unable to analyze non-volatile, polar or thermally labile compounds (Emwas, 2015), while in LC-MS there is no such problem since this technique can analyze samples over a wide polarity range and compounds with high molecular weight (e.g., proteins) (Ata-ur-Rehman et al., 2015). GC-MS has a high resolution and a very high accuracy and repeatability of results. Furthermore, this technique has the ability to discriminate between molecules with similar structure (Mussap et al., 2013). On the other hand, LC-MS has a high sensitivity but lacks reproducibility (Mussap et al., 2013). To monitor the reliability and the reproducibility of the analysis, quality control (QCs) samples are used (Broadhurst et al., 2018; León et al., 2013). Capillary electrophoresis can also be coupled to MS (CE-MS), but it is less used than the techniques mentioned above (Ramautar, 2018). MS requires a smaller sample volume, of just a few microliters, when compared to about 600 µL in NMR. Although a single technique is usually used in metabolomics experiments, the combination of NMR and MS can lead to the identification of novel biomarkers (Zhang et al., 2018).

1.2.4. Statistical analysis

When performing a metabolomics-based study, a vast quantity of raw data is obtained depending upon the analytical technique that was chosen. Thus, the starting point should be a data pre-processing method by specific tools to facilitate a semi-quantitative metabolite analysis. Normally, *ad hoc* software tools are used to perform noise filtering, peak detection, alignment and normalization (Alonso et al., 2015). Throughout these methods, we are able to normalize data and reduce the possibility of systematic biases, reducing the potential for mistakes while comparing samples, and avoiding misidentification of key metabolites that could alter the course of the study (Cambiaghi et al., 2017).

Typically, data analysis is performed using multivariate and univariate statistics to investigate which metabolites had the highest impact depending on the goal of the study (Cambiaghi et al., 2017). With regards to multivariate analysis, it involves the analysis of multiple variables simultaneously. It is normally employed in untargeted metabolomic studies as it tends the produce a higher amount of metabolites, facilitating the interpretation of the large quantities of data (Worley and Powers, 2013). Also, most modern multivariate statistical methods are able to extract the maximum possible information from the available data, reducing significantly the dimensionality of the data produced from NMR or MS methods. Normally, multivariate data can split into two categories, supervised and unsupervised analysis (Gowda et al., 2008). Unsupervised multivariate statistical analysis

includes principal component analysis (PCA) (Lamichhane et al., 2018). PCA does not require prior knowledge of the sample groups under the study, being mostly dependent upon data dimension reduction (Ren et al., 2015). Through this method, we are able to generate new combinations from the original data, that still contain all the information regarding the main source of data. The new originated variables, called principal components, need to form a new coordinate system in order to promote the separation of samples. Thus, PCA is ideal to identify potential outliers and data patterns (Ren et al., 2015). On the other hand, partial least squares-discriminant analysis (PLS-DA) is a supervised method, thus requiring prior knowledge of the sample group under study in order to adequately establish a classification. It is based upon a PLS regression and the connection between two data points form (i.e., X being metabolite information and Y being categorical outcome). In general, it is used to maximize the covariance between different groups (Antonelli et al., 2019). The main issue faced upon PLS-DA is due to the potential to find certain variables that are not intrinsically related to the subject under study. Thus, PLS-DA has to be validated by specific cross-validation tools and/or permutation tests. A cross-validation repeatedly analyses different sections of the data in order to ensure optimized data with minimal variability, whereas permutation allows the use of Q^2 values from the original data to be compared with Q^2 that were randomly assigned (Triba et al., 2015).

On the other hand, univariate approaches focus purely on a single variable, and they require prior information about the metabolite in question. It stems from the comparison of the relative concentrations of a particular metabolite in the control study group to another group with a specific pathology. Most commonly, the *t*-test, Wilcoxon rank-sum test and the analysis of variance (ANOVA) are employed. The *t*-test is one of the most well-known statistical tools. It can be split in unpaired *t*-test, which compares two groups that are independent, and paired *t*-test, where the two selected groups are dependent upon each other (Kim, 2015). The Wilcoxon rank sum test is a non-parametric test that is used to establish comparison between two independent groups. These two tests are only able to compare two groups, hence for three or more groups an ANOVA test is required, with subsequent tests to assess the variability between the groups (Mishra et al., 2019). The main issue associated with the univariate analysis is the high potential for false-positive results due to the high number of multiple comparisons usually performed. To circumvent this, multiple correction techniques are employed such as the Bonferroni correction. This correction method is based on the division of the selected threshold of the statistical analysis (e.g., 0.05) by the number of metabolites to be tested. An alternative to the Bonferroni correction is the false discovery rate (FDR) correction (Cambiaghi et al., 2017).

Following statistical analyses, it is critical to assess the functional and biological relevance of all metabolites identified using metabolomics pathway analysis or enrichment analysis (Xia and Wishart, 2011).

1.2.5. State of art of metabolomics studies evaluating the impact of sunitinib and pazopanib in RCC

A wide range of drugs have been studied over the years in order to find better treatments for RCC and to improve patients' quality of life. Sunitinib and pazopanib are two drugs extensively studied in this field that have demonstrated efficacy against the disease, being now first-line drugs for progressive RCC (Sato et al., 2019a).

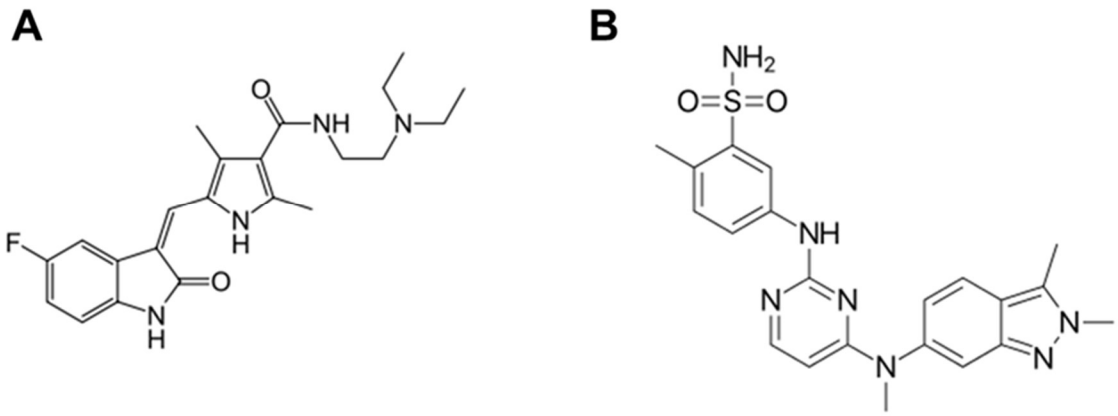


Figure 4 - Molecular structure of Sunitinib (A) and Pazopanib (B)

Sunitinib (**Figure 4A**) is an oral multi-targeted receptor tyrosine kinase inhibitor (TKI), approved by the Food and Drug Administration (FDA) in 2006, that was used as standard first-line treatment for metastatic renal cell carcinoma (mRCC) (Motzer et al., 2017). It also exerts an antiangiogenic effect through inhibition of PDGF receptors and VEGF receptors (Moran et al., 2019b). The approved dose and schedule for treatments with this drug is 50 mg/day using a schedule 4/2 (4 weeks on / 2 weeks off) (Motzer et al., 2017). Pazopanib (**Figure 4B**) is another TKI that primarily inhibits VEGF receptors-1, -2 and −3, as well as the PDGF receptors -α and -β, and the stem cell factor receptor c-Kit (Frampton, 2017). It was used as a reference for first-line advanced RCC therapy in spite of its high cost and

toxicity, especially in the liver. The approved oral dose for this drug is 800 mg per day (Miyamoto et al., 2018).

Metabolomics has been important in the identification of the mechanisms of action and the signal transduction mechanics of targeted therapies in multiple cancers (Johnson et al., 2016; Pang et al., 2019). Particularly in the scope of this thesis, we aimed at investigating the impact of sunitinib and pazopanib in RCC cells. In fact, there are several metabolomics studies investigating the impact of sunitinib as an RCC therapy in mice (Zhao et al., 2019) and in humans (clinical trials) (Jobard et al., 2015; Sato et al., 2019b). However, only few *in vitro* studies have been reported and these are focused on its mechanisms of action (Sato et al., 2019b) and resistance (Hatakeyama et al., 2018; Sato et al., 2020). In 2019, Sato et al. investigated whether trichostatin A (TSA), a potent histone-deacetylase (HDAC) inhibitor, alters sunitinib-induced cytotoxicity and metabolism in RCC cells at epigenetic regulatory concentrations (Sato et al., 2019b). Three RCC cell lines were used in the study: 786-O, ACHN and Caki-1. The results showed that the combination of sunitinib and TSA in the established sunitinib resistant-RCC cell (786-O Res) led to a significant cellular growth inhibition. The authors suggested that TSA may reduce sunitinib resistance by triggering intracellular metabolome shifts regarding energy metabolism, that is the first recognized mechanism as an HDAC inhibitor (Sato et al., 2019b). In the other hand, in 2018, Hatakeyama et al. studied the metabolic changes between sunitinib-resistant RCC and parental 786-O cell line, using Capillary Electrophoresis-Time of Flight Mass Spectrometry (CE-TOFMS) (Hatakeyama et al., 2018). It was concluded that the glycolysis, the pentose phosphate pathway (PPP) and the tricarboxylic acid (TCA) cycle were up-regulated in sunitinib-resistant 786-O cells, suggesting a sunitinib-resistance mechanism. In addition, the energy-producing pathways, the arginine and proline pathways and the urea cycle were also found to be up-regulated in sunitinib-resistant 786-O cells (Hatakeyama et al., 2018). In 2020, Sato et al., used a metabolomics analysis to elucidate the mechanisms of sunitinib resistance in RCC (Sato et al., 2020). LC-MS was used to evaluate the intracellular metabolites involved in sunitinib resistance, considering three RCC cell lines: 786-O, ACHN and Caki-1. Among all metabolites identified, glutamine, glutamic acid, and α-ketoglutaric acid (α-KG) (involved in glutamine uptake into the TCA cycle for energy metabolism); fructose 6-phosphate, D-sedoheptulose 7-phosphate, and glucose 1-phosphate (involved in increased glycolysis and its intermediate metabolites); and glutathione and myo-inositol (antioxidant effects) were significantly increased in the sunitinib-resistant RCC cell line. As main conclusion, the authors considered that glutamine metabolism regulation may help to overcome sunitinib resistance in RCC (Sato et al., 2020).

Regarding pazopanib, there has been little research into the use of this drug in the treatment of RCC using metabolomics approaches. Until now, the majority of these studies have been conducted in humans (clinical trials) (Adkins et al., 2018) and mice (Wang et al., 2019b). There are no *in vitro* studies evaluating the impact of this drug on RCC cell lines in the literature.

2. Aims of this thesis

The work carried out throughout this dissertation aimed at performing an *in vitro* untargeted metabolomics approach, based on NMR spectroscopy, to unravel the impact of sunitinib and pazopanib on the metabolic response of a metastatic RCC cell line (Caki-1). A deeper knowledge on the mechanism of action of these drugs at the molecular level may contribute to improve the treatment guidelines for metastatic RCC. The specific aims of this thesis were:

- To obtain a comprehensive metabolic profiling of the metabolites present in the intracellular (endometabolome) and extracellular (exometabolome) domains of Caki-1 cells, by proton (^{1}H) NMR spectroscopy;
- To investigate the intracellular and extracellular metabolites changing in Caki-1 cells in response to clinically relevant concentrations of sunitinib and pazopanib and elucidate their statistical significance and magnitude of variation;
- To interpret the alterations induced by drug exposure in terms of a potential impact on the metabolic pathways of metastatic RCC cells.

3. Experimental Section

3.1. Chemicals

All chemicals were of analytical grade and dissolved in deionized water unless otherwise indicated. RPMI-1640 medium and trypsin-EDTA solution (0.25%) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Heat-inactivated fetal bovine serum (FBS) and antibiotic mixture of penicillin/streptomycin (10.000 U/mL/10.000 mg/mL) were obtained from GIBCO Invitrogen (Barcelona, Spain). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium pyruvate, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (β-NADH), β-nicotinamide adenine dinucleotide 2´-phosphate reduced tetrasodium salt hydrate (β-NADPH), dimethyl sulfoxide (DMSO), chloroform, methanol, sunitinib malate, pazopanib, and deuterium oxide (D_2O) containing 0.05 wt% 3-(trimethylsilyl)propionic-2,2,3,3-d 4 acid (TSP) sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). D_2O (99.96%) was purchased from Eurisotop (Eurisotop, Saint-Aubin, France).

3.2. Cell lines and culture conditions

Caki-1, an immortalized cell line obtained from a 49-year-old male Caucasian patient diagnosed with clear cell renal carcinoma, was used in this project due to its phenotypic similarity to proximal tubule epithelium. In fact, these cells are a widely accepted and reliable *in vitro* model for proximal tubule epithelium research (Glube et al., 2007). The cells were routinely cultured in 75 cm^2 flasks using RPMI cell culture medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in a 5% CO_2-95% air atmosphere at 37 °C. Cultures were passed weekly by trypsin-EDTA solution. The cells used for all the experiments were taken between the 10^{th} and 16^{th} passages, and they were tested for *mycoplasma* at IPO-Porto (TaKaRa PCR Mycoplasma Detection Set, Clontech Laboratories, Mountain View, CA, USA).

3.3. Cell viability assays

Sunitinib and pazopanib cytotoxicity in Caki-1 cells was evaluated using two different assays: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay, which assesses mitochondrial dehydrogenase activity (Morgan, 1998), and the Lactate Dehydrogenase (LDH) release assay, which assesses plasma membrane integrity.

3.3.1. MTT reduction assay

MTT is a yellow tetrazolium salt and a water-soluble dye. When in contact with the mitochondrial dehydrogenases of living cells, the tetrazolium ring is cleaved, reducing the yellow tetrazolium dye into a purple-colored water-insoluble formazan product. Therefore, the formed formazan crystals need to be dissolved in an appropriate organic solvent for the colorimetric measurement. Furthermore, it is expected that the absorbance of the reduced product obtained is directly proportional to the number of metabolically viable cells in culture, since this method is based on the mitochondrial activity (Morgan, 1998).

Caki-1 cells were seeded at a density of 7.5×10^4 cells/mL in 96-well plates and, after reaching confluence, exposed to increasing concentrations of sunitinib (0.1, 0.2, 1, 2, 5, 10, 25, 50, and 100 μM) or pazopanib (0.1, 1, 5, 10, 25, 50, 100, and 200 μM) in fresh cell culture medium for 48 hours, at 37 °C, in a humidified 5% CO_2 - 95% air atmosphere. After the incubation period, the cell culture medium was removed, 200 μL of fresh cell culture medium containing 0.5 mg/mL MTT were added, and the plates incubated for 120 min, at 37 °C. Subsequently, the cell culture medium was removed, and 100 μL of DMSO were added to dissolve the formed formazan crystals. The absorbance was measured at 545 nm in a multi-well plate reader (BioTek Instruments, VT, USA). The percentage of MTT reduction relative to that of the control cells (non-exposed) was used as the cytotoxicity measure. Seven independent assays, with three technical replicates each, were performed. Statistical analysis was performed using one-way ANOVA analysis (GraphPad Prism, version 8.01, GraphPad Software Inc., La Jolla, CA, USA). Significance was accepted for p-value < 0.05.

3.3.2. Lactate dehydrogenase (LDH) release assay

The LDH release assay is based on measuring the activity of LDH enzymes released by damaged cells (Kumar et al., 2018). LDH enzyme is stable and is found in the cytoplasm of all cells. LDH enzymatically converts pyruvate and NADH to lactate and NAD+ in a reversible biochemical reaction. Because NADH absorbs at 340 nm, the basis of this kinetic assay is the decrease of optical density at the specific wave length caused by a decrease in NADH.

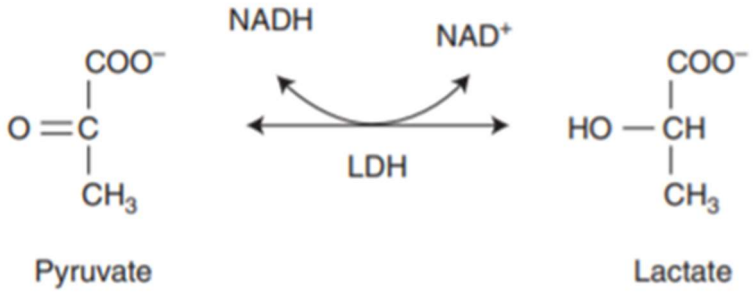


Figure 5 - Reaction catalyzed by lactate dehydrogenase. Adapted from (Kumar et al., 2018)

Caki-1 cells were seeded at a density of 7.5×10^4 cells/mL in 96-well plates and, after reaching confluence, exposed to sunitinib (0-100 µM) or pazopanib (0-200 µM) in fresh cell culture medium for 48 hours. After the incubation period, 20 µL of supernatant were transferred to a 96-well plate and it was diluted in 30 µL phosphate buffer. Then, 200 µL β-NADH were added followed by immediate addition of 25 µL of sodium pyruvate. The absorbance was measured at 340 nm, for 3 min, performing readings from 10 in 10 seconds, at 30ºC in a multi-well plate reader (BioTek Instruments, VT, USA). The percentage of LDH release relative to that of the control cells (non-exposed) was used as the cytotoxicity measure. Statistical analysis was performed using one-way ANOVA analysis (GraphPad Prism, version 8.01, GraphPad Software Inc., La Jolla, CA, USA). Significance was accepted for p-value < 0.05.

3.4. Metabolomics Assay

3.4.1. Sample collection and preparation for NMR analysis

Caki-1 cells were seeded at a density of 22.5 × 10^4 cells/mL in 60 mm dishes and the next day exposed for 48 hours to 2 μM of sunitinib or 50 μM of pazopanib. After incubation, the cell quenching and extraction of metabolites were performed. Five independent experiments from different passages were performed considering one sample for each condition under study. Medium aliquots were collected from each dish, centrifuged (1200g, 5 min, 4 ºC), and the supernatant stored at −80 °C until NMR analysis. Aliquots from medium without cells (blanks), incubated under the same conditions, were also collected. To collect cell samples, the remaining medium was aspirated, and each dish was washed twice with PBS. Then, intracellular aqueous metabolites were extracted using a dual-phase extraction, with methanol/ chloroform/ water (4:4:2.85). This protocol was adapted from literature (Teng et al., 2009). Briefly, 800 μL of cold methanol was added to stop cellular metabolism and the cells were scrapped and transferred to a microcentrifuge tube containing 0.5 mm glass beads to facilitate cell breakage using the vortex for 2 min. Then, 320 μL of chloroform (added twice) and 320 μL of water were added (the samples were vortexed 2 min between each solvent addition). After 10 min of rest on ice, the samples were centrifuged at 2000× g for 15 min plus 10,000× g for 2 min at 4 ºC. The upper phase containing the polar metabolites was collected, lyophilized, and stored at −80 ◦C until analysis.

For NMR analysis, dried extracts were reconstituted in 650 μL of D_2O and centrifuged (10,410×*g*, 5 min, 4 ºC). Then, 600 μL of each sample were transferred to 5 mm NMR tubes. For analysis of extracellular metabolites, 585 μL of culture medium were mixed with 65 μL of D_2O containing 0.25% TSP, followed by centrifugation (10,410×*g*, 5 min, 4 ºC), and transfer of 600 μL of the supernatant to a 5 mm NMR tube.

3.4.2. NMR acquisition and processing parameters

^{1}H NMR experiments were performed on a Bruker Avance III HD 600 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a cryoprobe (T= 300 K). The acquisition conditions for intracellular extracts and culture medium were selected based on previous literature (Carrola et al., 2016) with some modifications. Briefly, a

standard pulse sequence (*noesypr1d*) was used with 4s relaxation delay, 100 ms mixing time, 256 transients, 64k complex data points, 10,080.646 Hz spectral width and 3.25 s acquisition time. Each free induction decay was multiplied by a 1.0 Hz exponential line-broadening function, manually phased and baseline corrected, and chemical shifts referenced internally to lactate at δ = 1.32 ppm for intracellular extracts and glucose at δ = 5.23 ppm for culture medium. The Biological Magnetic Resonance Bank (Ulrich et al., 2008) and reported literature (Carrola et al., 2016) were used for identification of metabolites.

3.4.3. Statistical analysis

Prior to statistical analysis, the data obtained from ^{1}H NMR experiments were converted into a manageable format using the NMRProwFlow platform (Jacob et al., 2017). The spectral region between 9.35–0.75 ppm was considered, after exclusion of residual water (5.20–4.67 ppm), ethanol (1.28–1.13 ppm and 3.67-3.62 ppm), methanol (3.36-3.34) and DMSO (2.74-2-69). Spectra were aligned using the Parametric Time Warping method (Bloemberg et al., 2010) and bucketing (uniform, 0.001 ppm, signal to noise ratio = 3) and normalized by the total area (TA) of the spectrum.

The pre-processed data matrix was firstly scaled to unit variance (UV) followed by multivariate analyses in SIMCA Software (version 17, Umetrics Umea, Sweden). PCA (unsupervised analysis) was applied to detect trends and outliers, followed by PLS-DA (supervised analysis) to discriminate classes and to identify the discriminant metabolites after drug exposure. The PLS-DA loadings plots were back transformed by multiplying the loading weights (*w*c)* by the standard deviation of each variable and colored according to variable importance to the projection (VIP) using the ggplot2 package (Wickham, 2016) in the R statistical software (R Foundation for Statistical Computing, Vienna, Austria). The PLS-DA model robustness was assessed by seven-fold cross validation in the SIMCA software (version 17, Umetrics Umea, Sweden). Due to the low sample size, no permutation testing was performed. Resonances with VIP > 1.0 were considered for univariate analysis (Mann–Whitney test) performed in GraphPad Prism (version 8.01, GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was considered when *p*-values were below 0.05. For all significantly altered metabolites, the magnitude of variation [effect size (ES) and standard error (SE)] were computed (Berben et al., 2012).

The significantly altered metabolites were used for pathway search in KEGG database (Kanehisa et al., 2023)

4. Results

4.1. Impact of sunitinib and pazopanib on cell viability

The cytotoxicity of sunitinib and pazopanib was evaluated by the MTT reduction and LDH release assays. The MTT reduction assay reflects interferences at the mitochondrial level, while the LDH release assay shows the loss of cell membrane integrity. Both assays were performed after 48 hours of drug exposure. As shown in **Figure 6** both TKIs caused a concentration-dependent loss of cell viability.

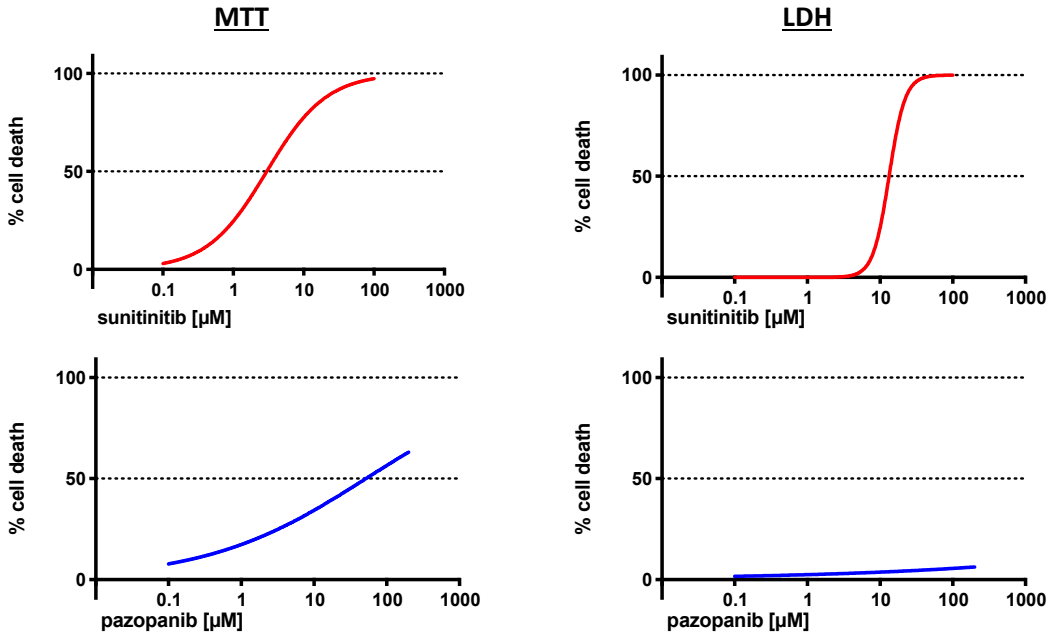


Figure 6 - Nonlinear regression models obtained for the cell death induced by sunitinib (red) and pazopanib (blue) in Caki-1 cells, as evaluated by the MTT and LDH assays after 48 hours of exposure.

The EC_{50} obtained for sunitinib exposure was 2.99 µM and 13.3 µM for MTT and LDH assays, respectively. For pazopanib, the EC_{50} obtained in the MTT assay was 51.9 µM, whereas the EC_{50} of the LDH assay was unable to be estimated from the concentration range tested. Based on these results, an exposure concentration of 2 µM sunitinib and 50 µM pazopanib were selected for the metabolomics experiment. These concentrations correspond to approximately 40–50% loss of cell viability (MTT assay) with no significant membrane damage (LDH assay). Indeed, our selection correspond to clinically relevant

concentrations as reported in literature for plasma and tissue collected from RCC patients (Gotink et al., 2011; Rausch et al., 2021; Suttle et al., 2014).

4.2. Impact of sunitinib and pazopanib on the endo- and exometabolome of metastatic renal cell carcinoma cells

4.2.1. Metabolic profile of intracellular and extracellular domains of Caki-1 cells

A typical ^{1}H NMR spectrum of the endometabolome (intracellular aqueous extract) of Caki-1 cells (non-exposed) is shown in **Figure 7**. As listed in **Table 5**, 23 metabolites were identified from different chemical classes, mainly amino acids, organic acids, choline-containing compounds, and nucleotides. At the low-frequency region (δ 0-3 ppm), resonances from several amino acids (valine, leucine, isoleucine, alanine, glutamate, aspartate, asparagine), organic acids (lactate, acetate) and glutathione were annotated. At the mid-frequency region (δ 3-5.5 ppm), along with the resonances of the mentioned amino acids, creatine, phosphocreatine, choline-containing compounds (choline, *o*-phosphocholine, glycerophosphocholine), ethanolamine, and myo-inositol were identified. At the high frequency region (δ 5.5-10 ppm), the signals detected were assigned mainly to one organic acid (formate) and nucleotides and derivatives (e.g., ADP, ATP, NAD^{+}, UDP-glucose, UDP-galactose).

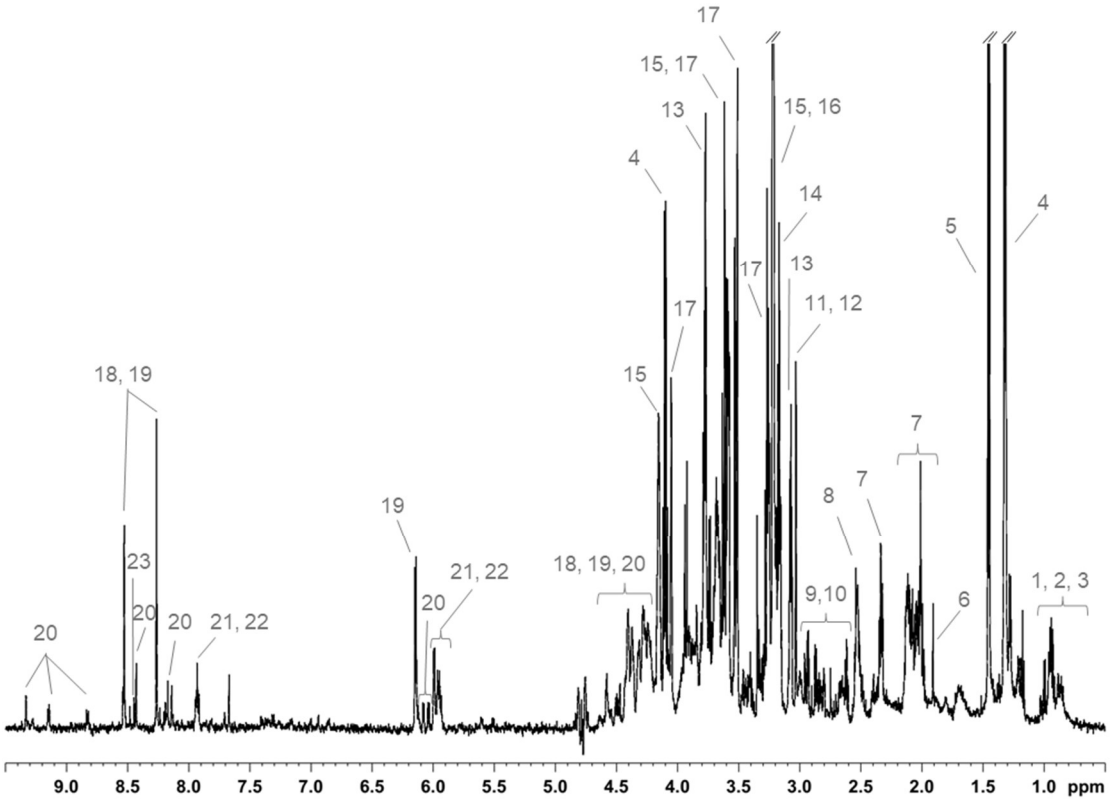


Figure 7 - Representative ^{1}H NMR spectrum of the endometabolome (intracellular aqueous extract) of non-exposed Caki-1 cells. The metabolites marked from 1 to 23 are described in **Table 5**.

Table 5 - Metabolites identified in the ^{1}H NMR spectra of the intracellular aqueous extracts (endometabolome) of Caki-1 cells.

No.	Compound	δ ^{1}H in ppm (multiplicity, assignment)
1	Isoleucine	0.93 (t); 1.00 (d); 1.25 (m); 1.46 (m); 1.97 (m); 3.66 (d)
2	Leucine	0.94 (d); 0.95 (d); 1.70 (m); 1.69 (m); 3.73 (d)
3	Valine	0.98 (d); 1.03 (d); 2.26 (m); 3.59 (d)
4	Lactate	1.31 (d); 4.09 (q)
5	Alanine	1.47 (d); 3.77 (q)
6	Acetate	1.90 (s)
7	Glutamate	2.04 (m); 2.11 (m); 2.34 (m); 3.74 (q)
8	Glutathione	2.15 (m); 2.54 (m); 2.94 (dd); 2.97 (dd); 3.76 (m); 4.55 (m)
9	Aspartate	2.67 (dd); 2.80 (dd); 3.88 (dd)
10	Asparagine	2.84 (dd); 2.94 (dd); 3.98 (q)
11	Creatine	3.02 (s); 3.91 (s)
12	Phosphocreatine	3.03 (s); 3.93 (s)

No.	Compound	δ ^{1}H in ppm (multiplicity, assignment)
13	Ethanolamine	3.13 (t); 3.81 (t)
14	Choline	3.19 (s); 3.50 (t); 4.05 (m)
15	*o*-Phosphocholine	3.21 (s); 3.58 (m); 4.15 (m)
16	Glycerophosphocholine	3.24 (s); 3.68 (m); 3.70 (m); 4.33 (m)
17	Myo-inositol	3.26 (t); 3.52 (dd); 3.61 (t); 4.05 (t)
18	ADP	4.22 (m); 4.38 (m); 4.61 (m); 6.15 (d); 8.28 (s); 8.54 (s)
19	ATP	4.22 (m); 4.29 (m); 4.41 (m); 4.62 (m); 6.15 (d); 8.28 (s); 8.55 (s)
20	NAD+	4.23 (m); 4.36 (m); 4,39 (m); 4.42 (m); 4.50 (m); 4.54 (m); 6.04 (d); 6.10 (d); 8.18 (s); 8.19 (t); 8.43 (s); 8.83 (d); 9.14 (d); 9.34 (s)
21	UDP-Glucose	3.49 (t); 3.54 (m); 3.74 (t); 3.76 (dd); 3.84 (d); 4.19 (m); 4.23 (m); 4.27 (s); 4.36 (m); 5.97 (d); 5.98 (d); 7.93 (dd)
22	UDP-Galactose	3.70 (dd); 3.74 (d); 3.90 (dd); 4.02 (dd); 4.16 (m); 4.22 (m); 4.29 (d); 5.97 (d); 7.99 (d)
23	Formate	8.44 (s)

s: singlet, d: doublet, t: triplet, dd: doublet of doublets, m: multiplet.

A typical ^{1}H NMR spectrum of the exometabolome of Caki-1 cells, i.e., the set of all metabolites existing in the medium where the cells were cultured, is shown in **Figure 8**. As listed in **Table 6**, 31 metabolites were identified including several amino acids, organic acids and derivatives, and carbohydrates. At the low-frequency region (δ 0-3 ppm), we can observe the resonances from several amino acids (isoleucine, leucine, valine, threonine, alanine, arginine, methionine, glutamate, glutamine, aspartate, asparagine, and lysine) and organic acids (lactate, acetate, pyruvate, succinate). At the mid-frequency region (δ 3-5.5 ppm), additional intracellular metabolites were detected, along with the resonances of the mentioned amino acids and some organic acids, along with creatine, phosphocreatine, choline, carbohydrates (glucose and glycogen), myo-inositol, and glycine. At the high frequency region (δ 5.5-10 ppm), the signals detected were assigned mainly to aromatic amino acids (tyrosine, 1-methylhistidine, and phenylalanine), organic acids (fumarate and formate), and 1-methylnicotinamide.

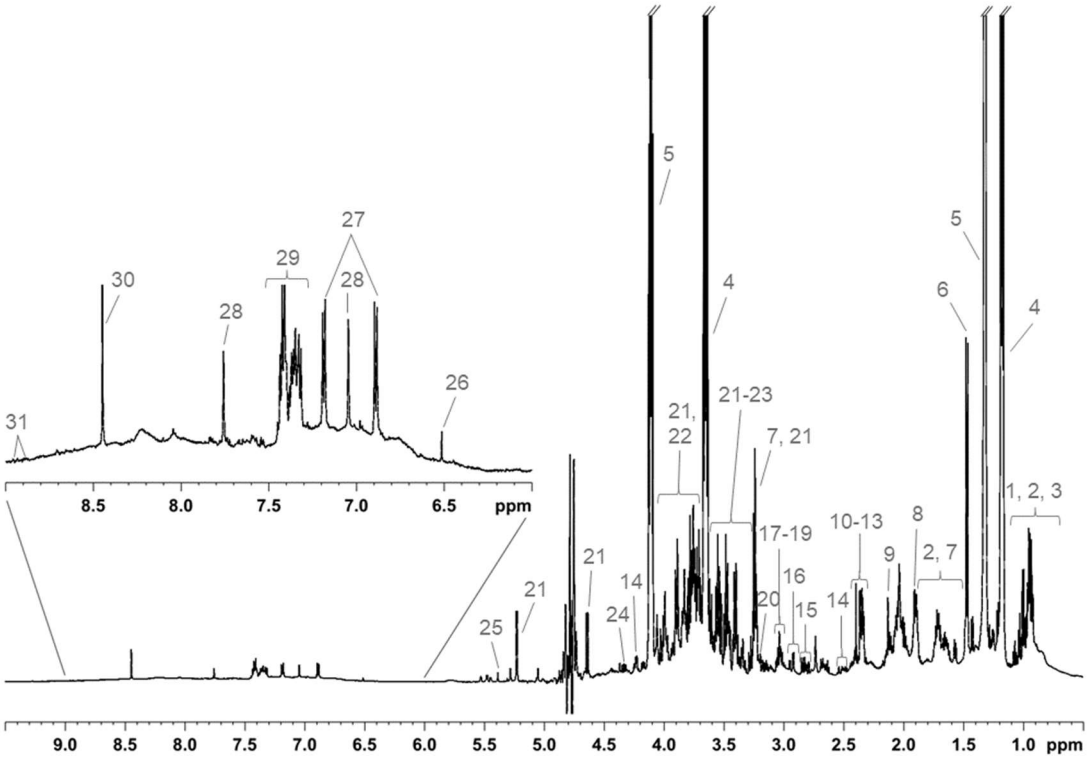


Figure 8 - Representative ^{1}H NMR spectrum obtained for the exometabolome (culture medium) of non-exposed Caki-1 cells. The metabolites marked from 1 to 31 are described in **Table 6**.

Table 6 - Metabolites identified in the ^{1}H NMR spectra of the culture media (exometabolome) of Caki-1 cells.

No.	Compound	δ ^{1}H in ppm (multiplicity, assignment)
1	Isoleucine	0.93 (t); 1.00 (d); 1.25 (m); 1.46 (m); 1.97 (m); 3.66 (d)
2	Leucine	0.94 (d); 0.95 (d); 1.70 (m); 1.69 (m); 3.73 (d)
3	Valine	0.98 (d); 1.03 (d); 2.26 (m); 3.59 (d)
4	Ethanol	1.19 (t); 3.67 (t)
5	Lactate	1.31 (d); 4.09 (q)
6	Alanine	1.47 (d); 3.77 (q)
7	Arginine	1.64 (m); 1.72 (m); 1.89 (m); 1.91 (m); 3.35 (t); 3.76 (t)
8	Acetate	1.90 (s)
9	Methionine	2.11(m); 2.12 (s), 2.18 (m); 2.63 (t); 3.84 (t)
10	Glutamate	2.04 (m); 2.11 (m); 2.34 (m); 3.74 (q)
11	Pyruvate	2.35 (s)
12	Succinate	2.38 (s)
13	Glutamine	2.13 (m); 2.44 (m); 3.76 (t)
14	Pyroglutamate	2.02 (m); 2.39 (t), 2.49 (m), 4.16 (q)

No.	Compound	δ ^{1}H in ppm (multiplicity, assignment)
15	Aspartate	2.67 (dd); 2.80 (dd); 3.88 (dd)
16	Asparagine	2.84 (dd); 2.94 (dd); 3.98 (q)
17	Lysine	1.46 (m); 1.72 (m) 1.89 (m); 1.92 (m); 3.01 (t); 3.74 (t)
18	Creatine	3.02 (s); 3.91 (s)
19	Phospho-creatine	3.03 (s); 3.93 (s)
20	Choline	3.19 (s); 3.50 (t); 4.05 (m)
21	Glucose	3.54 (dd); 3.70 (m); 3.72 (m); 3.76 (q); 3.84 (m); 3.85 (dd); 3.91 (dd); 4.65 (d); 5.24 (d) 3.23 (t); 3.39 (m); 3.45 (m); 3.50 (dd); 3.71 (m); 3.81(m); 3.88 (dd); 4.63 (d)
22	Myo-inositol	3.26 (t); 3.52 (dd); 3.61 (t); 4.05 (t)
23	Glycine	3.55 (s)
24	Threonine	1.31 (d); 3.57 (d); 4.24 (m)
25	Glycogen	3.40 (m); 3.60 (m); 3.80 (m); 3.96 (s); 5.50 (s)
26	Fumarate	6.50 (s)
27	Tyrosine	3.05 (q); 3.18 (dd); 3.92 (m); 6.89 (d); 7.18 (d)
28	1-Methylhistidine	3.06 (q); 3.17 (dd); 3.68 (s); 3.96 (q); 7.05 (s); 7.76 (s)
29	Phenylalanine	3.12 (m); 3.27 (dd); 3.98 (m); 7.32 (d); 7.36 (m); 7.41 (m)
30	Formate	8.44 (s)
31	1-Methylnicotinamide	4.47 (s); 8.17 (t); 8.89 (d); 8.96 (d); 9.27 (s)

s: singlet, d: doublet, t: triplet, dd: doublet of doublets, m: multiplet.

4.2.2. Alterations induced on the endometabolome

The endometabolome of Caki-1 cells exposed to 2 μM sunitinib or 50 μM pazopanib for 48 hours, as well as non-exposed (control) cells, were analyzed by ^{1}H NMR spectroscopy. Moreover, cells exposed to DMSO were also analyzed to access the alterations/contributions on the metabolome caused by the use of this vehicle (solvent). Pairwise multivariate supervised analysis (PLS-DA) was performed between exposed and non-exposed cells for sunitinib and pazopanib as shown in **Figure 9**. The models showed a good separation for both comparisons; nonetheless, a more robust discrimination was found for the cells exposed to sunitinib vs. controls since a higher predictive power was achieved (Figure 9A left, Q^2 = 0.77) when compared with pazopanib exposure (Figure 9B left, Q^2 = 0.55). According to the PLS-DA loadings plots, a higher number of metabolites

was also found for sunitinib-exposed cells (Figure 9A right) when compared with pazopanib-exposed cells (Figure 9B right).

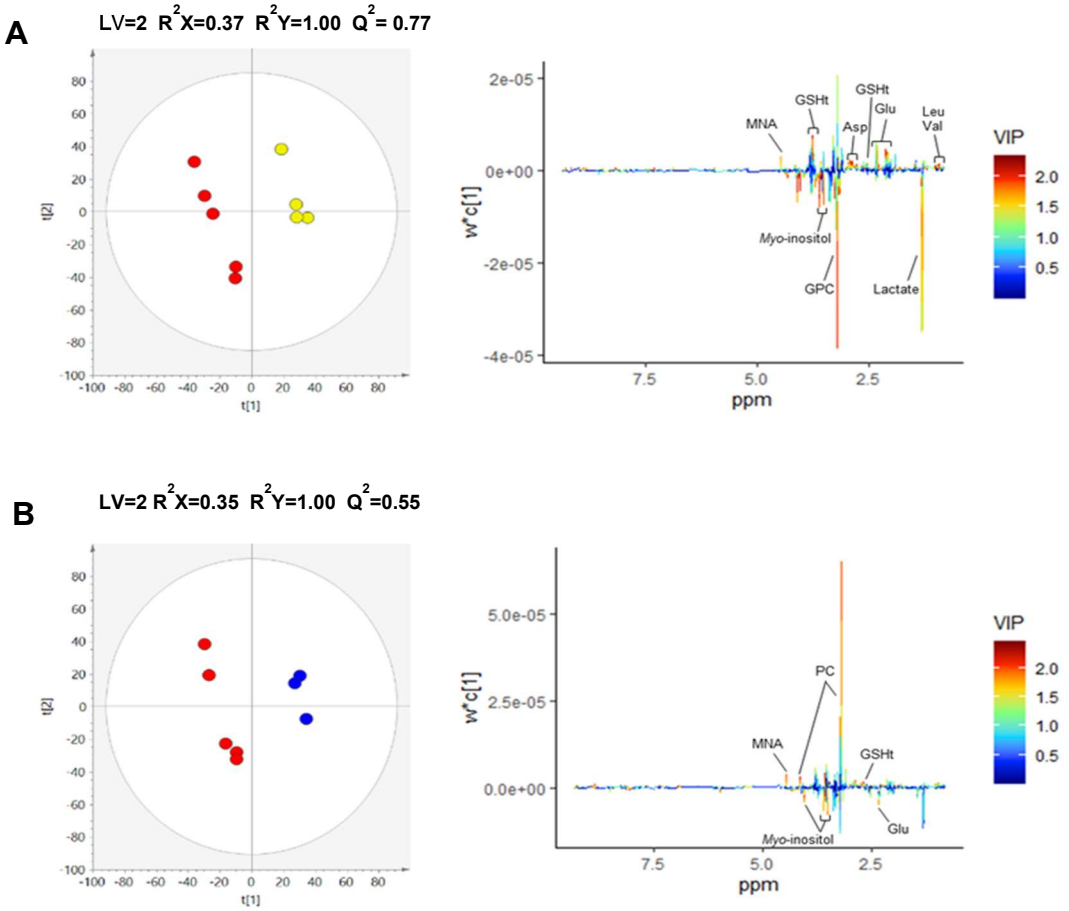


Figure 9 - Partial least squares-discriminant analysis (PLS-DA) scores scatter plots (left) and loading plots (right) obtained for the ^{1}H NMR data of the endometabolome of A) non-exposed cells (red) vs. cells exposed to 2 μM sunitinib (yellow); b) non-exposed cells (red) vs. cells exposed to 50 μM pazopanib (blue).

The **Table 7** discloses the intracellular metabolites responsible for the discrimination between cells exposed to both TKIs compared with non-exposed cells. Overall, the results obtained for sunitinib exposure revealed statistically significant decreased levels of glycerophosphocholine and myo-inositol as well as increased levels of several amino acids and derivatives (asparagine, aspartate, leucine, valine, isoleucine, and glutathione). Regarding the exposure to pazopanib (**Table 7**), a similar decrease on the levels of myo-inositol and, additionally, in glutamate were observed in Caki-1 cells.

Table 7 - List of the intracellular metabolites found significantly altered in Caki-1 cells after exposure to 2 μM sunitinib or 50 μM pazopanib in comparison with control (non-exposed) cells.

Metabolites	Sunitinib		Pazopanib		Dysregulated metabolic pathways
	ES ± SE	*p*-value	ES ± SE	*p*-value	
Amino acids and derivatives					
Asparagine	1.85 ± 1.45	0.0292	-	-	Aminoacyl-tRNA biosynthesis
Aspartate	1.77 ± 1.43	0.0340	-	-	Aminoacyl-tRNA biosynthesis
Glutamate	-	-	-2.44 ± 1.73	0.0145	Aminoacyl-tRNA biosynthesis, glutathione metabolism
Glutathione	1.71 ± 1.41	0.0317	-	-	Glutathione metabolism
Leucine	2.49 ± 1.64	0.0159	-	-	Aminoacyl-tRNA biosynthesis
Valine	2.78 ± 1.74	0.0159	-	-	Aminoacyl-tRNA biosynthesis
Glycerophospho-lipids					
Glycerophospho-choline	-1.87 ± 1.45	0.0317	-	-	Glycerophospholipid metabolism
Sugars					
Myo-inositol	-2.19 ± 1.55	0.0159	-1.96 ± 1.57	0.0357	Galactose metabolism, phosphatidylinositol signaling system, inositol phosphate metabolism

ES: effect size, SE: standard error. Statistical significance assessed using the Mann-Whitney test.

4.2.3. Alterations induced on the exometabolome

To investigate the metabolic alterations on the exometabolome of the metastatic ccRCC cells after exposure to both TKIs, the same strategy described for endometabolome was applied to compare the extracellular culture medium of exposed *versus* non-exposed Caki-1 cells (**Figure 10**). The PLS-DA model (**Figure 10A**) obtained for sunitinib exposure showed a reasonable separation between the extracellular culture media of exposed and non-exposed cells (Q^2 = 0.35). Concerning the exposure to pazopanib, as demonstrated in **Figure 10B**, a good discrimination was obtained for exposed *versus* non-exposed cells with higher predictive power value (Q^2 = 0.45).

The **Table 8** depicts the statistically significant alterations among the metabolites found in the extracellular culture media of Caki-1 cells. The only alteration found on the culture medium of Caki-1 after exposure to sunitinib comprises a small increase in the levels of tyrosine. The comparison with blanks (culture medium analyzed under the same conditions but without cells) shown in **Figure 11** allowed the elucidation of dysregulations occurring in cells in terms of excretion or consumption of culture medium components.

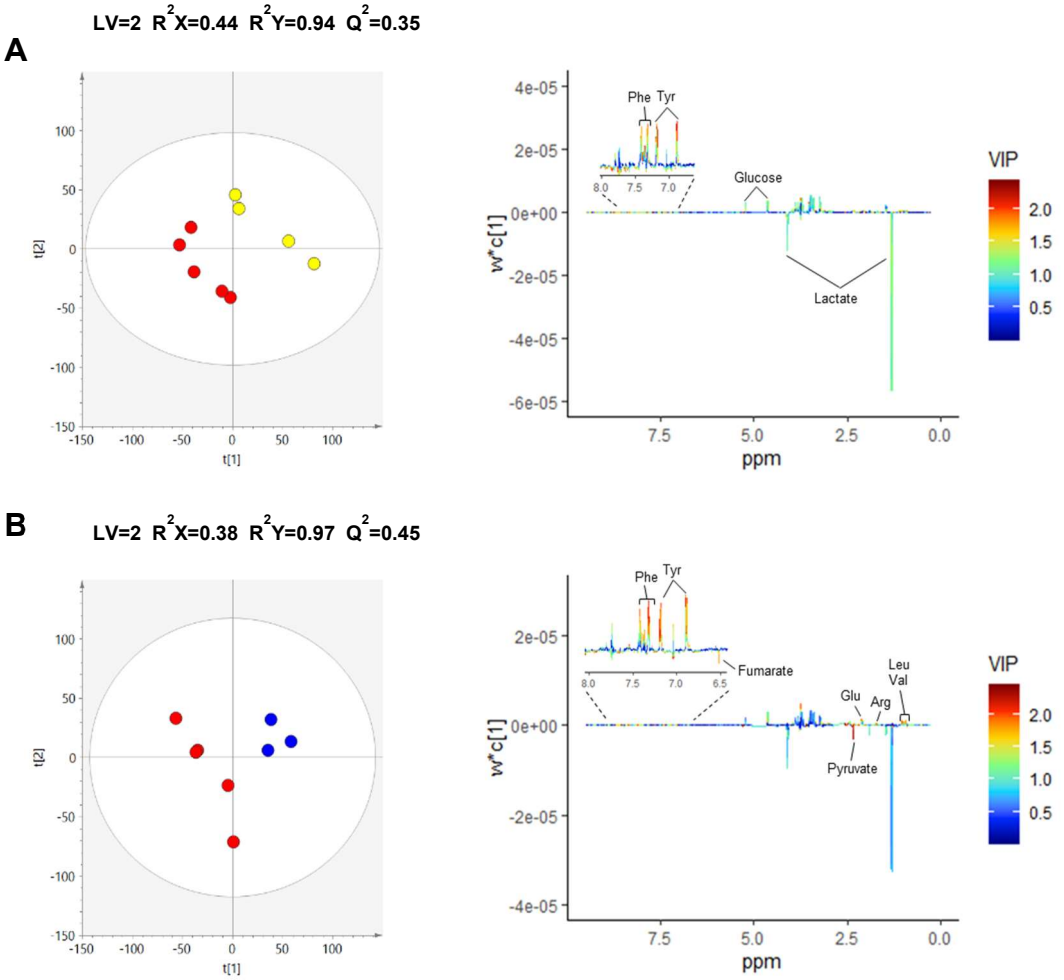


Figure 10 - Partial least squares-discriminant analysis (PLS-DA) scores scatter plots (left) and loading plots (right) obtained for the ^{1}H NMR data of the exometabolome of A) non-exposed cells (red) vs. cells exposed to 2 μM sunitinib (yellow); and B) non-exposed cells (red) vs. cells exposed to 50 μM pazopanib (blue).

Table 8 - List of the extracellular metabolites found significantly altered Caki-1 cells after exposure to 2 μM sunitinib or 50 μM pazopanib in comparison with control (non-exposed) cells.

Metabolites	Sunitinib		Pazopanib		Dysregulated metabolic pathway
	ES ± SE	*p*-value	ES ± SE	*p*-value	
Amino acids and derivatives					
Arginine	-	-	3.42 ± 2.09^{C}	0.0357	Aminoacyl-tRNA biosynthesis
Glutamate	-	-	2.03 ± 1.59 ^{C}	0.0353	Aminoacyl-tRNA biosynthesis, glutathione metabolism
Leucine	-	-	2.31 ± 1.68^{C}	0.0357	Aminoacyl-tRNA biosynthesis
Tyrosine	1.58 ± 1.38^{C}	0.0476	2.56 ± 1.77^{C}	0.0357	Aminoacyl-tRNA biosynthesis
Valine			1.78 ± 1.52^{C}	0.0357	Aminoacyl-tRNA biosynthesis
Organic acids					
Fumarate	-	-	-3.04 ± 1.94^{E}	0.0179	Energy metabolism
Pyruvate	-	-	-3.88 ± 2.27^{E}	0.0357	Energy metabolism

ES: effect size, SE: standard error. Statistical significance assessed using the Mann-Whitney test. [E,C] Metabolites excreted and consumed, respectively, when compared with blanks (culture medium without cells).

Thus, we can verify that tyrosine was consumed by non-exposed cells, but the sunitinib exposure led to a lower cellular intake of this metabolite compared with non-exposed cells. Contrastingly, the alterations on the extracellular culture medium of Caki-1 after exposure to pazopanib comprised decreased levels of fumarate and pyruvate, and increased levels of several amino acids (arginine, glutamate, leucine, tyrosine, and valine). Comparing with blanks, fumarate and pyruvate were excreted by cells, however, the pazopanib exposure induced a lower excretion of fumarate and pyruvate comparing with sunitinib exposure. A lower consumption of the remaining amino acids was also observed after treatment with this TKI.

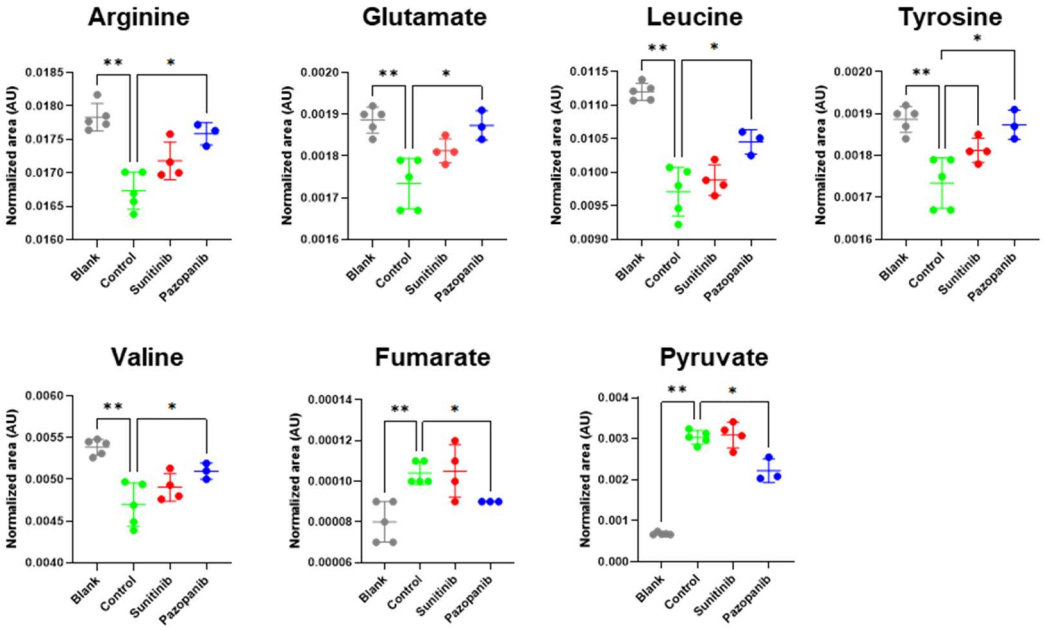


Figure 11 - Boxplots of the extracellular metabolites found significantly altered in Caki-1 cells after exposure to 2 μM sunitinib or 50 μM pazopanib in comparison with blanks (culture medium without cells). *- *p*-value < 0.05, **- *p*-value < 0.01, ***- *p*-value < 0.001, ****- *p*-value < 0.0001.

5. Discussion

With the development of new techniques and methods, cancer therapies have been in constant update. The introduction of novel anticancer drugs allowed to improve the treatments administered as well as the quality of life of patients. However, not all treatments have the expected outcome and, therefore, it is necessary to resort to more effective and specific treatments (De Santis et al., 2021). Since pharmacometabolomics enables the comprehension of the metabolic alterations due to drug exposure (Gomez-Cebrian et al., 2021), the present study provided a better understanding of the impact of sunitinib and pazopanib, two widely used TKIs, on the metabolome of metastatic ccRCC cells (Caki-1) through the identification of significant changes on the levels of intracellular and extracellular metabolites. To the best of your knowledge, this is the first *in vitro* study that explore the effects of both TKIs on cell viability and metabolic status of metastatic ccRCC cells. Notwithstanding, previous studies reported the use of a metabolomics approach to assess the metabolic changes associated with the development of resistance to sunitinib (Hatakeyama et al., 2018; Sato et al., 2020), unveiling dysregulations on energy metabolism and in the antioxidant activity of cells. Instead, the impact of pazopanib on cell metabolism has not yet been studied. Moreover, there is a lack of literature on studies that investigate the metabolic changes associated with drug exposure on the exometabolome of cells (Rattner and Bathe, 2017) whose potential should be explored since the analysis of the extracellular compartment offers an comprehension of the dynamics of some metabolites, namely in terms of consumption and excretion.

Before starting the investigation of the influence of sunitinib and pazopanib on the metabolic status of Caki-1 cells, a study on the effects of the two TKIs on cell viability was carried out**.** The results demonstrated important differences in the cytotoxicity profiles of these agents. A previous study performed by Canter *et al.* (Canter et al., 2011) investigated the cellular effects of both sunitinib and pazopanib at 24 and 48 hours of exposure using concentrations ranging from 10 µM to 750 µM on several human RCC cell lines (769-P, 786-O, HRC-24, HRC-31, HRC-45, HRC-78, RCC-26B, and SK-45) demonstrating some differences in the biological activity of these two agents: sunitinib seems to have a more cytotoxic effect (lower EC_{50} in MTT assay and membrane rupture in LDH assay) on RCC cell lines, whereas pazopanib seems to have only a cytostatic effect (higher EC_{50} in MTT assay and no significant membrane rupture in LDH assay). In our investigation, pazopanib does not cause membrane rupture at the concentrations tested (up to 200 µM), which agrees with the observation made by Canter *et al.* (Canter et al., 2011). Considering the EC_{50} obtained for Caki-1 cells, 2 µM sunitinib and 50 µM pazopanib were selected for further

^{1}H NMR metabolomics experiments, corresponding also to clinically relevant concentrations. It is important to note that the selected concentrations do not cause cell death since no cell membrane disruption is observed, which allows to investigate the early mechanisms of cell response.

As summarized in the heatmaps of **Figure 12**, the impact of the two TKIs on the metabolome of cells was different, since sunitinib seems to have a higher influence on the endometabolome of Caki-1 cells (**Figure 12A**), while pazopanib was responsible for a higher number of altered compounds in the extracellular medium (**Figure 12B**). Regarding the endometabolome changes (**Figure 12A**), a significant increase in the levels of two branched chain amino acids (leucine and valine), and asparagine and aspartate were observed in Caki-1 cells after exposure to sunitinib. These results suggest a dysregulation on amino acid metabolism and the higher levels observed can be related with a possible intracellular accumulation. Since a high demand of protein synthesis is necessary for cell proliferation and tumor growth (Poliakova et al., 2018), these results can indicate a slowdown in protein synthesis and, consequently, a decrease in cell proliferation and cancer progression. A similar finding was observed by Lodi *et al*. (Lodi and Ronen, 2011) that carried out an *in vitro* study to investigate the metabolic changes in prostate and breast cancer cell lines after treatment with a phosphoinositide 3-kinase inhibitor. Furthermore, a significantly higher level of glutathione was also observed which indicates an alteration on the antioxidant activity of cells. Glutathione constitutes the most important protector against oxidative stress in order to reduce the number of ROS levels (Liu et al., 2020; Valente et al., 2016). Additionally, though not statistically significant, a tendency of lower glutamate levels (**Figure 12A**), a precursor on glutathione synthesis, was also observed which may indicate a possible use for glutathione synthesis to balance ROS levels. The amounts of membrane precursors were found in lower levels after exposure to sunitinib namely myo-inositol and glycerophosphocholine. This data indicates an alteration on fatty acid and phospholipid metabolisms (Gupta et al., 2020), important pathways for tumor cell proliferation, migration, and invasion (Fernandez et al., 2020). These compounds are usually found increased in different types of tumors such as breast (Eliyahu et al., 2007), colon (Mori et al., 2004) or prostate (Lima et al., 2021), justifying the higher rate of cell proliferation observed during carcinogenesis. Regarding the impact of sunitinib exposure on the exometabolome, only a significant increase on the levels of tyrosine was observed (Figure 12B), which may be explained by a slowdown in protein synthesis resulting in an accumulation of this metabolite in the extracellular medium.

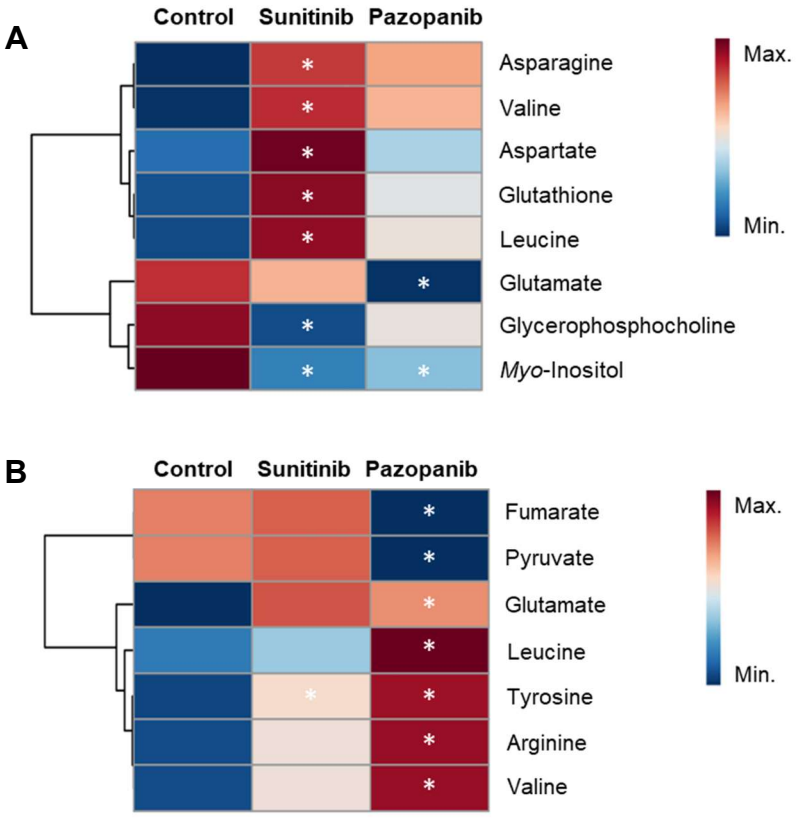


Figure 12 - Heatmaps summarizing the mean levels of intracellular (A) and extracellular (B) metabolites changing in Caki-1 cells after exposure to 2 μM sunitinib or 50 μM pazopanib. Rows correspond to the mean normalized peak area of each metabolite colored from minimum value (dark blue) to maximum value (dark red), while the columns represent each sample group. *- *p*-value < 0.05

Pazopanib is a more recent TKI used in RCC therapy, showing similar mechanism of action and efficacy in comparison with sunitinib, but giving better results in terms of safety and improvement of patient outcomes (Motzer et al., 2013; Stamati et al., 2020). However, few *in vitro* studies investigating its toxic effects have been performed, and none has been done with the aim to investigate its impact on the metabolome. Thus, our results revealed that this TKI has a lower impact on the endometabolome of metastatic ccRCC cells in comparison with sunitinib since it only induced a decrease on the levels of glutamate and myo-inositol (**Figure 12A**). Interestingly, these alterations were found in common between the two TKIs. In addition, these two metabolites were previously found in higher levels in RCC cells before exposure to sunitinib (Sato et al., 2020) and in human samples (tissue and plasm) derived from RCC patients (Catchpole et al., 2011; Gao et al., 2012; Poplawski et al., 2017; Zira et al., 2010) which might indicate that they are metabolic features of the disease. Indeed, to follow the dynamic of these compounds may be useful to monitor the therapeutic response to TKIs. A significant lower consumption from culture medium (**Figure 12B**) of important nutrients from protein synthesis (arginine, leucine, tyrosine and valine)

and TCA cycle (fumarate and pyruvate) was observed which might be related with a decrease on cell proliferation and growth (Poliakova et al., 2018).

6. Conclusion and future perspectives

Throughout this work, a comprehensive analysis of the metabolome of Caki-1 cells was performed after exposure to sunitinib and pazopanib, which are two TKIs widely used in the treatment of metastatic RCC. Through the use of NMR spectroscopy, we were able to obtain a comprehensive metabolic profile of the intracellular and extracellular domains of these cells, identifying potential altered metabolites after exposure to the selected compounds. Particularly, the metabolomic analysis indicated that sunitinib has a higher influence on the endometabolome of Caki-1 cells while pazopanib was responsible for a higher number of altered compounds in the extracellular compartment. Sunitinib-exposure caused alterations on the metabolism of amino acids, which is suggestive of a preferential intracellular accumulation. The particular importance of these findings suggests that sunitinib can be a viable candidate to halt the tumor microenvironment machinery and promote tumor remission. In addition, sunitinib also showed a particular decrease in membrane precursor compounds, demonstrating a particular impact on fatty acid and phospholipid metabolisms, which are critical pathways in tumor invasiveness. This suggests that sunitinib is a particularly effective candidate at suppressing the metastatic potential of these cancer cells. On the other hand, pazopanib revealed a lower impact on the endometabolome of Caki-1 cells, showing only dysregulations in the expression of glutamate and myo-inositol. Nonetheless, cells exposed to pazopanib reduced their nutrient consumption, potentially due to a significant decrease on their proliferation and growth. To confirm these findings, further studies should be performed to investigate the safety of these drugs in non-tumor cells, as well as, whether the fluctuations in metabolite levels induced by sunitinib and pazopanib *in vitro* can be candidate biomarkers to monitor *in vivo* therapeutic efficacy and safety.

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