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Monocarboxylate transporters (MCTS) in kidney cancer: the role of epigenetic mechanism

Ana Luísa Pinto

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Monocarboxylate transporters (MCTs) in kidney cancer: the role of epigenetic mechanism

Dissertação de candidatura ao grau de **Mestre em Oncologia** submetida ao Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto

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"The greatest moments in life are not concerned with selfish achievements but rather with the things we do for the people we love and esteem"

Walt Disney

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Resumo

Introdução: Cancro no rim encontra-se entre os quinzes cancros mais comuns, a nível mundial, no qual o carcinoma de células renais (RCC) corresponde a 90% dos tumores renais. O carcinoma de células claras do rim (ccRCC) é o subtipo histológico mais frequente e representa cerca de 75-80% dos RCC. Apesar dos progressos feitos nas terapias derigidas, a progressão da doença é uma característica típica deste tumor. Neste contexto, potenciais novos alvos moleculares são uma área importante de investigação. Sabe-se que o gene *VHL* está frequentemente inativado por mutação ou hipermetilação do promotor em ccRCC o que permite a acumulação de factores indutores de hipoxia, causando um estado de pseudo-hipoxia. Consequentemente verifica-se um aumento da taxa glicolítica em condições de normoxia – Efeito de Warburg e concomitante aumento da quantidade de lactato produzido. MCT1 e MCT4 são transportadores de monocarboxilatos (MCTs) responsáveis pelo efluxo do lactato para o microambiente tumoral e por isso, são fundamentais para a manutenção da alta atividade glicolítica, bem como para a proliferação e invasão tumoral. A expressão dos MCTs já foi descrita em vários tumores sólidos, contudo o exato mecanismo de regulação permanece por esclarecer. Nomeadamente, a regulação epigenética dos genes dos MCTs ainda não foi estudada, bem como a sua associação com a inativação do *VHL* em ccRCC.

Objetivos: Assim, nesta dissertação, pretendemos investigar quais os mecanismos subjacentes à regulação dos MCTs.

Materiais e métodos: Uma série de 241 amostras de tecido congelado de carcinoma de células claras de rim foram obtidas de doentes diagnosticados e tratados no Instituto Português de Oncologia do Porto. Vinte e cinco amostras de rim normal foram incluídas como controlos. Linhas celulares de rim tratadas com agente desmetilante foram testadas para confirmação do estado de metilação do promotor dos MCTs. Efetuou-se imunohistoquimica de 223 casos para avaliação da expressão do MCT1, MCT4, VHL e HIF-1α.

Resultados e discussão: Os MCTs estão sobreexpressos em ccRCC. Embora, a metilação do *VHL* e o transcrito do HIF-1α estejam significativamente associados com os transcritos dos MCTs, nenhuma associação foi encontrada entre o transcrito do VHL e do HIF-1α. A nível proteico, não se encontrou nenhuma correlação entre o VHL, o HIF-1α, e os MCTs. Além disto, o *MCT1* não é regulado por metilação, ao contrário do *MCT4*,

para o qual este mecanismo epigenético não afeta a sua expressão, em ccRCC. Por oposição, a metilação surgiu como mecanismo de regulação do MCT4 em pRCC.

Conclusões: É necessário ter em consideração que estes transportadores podem ser alvo de vários mecanismos inexplorados que alteram a sua expressão e atividade. Finalmente, pRCC é um melhor modelo para o estudo da regulação do *MCT4* por metilação do DNA.

Abstract

Introduction: Kidney cancer is among the fifteen most common cancer worldwide, where renal cell carcinoma (RCC) corresponds to 90% of renal tumors. Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype that represents about 75-80% of RCC. Besides developments in targeted therapies, disease progression is a presented characteristic of these tumor. Thus new putative molecular targets emerge as an important field of research. VHL gene is known to be frequently inactivated due to mutation or hypermethylation in its promoter in ccRCC which allows the accumulation of the hypoxia inducible factors, creating a pseudohypoxia state. This leads to increased glycolysis rates in normoxia conditions – "Warburg effect" and consequently increased amount of lactate production. MCT1 and MCT4 are monocarboxylate transporters (MCTs) that are responsible for lactate efflux to tumor microenvironment, being fundamental for the maintenance of high glycolytic rate and tumor cell proliferation and invasion. MCTs expression has been described in different solid tumors, however it is not well understood how MCTs could be regulated. Regarding this field, little is known about epigenetic MCTs regulation, as well as their association with *VHL* inactivation in ccRCC.

Aims: In this dissertation we intend to investigate what mechanisms underlie MCTs regulation.

Matherials and methods: A series of 241 samples of fresh frozen tissues of clear cell renal cell carcinoma obtained from patients diagnosed and treated at the Portuguese Oncology Institute of Porto were used in this work. Twenty five normal kidney samples were included as controls. Kidney cells lines treated with demethylating agent are also evaluated to confirm methylation status of MCTs promoter. The expression of MCT1, MCT4, VHL and HIF-1α were assessed by IHC in 223 cases.

Results and discussion: MCTs are overexpressed in ccRCC. Although *VHL* methylation and HIF-1α transcript were significantly associated with MCTs transcripts, none association was found between VHL and HIF-1α transcripts. At protein level, no correlation was found between VHL, HIF-1α and MCTs. Furthermore, *MCT1* is not regulated by DNA methylation, contrary to *MCT4*, where this epigenetic mechanism does not affect its expression, in ccRCC. By opposition, methylation emerged as a regulation mechanism in pRCC.

Conclusions: We need to take into consideration that this transporters can be target of of many unexplored mechanism that alter their expression and activity. Finally, pRCC is a better model for study of *MCT4* regulation by DNA methylation.

Index

Figures Index

Tables Index

List of Abbreviations

3'UTR - 3'untranslated region

5-Aza-CdR - 5-aza-2'deoxycytidine

β-Actin - Beta-actin

AJCC - American Joint Committee on Cancer

BAP1 - BRCA1-associated protein-1

BDNF - Brain-derived neurotrophic factor

bFGF - Basic fibroblast growth factor

BHD - Birt–Hogg–Dubé

ccRCC - Clear cell renal cell carcinoma

cDNA - Complementary DNA

CpG – Cytosine phosphate guanine

DAB - 3'-diaminobenzidine

DFS - Disease-free survival

DSS - Disease-specific survival

DNA – Deoxyribonucleic acid

DNMT1 - DNA methyltransferase 1

DNMT3a - DNA methyltransferase 3a

DNMT3b - DNA methyltransferase 3b

DNMT3L - DNA methyltransferase 3L

FBS - Fetal Bovine Serum

FFPE - Formalin-fixed paraffin-embedded

GUSβ - Beta-glucuronidase

H&E – Haematoxylin and eosin

HATs - Histone acetylases

HIF-α - hypoxia inducible factors α

HLRCC - Hereditary leiomyomatosis and renal-cell cancer

HMTs - Histone methylases

HPRC - Hereditary papillary renal carcinoma

HRE - Hypoxia response elements

IL-8 - Interleukin-8

LOH - Loss of heterozygosity

MBDs - Methyl-CpG-binding domain proteins

miRNA – MicroRNA

mRNA – Messenger RNA

NF- kB - Nuclear factor-κB

PBS - Phosphatebuffered saline

PBRM1 - Polybromo-1

PDGF - Platelet-derived growth factor

pRCC - Papillary renal cell carcinoma

RCC - Renal Cell Carcinoma

SEER - Surveillance, Epidemiology, and End Results

SETD2 - SET domain-containing 2

siRNA - Small interfering RNA

TGFα - transforming growth factor α

TNM – Tumor Node Metastasis Classification

TMs - Transmembrane helices

TSH - Thyroid stimulating hormone

TRD - Transcriptional repressive domain

VEGF - Vascular endothelial growth factor

VHL - Von Hippel-Lindau

WHO/ ISUP – World Health Organization/ International Society of Urological Pathology

INTRODUCTION

1. Epidemiology of kidney cancer

1.1. Incidence and mortality

According to GLOBOCAN 2012, kidney cancer is the 14th most common cancer worldwide and the 10th most frequent in Europe. It displays higher indicence rates in North of America, Europe and Australia and lower values in Asia and Africa. As for mortality, this cancer is responsable for 143.406 and 52.816 death cases, worldwide and in Europe, respectively. In Portugal, 1004 new cases and 368 deaths were reported for both sexes, in 2012[1].

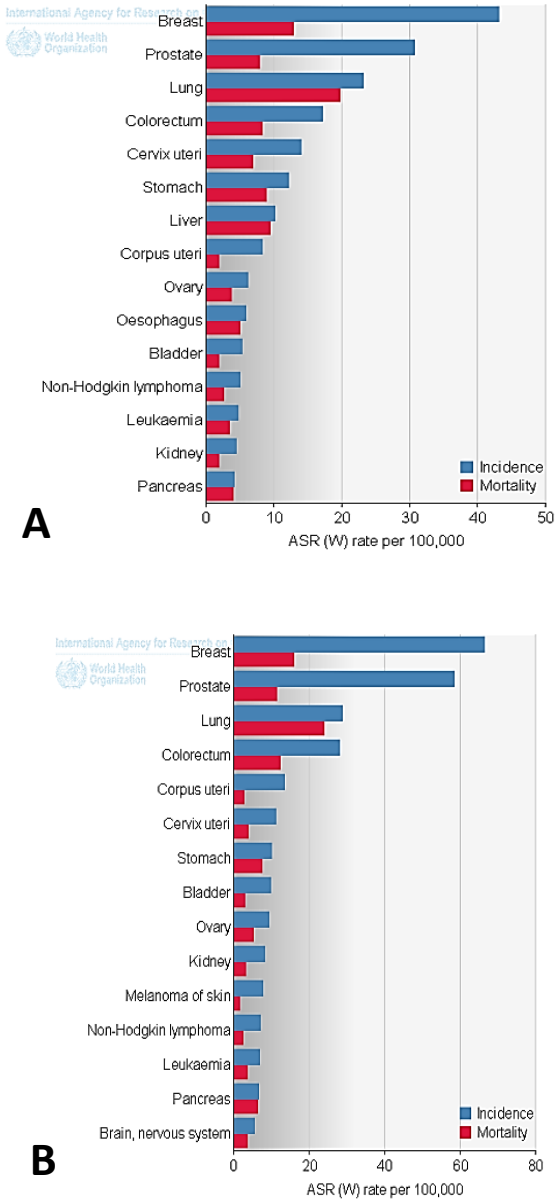


Figure 1: Incidence and mortality of the most common cancers worlwide **(A)** and in Europe **(B)** for both sexes [ref.1].

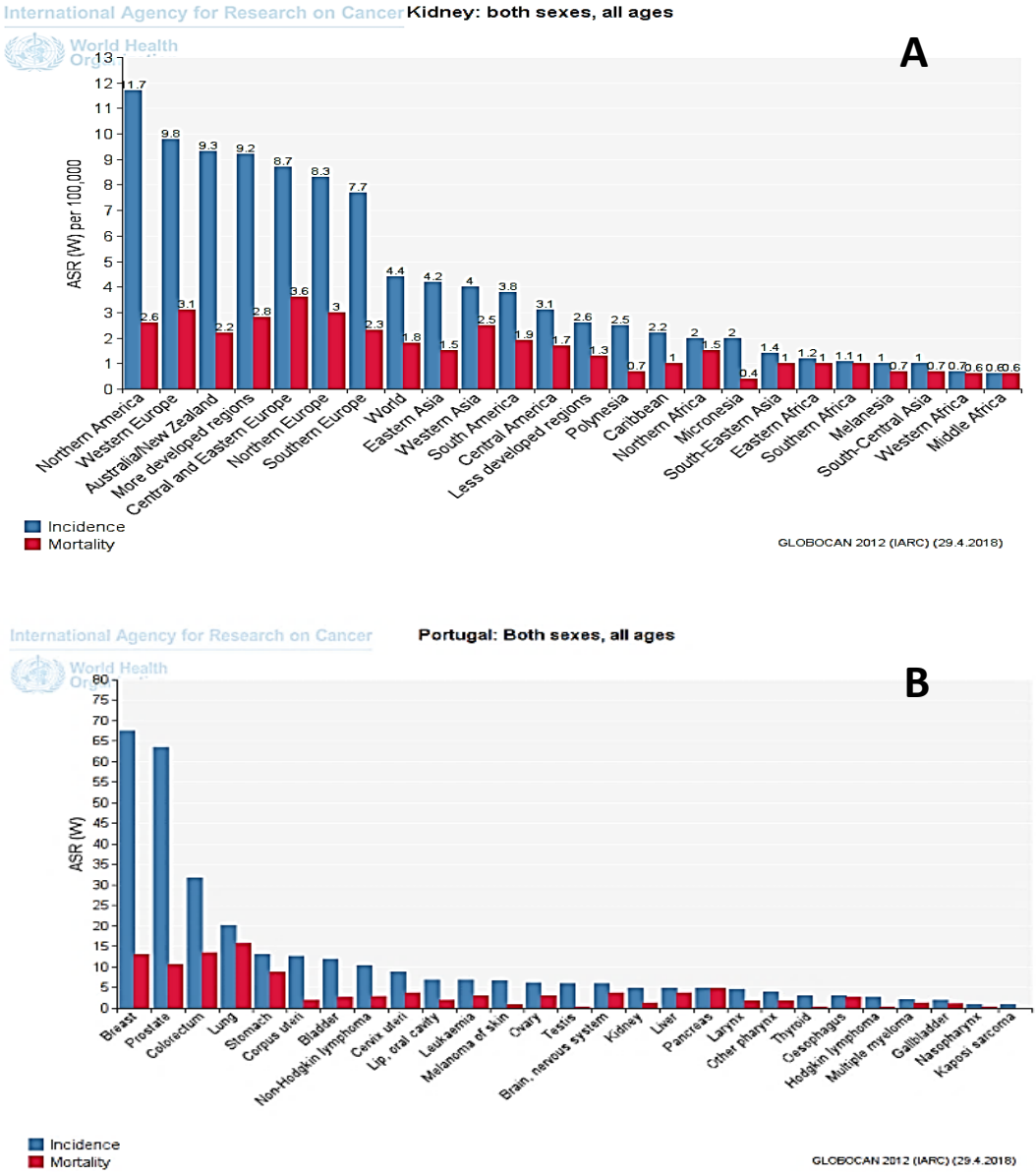


Figure 2: Incidence and mortality, for both sexes, of kidney cancer worldwide **(A)** and of different types of cancer in Portugal **(B)** [ref.1].

From SEER data, 74.5% of patients survive five years or more after being diagnosed with kidney cancer and that patients with localized disease have better five-year relative survival values (92.6%) compared with patients that have regional (68.7%) or distant metastasis (11.6%). It is expected, in the year 2035, an increment of both incidence and mortality, having more 240.083 of new cases and 115.805 of deaths, in comparison to the year 2012[1].

1.2 Risk Factors

1.2.1. Gender and ethnicity

Men appear to have a greater risk of developing this malignancy in comparison to women. In terms of ethnicity groups, African-americans have the smallest survival rates, even though the cancer is detected in a early stage and at younger age. Hispanic patients also display low levels of survival compared to other races[2].

1.2.2. Cigarette smoking, hypertension and obesity

Smoking is a well known risk factor, since the risk increases 50% in males and 20% in females smokers in relation to non-smokers. There is an evident dose-response association and is possible the diminution of the risk after at least ten years without smoking. Tobacco consumption increases the risk of developing kidney cancer due to exposure to carbon monoxide that creates a chronic tissue hypoxia. Furthermore, the presence of a carcinogenic N-nitrosamine, in the cigarettes, provokes DNA damage in lymphocytes of the peripheral blood and the same goes with the other major component, benzo-α-pyrene-diol epoxide, that leads to alterations in the chromossome 3p[2,3].

Individuals with hypertension have double the risk of having kidney cancer. Several studies showed that the risk enhances as the blood pressure rises, comproving a dose-response association, but the explanation behind this relation is yet to be found[2,3]. When it comes to obesity, is a risk factor recognised to be responsable for around 40% of kidney cancers in the United States and around 30% in Europe. It is acknowledged that the risk increases 34% for women and 24% for men for each 5kg/m^2 augmentation in body mass index. Individuals that have obesity and hypertension display higher risk for developmenting kidney cancer compared to others that only suffer from one of these health issues[3].

1.2.3 Inherited Syndromes

Although the majority of the kidney tumors are sporadic, some subtypes are developed due to germline mutations. Moreover, it is recognized that close to 2-3% of the cases are result of familial and autosomal dominant syndromes[4,5].

Von Hippel-Lindau (VHL) disease is a syndrome characterized by the inheritance of one mutated VHL allele and then acquisition of an alteration in the other allele due to deletion in most of the cases and less frequently due to promoter hypermethylation. It is responsable for the appearance of retinal angiomas, central nervous system's hemangioblastomas, pheochromocytomas and clear cell renal cell carcinoma. Penetrance for each condition is not complete since, for instance, Renal Cell Carcinoma (RCC) was found solely in 40-50% of the individuals with VHL mutation[4–7]. Patients with

this syndrome have kidney cists and multifocal bilateral ccRCC at an age close to 37 years old[7].

Hereditary papillary renal carcinoma (HPRC) results from mutations in the *MET* gene present in chromossome 7 that leads to the growth of multifocal and type 1 of these tumors[4].

Hereditary leiomyomatosis and renal-cell cancer (HLRCC) syndrome occurs due to mutations in the *FH* gene and causes the appearance of solitary papillary renal-cell carcinoma and leiomyomas in the uterus and in the skin. Sometimes can provoke collecting duct or clear cell renal cell carcinoma[4].

Birt–Hogg–Dubé (BHD) syndrome is a rare condition characterized by the presence of hair-follicle hamartomas in the face and the neck. Around 15% of the patients display chromophobe or mixed chromophobe–oncocytomas. Sometimes, papillary and clear-cell renal-cell carcinoma can develop in the same patient[4].

2. Kidney Cancer

2.1 Clinical presentation and diagnosis

Kidney cancer can be divided in two hystological types, based in the site of origin of the tumor in the kidney. Renal cell carcinomas (RCC) arise from the kidney parenchyma, constituing about 90% of kidney cancer, while the renal pelvis tumors are of transitional type with only 10% being confirmed as kidney carcinomas[3]. Usually, renal cell carcinoma, is more frequent in men than that in women[6]. Incidence starts augmenting in patients with 40 years old until patients with 75 years old and sometimes declines at this age, being 64 years old the median age of presentation. RCC is incidentally detected in the majority of the times (60%) by the use of abdominal image. Only 10% of the patients with RCC have the classic triad of hematuria, flank pain and abdominal mass while more than 40% show systemic symptoms like weight loss, abdominal pain, anorexia and fever. Hematuria also is a sign to take in consideration and it needs to be analysed with the help of computed tomography scan of the urinary tract. In the case of patients with more than forty years old should be performed a cystoscopy to exclude bladder cancer[4]. When diagnosticated, 25% of the patients present advanced disease, where it is locally advanced or metastasized, which associates with a median survival of about thirteen months[4,7]. In local disease patients', recurrence is rather common after surgery.

2.2 Hystological subtypes of RCC

Renal cell carcinoma acomprises a group of heterogeneous malignant tumors that differ in morphology, in genetics and in behaviour. Clear cell renal cell carcinoma (ccRCC) it is the most common and constitutes 75-80% of RCC while the papillary renal cell carcinoma (pRCC) and the chromophobe renal cell carcinoma (chRCC) constitute about 15% and 5% respectively. Collecting duct carcinoma is a rare histological subtype that it is worth less than 1% of RCC. In around 4% of the times it is dificult to distinguish the different subsets of RCC since some tumors present mixed morphologies and sometimes have irrecognizable characteristics which prevents a congruous diagnosis and are named renal cell carcinoma unclassified. Heidelberg classification is the guideline for the differentiation of RCC histologic types[7–9].

Different studies have shown that ccRCC has the worst outcome out of the main histological types. It is notable that when the analysis of survival is adjusted for tumor grade and for TNM stage, patients with chromophobe carcinoma grade 3-4 or T3-4 have the best outcome while patients with grade 1-2 or T1-2 tumors do not show statistical difference in outcome between the principal subtypes. Papillary tumor seems to appear at an earlier stage compared to clear cell tumor and has the lowest mean size of the main histological types. It is known that chromophobe tumor comes on in younger patients that are mainly women in comparison with papillary and clear cell RCC. The most acknowledge prognostic factors used for stratification of the patients representing an ally in the correct therapy administration are the pathologic stage, the ECOG performance status and the Furhman grade[9,10].

2.2.1 Clear Cell Renal Cell Carcinoma

CcRCC is the most frequent hystological subtype of RCC that show high vascularization and a characteristic yellow surface due to the accumulation of lipids. It is common the cystic presentation because of necrosis and the existence of hemorrhagic zones. Microscopically it can be observed that neoplastic cells show a round nucleus and clear cytoplasm, since they have a great quantity of glycogen and lipds like cholesterol and phospholipds. Usually cells display a solid, tubular or microcystic disposition. Inflammatory processes can exist like it is the case of lymphocitic infiltration and some cases can present sarcomatoid differentiation which is associated with worst prognostic. These type of tumors are known to be able to metastasize to uncommon sites and metastases can appear ten years after diagnosis of the primary tumor. Metastization occurs through hematogenous dissemination via the vena cava directly to the lung and also via paravertebral veins, vena testicularis/ vena ovarii, intra-renal veins and throughout the ureter[8,11].

Sporadic clear cell renal cell carcinoma normally appears in individuals with 61 years old. Loss of the short arm of chromosome 3 is the most common genetic alteration and it is essencial for the transformation in malignant neoplastic cells[7,8,12,13]. Various genes are mapped in the deleted arm, such as polybromo-1 (*PBRM1*), SET domain-containing 2 (*SETD2*), BRCA1-associated protein-1 (*BAP1*) and *VHL*, a tumor supressor gene that is the one most frequently inactivated in ccRCC. Eighty seven percent of ccRCCs have loss of heterozygosity (LOH) of chromosome 3p, whereas 2/3 of the sporadic cases have VHL inactivation in both alelles, and about 30% of the cases habour *VHL* promoter hypermethylation[14]. VHL inactivation is considered the start event in clear cell RCC but there is lack of information regarding further alterations that are responsable for tumor progression and are associated with prognostic[7,12,13]. Nonetheless, it is acknowledged that when a subunity of the SWI/SNF chromatin remodeling complex, PBRM1, expression is compromised by small interfering RNA (siRNA) knockdown increases proliferation and migration in most of the ccRCC cell lines. Also, the discoverement of truncating mutations in PBRM1 is important since its complex participates in cells response to hypoxia which implicates chromatin remodeling genes in ccRCC[12]. An analysis of the sequencing of more than 3000 genes in 101 ccRCC allowed detection of new mutations in genes involved in histone modifications like *JARIDIC* and *UTX*[7,12].

2.2.1.1 VHL/HIF-1α pathway

As mentioned above, *VHL* gene is inactivated in most of ccRCC cases, which leads to protein loss, that together with elongin B and elongin C belongs to the E3 ubiquitin ligase complex. This is responsable for ubiquitination of hypoxia inducible factors α (HIF-1α and HIF-2α) resulting in their consequent degradation. In normoxia conditions, prolyl hydroxylases cause hidroxylation of proline residues of HIF-α, allowing VHL activity, but in hypoxia the hydroxylases are inactivated and as a consequence HIF-1α and HIF-2α will not be recognized by VHL, which allows them to interact with HIF-1β, present in the nucleus, to posteriorly connect with hypoxia response elements (HRE) existing in the target genes. In ccRCC, VHL default will grant HIF-α stabilization, their accumulation and thereafter constant activation of genes involved in angiogenesis, in division and cell growth, in acid-base balance and in glucose uptake which explains the overexpression of vascular endothelial growth factor (VEGF), transforming growth factor α (TGFα), platelet-derived growth factor (PDGF) and GLUT-1 proteins, respectively. In this situation, cells display a hypoxic behavior when they possess normal O_2 levels which is recognized as pseudohypoxic condition and present a glycolytic phenotype over a oxidative, performing aerobic glycolysis also known as “Warburg effect”. The

deregulation of HIF-α creates a perfect microenvironment for tumor proliferation, migration and invasion[4,13,15,16].

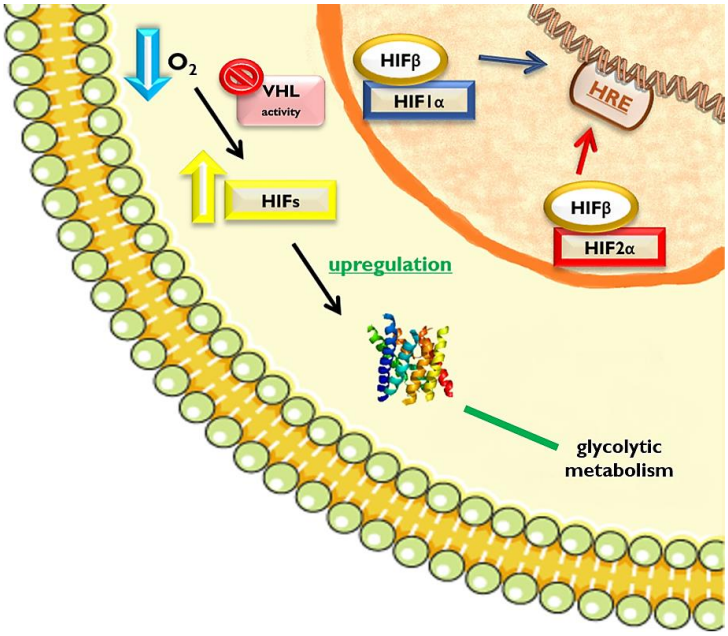


Figure 3: HIFs permanent activity due to VHL activity absence cause an upregulation of proteins that promote glycolytic metabolism through interaction with HRE of target genes after conection with subunity HIFβ in the nucleus.

2.3 Staging and grading

Stage assessment is based on tumor's size and extension, being important for prognosis evaluation. The American Joint Committee on Cancer (AJCC) guidelines for TNM staging system specifies local extension of primary tumor (T), implication of regional lymph nodes (N) and presence of distant metastases (M). CcRCC and pRCC are graded based the WHO/ISUP grading system, which is essentially, based on nuclei categorization of size, shape and prominence[2,17].

Table 1: WHO/ISUP grading system for RCC. Adapted from [14].

Definition of Histologic Grade (G)	
GX	Grade cannot be assessed
G1	Nucleoli absent or inconspicuous and basophilic at 400x magnification
G2	Nucleoli conspicuous and eosinophilic at 400x magnification, visible but not prominent at 100x magnification
G3	Nucleoli conspicuous and eosinophilic at 100x magnification
G4	Marked nuclear pleomorphism and/or multinucleate giant cells and/or rhabdoid and/or sarcomatoid differentiation

Table 2: TNM staging system for RCC. Adapted from [14].

Definition of Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor ≤ 7 cm in greatest dimension, limited to the kidney
T1a	Tumor ≤ 4 cm in greatest dimension, limited to the kidney
T1b	Tumor > 4 cm but ≤ 7 cm in greatest dimension, limited to the kidney
T2	Tumor > 7 cm in greatest dimension, limited to the kidney
T2a	Tumor > 7 cm but ≤ 10 cm in greatest dimension, limited to the kidney
T2b	Tumor > 10 cm, limited to the kidney
T3	Tumor extends into major veins or perinephric tissues, but not into the ipsilateral adrenal gland and not beyond Gerota's fascia
T3a	Tumor extends into the renal vein or its segmental branches, or invades the pelvicalyceal sistem, or invades perirenal and/or renal sinus fat but not beyond Gerota's fascia
T3b	Tumor extends into the vena cava below the diaphragm
T3c	Tumor extends into the vena cava above the diaphragm or invades the wall of the vena cava
T4	Tumor invades beyond Gerota's fascia (including contiguous extension into the ipsilateral adrenal gland)
Definition of Regional Lymph Node (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
Definition of Distant Metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

2.4 Treatment

For RCC there are available various treatments for different stages of the disease. However, it is perceived that even with the standard procedures, like surgery, an important number of the patients have recurrence and even develop metastases that cannot be controled with chemotherapy or radiotherapy due to tumor resistence[12,18]. Other approaches like imunotherapy or targeted therapy have shown limited efficacy. For this reason it is really important to take in consideration RCC metabolism, its heterogeneity along with its microenvironment to discover new molecular targets for a better outcome[12,18].

In early stages in which tumor it is confined to the kidney, active vigilance, surgery and ablation are treatment options. Vigilance is proposed when patients show small masses, around 3 cm, that expand slowly with no symptoms that do not present a risk of metastizing (close to 30% do not evolve). The goal is to watch closely its development in order to perform surgery in case the tumor achieves a fast progression to avoid negative effects and to maintain kidney integrity[7,13].

Surgery is a common practice to control localized disease. There are two options: radical or partial nephrectomy. The first one implies the removal of kidney together with gerota's fascia in addition to the ipsilateral adrenal gland and regional lymph nodes whereas the second is performed in tumors smaller than 4 cm with the objective of sparing the kidney to preserve the maximum of its funtion. To understand which of the these approaches is propper for the patient it is fundamental to determine the TNM stage and to analyse the individual physical condition[4,6].

When patients can not undergo surgery or are elder or present multifocal tumors there are other treatment options, like ablation. In general, this is based in the introduction of a needle or more in the tumor, that is guided through image and that it will destroy tumor cells due to liberation of heat, cold or electromagnetic waves. In the case of radiofrequency ablation the needle will heat up to a temperature between 50-100°C, opposite to cryoablation in which it cools down to a maximum of -195°C. As for the microwave ablation the needle will liberate electromagnetic waves. Compared to surgery, this procedure presents a higher local recurrence, an equal disease-free survival and it can not be executed in tumors bigger than 3 cm[4,6,13].

For tumors in advanced stage or that are impossible to operate, immunotherapy or targeted therapy are an alternative. Radical nephrectomy can be taken into account when there are metastases, since the median overall survival is higher when this cytoreductive approach is combined with interferon alfa compared to when this is admnistrated alone. It is well stablished that RCCs have a huge vascular network, being quiet dependent on VEGF action. Sunitinib, sorafenib, pazopanib, axitinib, lenvatinib and

cabozantinib are tyrosine kinase inhibitors that will interact with VEGF receptors. In addition to these, there was also developed an antibody that neutralizes VEGF protein named bevacizumab and better results are achieved when patients receive a combination of this with interferon. Sunitinib, pazopanib and bevacizumab with interferon constitute the first line of treatment while axitinib and cabozantinib belong to second line. There were inhibitors already designed for the mammalian target of rapamycin (mTOR) family like, temsirolimus and everolimus that are able to diminuish the ammount of HIF and that together make part of the second line treatment. Temsirolimus administration allowed patients to have a greater overall survival compared to interferon. Furthermore, in immunology context, antibodies that inhibit CTLA4 (ipilumumab), which negatively regulates T cells function, and for PD-1 (avelumab and atezolizumab), a protein extensively expressed in T cells and for its ligand, PD-1L (nivolumab and pembrolizumab) that is overexpressed in tumors in order to block imune response. Moreover, it was noticed that antigen CA9 is highly expressed in ccRCC and represents a potencial target for vaccine therapies that are based on the use of dendritic cells, that necessitate to be more investigated[4,6,7,12,13,18].

3 Epigenetic mechanisms

Epigenetics results in information that is not codificated in the DNA sequence but it allows regulation of gene expression by certain mechanisms of action. These are known as DNA methylation and histone modifications and are responsible for heritable and reversible alterations that are fundamental for normal cell growth, development and identity. The good maintenance of tissue-specific epigenetics patterns allows correct gene expression while abnormal changes result in apoptosis and in corrupted expression which can be the foundation for cancer. The complexity of this disease it can not be explained only on genetics bases since throughout the tumor progression, cells achieve different behaviour. The deregulation of the epigenetics mechanisms promote genetic instability which creates new mutations. Overall, epigenetics modifications are dynamic alterations, that are susceptible to tumor microenvironment. Ultimately, they activate oncogenes and repress tumor suppressor genes, being important in malignant transformation[19–22].

Thus, a better comprehension of the epigenetics processes is crucial to the discovery of new biomarkers as well of new pathways that can be object of target therapies. In this way, it would be possible to generate new approaches for diagnosis, and prognosis evaluation, as well for therapy to control the disease.

3.1 DNA methylation

DNA methylation is characterized by addition of a methyl group in carbon 5 of the cytosine ring in the major of the times at dinucleotides CpG, approximately 75%. These CpG are heterogeneously distributed in the human genome but are highly concentrated in some regions, the CpG islands (CGIs) that have close to 50% of dinucleotides CpG. These islands lay in about 60% of the promoter regions of the genes[20,21]. Overall, in normal tissues the islands of CpGs are unmethylated which opens the access to transcription factors to initiate transcription and thereby the initiation of genetic expression. Some promoters have dinucleotides CpG methylated due to tissue differentiation that creates a tissue-specific methylation pattern and arises in a genetic silencing. It is known that repetitive regions of the genome are also methylated to keep the genomic stability. Deregulation of these epigenetic event leads to a defective phenotype and to an higher predisposition to cancer in which there is an activation of oncogenes by hypomethylation and an inactivation of tumor supressor genes by hypermethylation of the CpG islands in the promoters.

The process whereby DNA methylation is mantained and spread througout the systematic cell divisions is not fully covered. Until now there is a group of enzymes that are known to manage and preserve this epigenetic action: DNA methyltransferase 1 (DNMT1), DNA methyltransferase 3a (DNMT3a), DNA methyltransferase 3b (DNMT3b) and DNA methyltransferase 3L (DNMT3L). The settlement of the new methylation pattern in DNA unmethylated is done by DNMT3a and DNMT3b[23,24] that are greatly expressed during embryogenesis in opposite to mature tissues, while DNMT1 is responsible for the conservation of the existing methylation template during successives mitosis having preference for hemimethylated DNA[24] being also able to mend DNA methylation[25,26]. DNMT3a and DNMT3b can be enlisted by transcription factors or implement methylation marks in all CpG islands that are not covered through the connection with the transcription factors[25]. These enzymes have both catalytic and regulatory domain except for DNMT3L, since it does not have enzymatic activity instead, it only associates physically to DNMT3a and to DNMT3b to promote methylation[23,24]. These epigenetic modifiers can affect DNA expression in two manners: one is directly by inhibiting transcription factors connection to the promotor and the other one is through the enrolling of methyl-CpG-binding domain proteins (MBDs)[26] that attract other proteins capable of modifying histones and modulate chromatin. MBD proteins like MeCP2, MBD1, MBD2, MBD3 and MBD4 identify methylated CpG islands and are capable to bond to repressive protein complexes by their transcriptional repressive domain (TRD)[24,25].

Concluding, the methylation deregulation in cancer, namely hypermethylation of tumor supressor genes and hypomethylation of oncogenes allows tumorigenesis[23,24,26,27–36] since cells adquire selective advantage[21,22].

3.2 Histone modifications

Chromatin organization and structure is essencial for appropriate genetic expression and nucleosomes are its basic unit. They are the conjunction of DNA wrapped around a complex of proteins called histones that form an octamer composed of doublets of each of one of the following: H2A, H2B, H3 and H4. These present a C-terminal domain and a N-terminal tail that it is charged and that its residues will be target of enzymatic activity like methylation, acetylation, ubiquitylation and phosphorylation that are performed by histone methylases (HMTs), histone acetylases (HATs), ubiquitin ligases and kinases, respectively. Alteration in transcription status is a result of these dynamic and covalent modifications since they modify chromatin organization through change in physical characteristics of the nucleosomes like neutralization or augmentation of charge in the aminoacids, that affect interaction between the DNA and the histones, leading to an more "open" or more "closed" chromatin[20,37]. This means that histone-modifying enzymes regulate the accessibility of transcription factors to certain regions of DNA so that genes are expressed when chromatin is more unrolled, named euchromatin and are not expressed when it is more condensed, named heterochromatin. When it comes to adjustments in the tails of the histones there is no absolute sure about their meaning but lysine shows to be the more critical residue[20–22], since it is the most targeted. Usually, acetylation of lysine drives to active transcription[20,22,37] and methylation depending on the aminoacid, on its position and on the number of times that it is methylated can represent an active mark or not[20,37]. In terms of methylation, Polycomb group proteins are one of the most discussed. They can repress gene transcription and retain it throughout cell division, being the founders of H3K27me3[20,37,38].

It can be assumed that for cancer growth, the uncontrollable expression of hystone-modifying enzymes and the differents outcomes in histones's tails residues affect prognostic and proliferation of tumors, leading to a more open spectrum of feasible new biomarkers for this complicated disease[39–46].

4 Monocarboxylate Transporters (MCTs)

Monocarboxylic acids like lactate, pyruvate and ketone bodies are fundamental for proper metabolism since they are synthesized and used in different biological pathways and reactions such as glycolisis, gluconeogenesis, transamination, among others, being key factors in the obtention of energy in cells. It was thought that, for a long time, this molecules would pass plasma membrane unassociated by diffusion, until it was found out that α-cyano-4-hydroxycinnamate is capable of blocking the movement of lactate and pyruvate through the cell membrane of erythrocytes[47] which compromises the participation of some kind of transporter. Nowadays, it is known that this conveyor it belongs to a big family of 14 proteins called solute carrier family 16 (SLC16) or monocarbolxylate transporter family in which the isoforms MCT1 (SLC16A1), MCT2 (SLC16A7), MCT3 (SLC16A8) and MCT4 (SLC16A3) are adept to transfer lactate, pyruvate and ketone bodies by a proton dependent mechanism. MCT7 (SLC16A6), MCT8 (SLC16A2), MCT9 (SLC16A9) and TAT1 (MCT10 or SLC16A10) are recognized for carrying β-hydroxybutyrate, thyroid hormones, carnitine and aromatic amino acid, respectively. As for the rest of the family members, theirs' substrate was not identified, so far. In general, all of these molecules present distinct characteristics, localization and expression, having in common conserved sequence motifs. It is important to refer that the existence of two nomenclatures, SLC16 and MCT, happens because the first one was stablished once the cDNA sequences were obtained, while the latter was attributed based on the order of functionality characterization[48–52].

4.1 Structure/Activity/Function

Overall, MCTs are thought to have twelve transmembrane helices (TMs) with a cytosolic amino-terminal and a cytosolic carboxi-terminal[53] that together with the intracellular loop in the midst of TMs 6 and 7 display the major variation in the sequence lenght. The regions that are greatly conserved in all MCTs isoforms are situated at TMs 1 and 5 and do not show glycosylation[54,55]. MCT1, MCT2, MCT3 and MCT4 are the only members able to transport monocarboxylates and they do it in a proton dependent manner, acting like symporters. It is known that the translocation is done without energy supply, since it is performed according to the substrate and proton concentratrion gradients and it is believed that the mechanism of action implies first the addition of the proton, being the monocarboxylate linked secondly. The connection is made thanks to an lysine (K38) residue, when the protein is in a open conformation state, since it becomes available for the proton and consequently allows the interaction of the monocarboxylate that, afterwards, it is transfered to an ion pair formed by aspartate (D302) and arginine (R306) present at the intracellular domain of the protein that are

fundamental for proper carrying[56]. This will cause the lysine to be deprotonated which enables the relaxation and subsequently obtention of the closed state. At the same time, this change in arrangement will consent the liberation of the molecules transportated towards the cytoplasmic matrix[49,50,52]. The lysine amino acid tend to have neutral charge in the protein closed conformation state, considering that it is localized at a hydrophobic site and its importance was already tested, because its replacement leads to an inactivation of MCT1[57]. Near the ion pair discussed above is situated phenylalanine 360 that it is recognized for its relevance in the selection of substrates carried by MCT1[58]. For MCTs 1 to 4 to be able to perform their function, it is indispensable the presence of auxiliary proteins like embigin (gp-70) and basigin (CD147) that work as chaperones and by that are adept to guide MCTs to the cell membrane, meaning that their default will prevent the expression and therefore the function of the proteins discussed. They both have one transmembrane domain that holds a conserved glutamate amino acid, a cytosolic carboxi-terminal and a huge extracelular domain that shows glycosylation and distinct immunoglobulin regions that vary in accordance with the alternative splicing[59,60]. Basigin is extensevelly expressed while embigin has a more restricted expression, being the first the favored by MCT1, MCT3 and MCT4[61,62] whereas the latter is the favourite of MCT2[63]. MCT1 is also able to connect with embigin if needed[64], MCT8 due to its action does not need supporter proteins[65] and as for the rest of the MCTs family it lacks information relative to the necessity of these kind of proteins. It is perceived that the transmembrane and cytoplasmatic regions in basigin, as well the presence of the preserved glutamate are essential for the contact with MCTs and they do it by affiliation with their TM3 and TM6[56,57,61].

MCTs 1 to 4, in general, present a ample variety when it comes to substrates, since they can translocate shortchain monocarboxylates suchlike lactate, that it is the most significant, shortchain fatty acids like acetate and ketoacids with hydrophobic groups obtained from transamination of amino acids. MCT1 has a clear preference for L-lactate compared to D-lactate, it does the uptake and the efflux of lactic acid and because of this exists in tissues that produce lactate and in the ones that use it for synthesis of biomolecules like lipids or glucose. MCT2 is responsable for the uptake of lactic acid and it has greater affinity for most of the substrates compared to the other isoforms so it has lower values of *K*m (mM), for exemple, for lactate and pyruvate, it has arround 0,7 and 0,1, respectively[50,52]. Due to its function, this carrier is immensely existent in cells that utilize lactate for gluconeogenesis or lipogenesis. Compared to MCT1, its smaller value for *K*m allows efficient uptake of the susbstrate when this exist poorly in the blood[66]. As for MCT4, it performs the efflux and it shows the lowest affinity,

having the biggest *K*m (mM) values, more than 100 for pyruvate and close to 20 for lactate, for instance. This isoform has preference for lactate and is vastly expressed, notably in tissues that depend a lot in glycolysis[67,68]. This means that this isoform will have a value role on export of lactic acid when cells reach high levels of this molecule. Little is known about MCT3 so far, since it has not been fully characterized. MCT6 seems to be capable of translocate bumetanide[69] and as to MCT7, 8, 9 and 10 theirs substrate was already referred above. MCT8 and MCT10 do their function in a proton independent manner[51]. The activity of the remaining members of the MCTs family is not known.

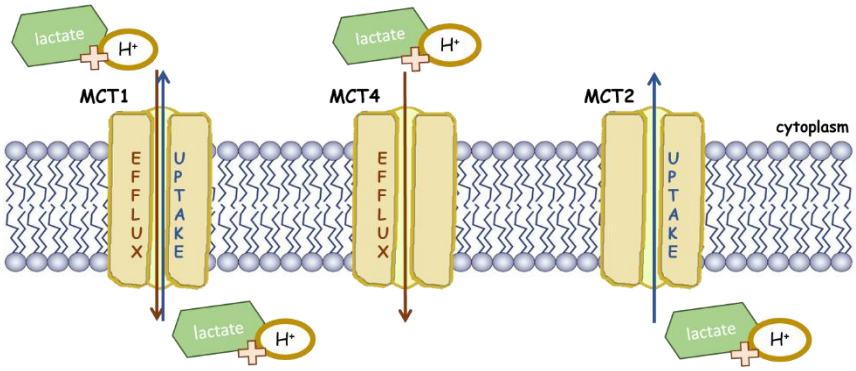


Figure 4: Monocarboxylate transporters 1, 2 and 4 activity, relative to lactic acid translocation.

4.2 Expression

MCT1, is expressed in both tissues that are able to produce monocarboxylates, like erythrocytes, or use them as it is the case of cardiac muscle and the red fibres in skeletal muscle. In the central nervous system, MCT1 is expressed, for exemple, in astrocytes, in endothelial cells, in neurons and in the hypothalamus. MCT1 is also detected in kidney[70] and largely divided along the gastrointestinal tract, with a increasing of expression throughout the intestine, while the stomach shows minor levels[51,71]. In few species, MCT1 and MCT2 are coexpressed in type I fibres of the skeletal muscle and the latter is also present in neurons for the importation of lactic acid[51]. MCT2 was found in rat hearts but not in human hearts, as well as in tissues that incorporate monocarboxylates like, for instance, kidney, testis, stomach, liver and lung[72]. As for MCT3, it is reported to be in the basolateral membrane of the retinal pigment epithelium in opposite to MCT1 that it is in the apical membrane, and to be in the choroid plexus, showing restrict expression[49,52]. This isoform suggest to ease the exportation of the lactic acid in the retina. Due to its function, MCT4 is expected to be only localized in cells that

have high glycolytic rate that consequently create a huge quantity of lactate, such as white fibers in skeletal muscle and astrocytes. It is reported to be also in chondrocytes, leukocytes, colon and in the kidney[48,51,70]. MCT8 is recognized for being largely expressed in different types of tissues that include liver, kidney, heart, brain and skeletal muscle like MCT10 and furthermore it exists in thyroid[49,51].

It is acknowledged that MCT1 and MCT4 are broadly expressed in cancer and are the most studied isoforms[73–75]. Also, it is important to refer that the pattern of expression of these proteins varies according to the metabolic profile of the malignant tumors[73]. MCTs are fundamental for the upkeep of the glycolytic rate since they are responsable for the transportation of lactate coupled with a proton and through that they can, additionally, prevent intracellular acidification, generating acid-resistant cells. This is of huge importance, because cancer cells rely greatly on glycolysis for obtention of energy[50,74,76]. Solid tumors tend to be quite heterogeneous developing various hypoxia regions with low amounts of nutrients as a result of the vast proliferation. In these conditions, neoplastic cells are able to switch their metabolism and adapt, becoming glycolytic cells, and gaining selective advantage. Some tumors cells, even in normoxia conditions, present huge glycolytic activity with consequent enormous production of lactate, that is known as “Warburg effect” or aerobic glycolysis. While the normal cells die due to microenvironment acidification, as a after effect of imense glycolytic activity, neoplastic cells survive by virtue of the protein machinery they evolve, like transporters, that maintain the intracellular pH homeostasis[51,52,74,76–78]. Furthermore, this acidosis activates the destruction of the extracellular matrix and promotes angiogenesis, that together aid in migration and in invasion of the tumor cells, promoting metastization[77,78]. Taken all of this into account, it makes perfect sense the role of the MCTs in cancer and it is the reason why they are overexpressed in diverse tumors types like non-small cell lung cancer[79], breast carcinoma[80], clear cell renal cell carcinoma[81], gliomas[82], pancreatic cancer[83], colorectal carcinomas[84], melanomas[85], among others[75]. The blockage of these proteins already exhibited as a outcome, intracellular acidification with interdiction in the tumor proliferation[52]. In order to cope with the unfavorable conditions that arise throughout the growth, tumors also show another strategy that is based on the interaction between glycolytic and oxidative neoplastic cells[86,87]. The lactate produced by the first ones, seems to be exported by MCT4 and it will be imported by the latters through MCT1, being used to obtain ATP molecules[74,78]. This happens because the oxidative cells will prefer lactate over glucose and will hand it over to the glycolytic cells that will use it to produce lactate in a great quantity[87].

According to MCTs functionalities already discussed, it is logical that these carriers are behind the development of distinct kinds of aggressive tumors with worse

prognosis[75,78,88]. For instance, in prostate cancer was discovered that MCT1 and MCT2 are necessary for the sustainment of the tumor, whereas MCT4 increases its aggressive behaviour[89]. In lung cancer, both MCT1 and MCT4 are behind its invansion[90]. In this context, not only the acidosis but also lactate is a key factor for the occurence of worst outcome[87,91]. This substrate is admitted to ease invasion, to ease escape from the immunological system, by, for exemple, asphyxiation of cytotoxic T cells, to induce endurance to therapy, to ease migration and proliferation, due to hyaluronan synthesis by fibroblast stimulation and due to formation of new blood vessels through VEGF production, that also shows lactate ability in angiogenesis promotion. Actually, this substrate when produced and liberated, moves into endothelial cells and into oxidative tumor cells, thanks to MCT1, and provokes activity of the hypoxia inducible factor 1α and nuclear factor-κB (NF-κB), generating expression of VEGF, basic fibroblast growth factor (bFGF) and interleukin-8 (IL-8)[74,78,91]. These actions, overall, will guarantee that tumor cells have enough oxygen, nutrients and a proper medium to keep on growing and metastasize.

Taking all of this together, the fundamental part of MCTs in cancer cells metabolism is becoming more obvious, as more studies get deeper in the comprehension of their phisiological activity. Their capacity to translocate lactic acid allows tumor cells to upkeep the glycolytic rate that is crucial for them to proliferate and confers immunity to the acid environment, since they prevent intracellular acidification. Through the substrate translocated, MCTs consent the establishment of a medium that is suitable for cancer cells proliferation, while the tissue host cells die for the lack of oxygen, nutrients and extracellular acidosis. It is undeniable the selective advantage cancer cells display over the normal cells, which demonstrates the necessity of understanding more about these transporters, to block their function for a better control of the disease.

4.3 MCTs regulation

There is little information about these transporters regulation, being MCT1 the most studied isoform. Alteration on expression levels of these proteins might result from changes in both transcription and post-transcriptional processes, along with post-translational modifications due to interaction with the chaperone proteins refered above.

Regarding MCT1, was previously demonstrated that is regulated through modifications on both calcium and AMP levels in the skeletal muscle[92]. As a result of intense exercise or chronic stimulation, the amount of calcium ion and AMP tend to be elevated causing the activation of the calcineurin, a protein phosphatase, and of the

AMPK, a protein kinase, respectively. Calcineurin is capable to dephosphorylate and by that activates the transcription factor NFAT that recognizes various sequences present in the promoter region of MCT1, provoking its expression augmentation[93]. This is verified, similarly, in the activation and proliferation of T-lymphocytes, whereas the upregulation of MCT1, due to NFAT action, is fundamental to mantain the glycolysis rate[92]. As for AMP, when its levels ascend, there is an increase in the expression of MCT1 through an AMPK-dependent mechanism[92]. Furthermore, it is recognized that AMPK and calcium are adept to instigate PGC1α[94,95], a transcription coactivator, that is accepted to raise MCT1 levels[96]. Additionally, it is known that pro-inflammatory cytokines IFN-γ and TNF-α down-regulate MCT1 expression[97]. Moreover, MCT1 expression is regulated at translation level, since MCT1 protein levels increase throughout the post-mitotic and G1 stages of the cell cycle, while there is no alteration in the mRNA quantity[92]. This is supported by the fact that MCT1's mRNA has a longer 3'untranslated region (3'UTR) compared to the other MCTs isoforms, and that displays a potential cytosolic polyadenylation element and hexanucleotide sequences[55]. Indeed, the sequences present in the 5'UTR and, more commonly, in the 3'UTR, are admitted to be target of initiation factors like eIF4e, that control translation process by stimulating or repressing it[98]. During the cell cycle, eIF4e and its inhibitor 4E-BP1 alterations in phosphorylation levels correlated with MCT1 expression, because when the 4E-BP1 phosphorylation reached its maximum, MCT1 presented the highest protein expression. Conversely, MCT4 do not show differences in the protein or mRNA levels[92]. It is aknownledged that MCT1 is regulated by its own substracte, because lactate, in high concentrations, leads to an increase in both MCT1's protein and mRNA expression and it was already proved both *in vitro*[99] and *in vivo*[100]. Butyrate is also another substracte that impacts on MCT1's regulation, because it provokes increased mRNA and protein expression, which consequently causes a increment in this short chain fatty acid transport, in the colon[101]. Hormones also seem to have a participation in the regulation of MCT1 and MCT4, like it is the case of the thyroid hormone (T3), in the skeletal muscle[102]. In addition, testosterone and thyroid stimulating hormone (TSH) can promote MCT1's protein expression[100]. Regarding MCT2, in brain and similarly to MCT1, its protein levels are increased by insulin, IGF-1[103] and noradrenalina[104], although no changes are apparent at mRNA levels. Moreover, in neurons, the brain-derived neurotrophic factor (BDNF) stimulates MCT2 protein expression[105].

Furthermore, several studies demonstrated the impact of hypoxia on MCTs regulation. HIF-1α is of huge importance in the control of the response of cells to hypoxia conditions, being active and connecting with the other subunity HIF-1β in the nucleus to further interact with the HRE present in the promoter target genes. Usually, as a

transcription factor, HIF-1α promotes the expression of proteins that enhance glycolysis over oxidative phosphorylation[106]. Specifically hypoxia up-regulates MCT4 expression, in skeletal muscle, while no alteration were found for MCT1[107]. At the same time, MCT1 and MCT2 overexpression was detected in brain in a recovery after a ischemic event[108]. In human adipocytes cell lines, MCT1 and MCT4 mRNA levels were increased, contrarily to MCT2, that was down-regulated in hypoxia, through an HIF-1α dependent mechanism[109]. Conversely, other study showed that only MCT4 was directly regulated by HIF-1α, due to the two hypoxia response elements presente in the promoter region, whereas MCT1 and MCT2, lacked this sequences. In this same study only MCT4 showed increased protein and mRNA levels, contrary to MCT1, supporting that MCT4 is overexpressed in hypoxia environment due to HIF-1α action[110].

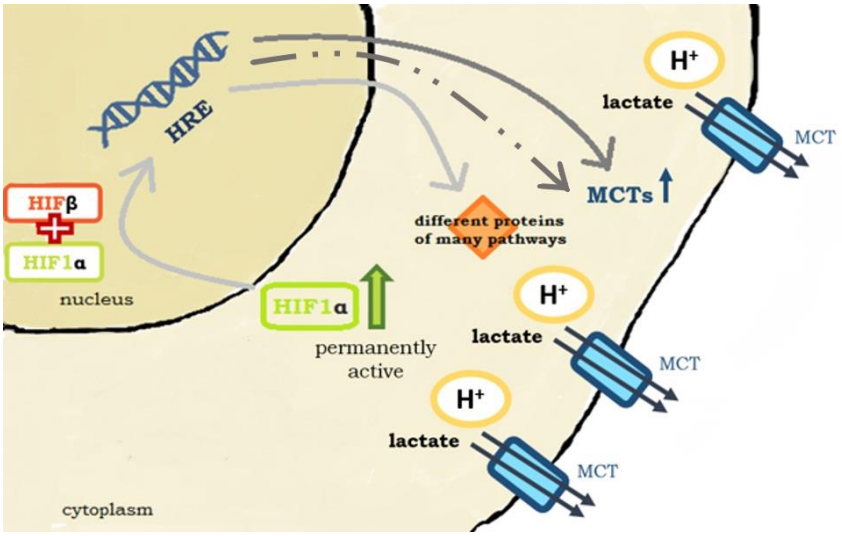


Figure 5: HIF-1α directly or indirectly upregulates MCTs in order to promote glycolytic metabolism, since these translocate lactate coupled with a proton.

Regarding MCT8, modifications on its expression levels throughout development have been reported[111].

4.3.1 MCTs regulation in cancer

As previously mentioned the impact of hypoxia in malignant tumors development is already acknowledge to influence MCTs regulation in cancer. Indeed, MCT4 overexpression, occurs in response to hypoxia conditions, both in bladder cancer[112] and in breast cancer[113]. Besides, it is also up-regulated in glioma cells[114] and trophoblast cells[115]. As for MCT1 higher levels were found in hypoxia due to lack of tumor suppressor gene p53 action and not due to HIF-1α activity in colorectal cancer[116]. Furthermore, both MCT1 and MCT4 isoforms were shown to be overexpressed in hypoxia, in both breast

cancer and glioblastoma cell lines, while MCT2 was only up-regulated in breast cancer, whereas reduced protein levels were observed in the brain cancer cells[117]. Another study showed that hypoxia can lead to increased MCT1 cell membrane expression in glioma cells, both *in vitro* and *in vivo.* Treatment with CHC led to MCT1 downregulation with consequent reduction in lactate production and tumor proliferation. Moreover, publish data suggest that MCT1 is regulated by hypoxia, not directly by HIF-1α, but, probably, by a pathway that depends on it[118]. Still in the glioma cells, mutations on the IDH1 gene drives to MCT1 and MCT4 expression reduction[119].

As previously discussed, the chaperone proteins, basigin and embigin, are fundamental for the proper expression of MCTs in the plasma membrane. In cancer, CD147 appears to be linked with MCT1's expression in ovarian cancer and with MCT4's both in lung and breast cancers[120]. In addition to the proteins already described, CD44 was also implicated in MCTs regulation, in human cancer[121], specifically in prostate[122] and in lung cancer[120]. Importantly, in a considered amount of cases, in which CD44 or CD147 was absent, MCT1 expression was still found, which raises the possible dependency of this protein's expression in an ancillary protein not yet determined[120].

4.3.2 Involvement of epigenetic mechanisms

In human breast cancer cell lines, MCT1 is promoter methylated was found in the CpG islands, whereas no expression was observed[123]. Additionally, MCT3 and MCT4 are known to be also regulated through DNA methylation, in atherosclerosis[124] and in clear cell renal cell carcinoma[125], respectively. Indeed, MCT4's mRNA levels were higher when the SLC16A3 promoter had lower methylation. Conversely, MCT3 presents lost of mRNA and protein associated with promoter hypermethylation[124]. Demethylation in a selective internal MCT2 promoter was associated with respective overexpression, in prostate cancer[126]. Furthermore, MCT1, is targeted by various miR-29 isoforms, both in human and in mouse, in pancreatic β cells, which also supresses MCT1 activity[127]. Similarly, miR-124 also affects MCT1 expression, in medulloblastomas, since this miRNA directly acts in the 3'UTR of MCT1's mRNA, blocking its translation reducing MCT1 protein levels[128].

AIMS

Taking into consideration the glycolitic phenotype of this tumor cells, due to VHL protein absence and consequent HIF-1α constant activation, is important to know how MCT1 and MCT4 are regulated to understand how their action can be blocked, since they have been implicated in the maintenance of malignant tumor cells metabolism, allowing these cells the acquirement of selective advantage to grow. There is limited knowledge regarding MCTs regulation, specially, whithin the epigenetics mechanisms context. To exploit this field we intend to perform two main tasks:

- Investigate MCT1 and MCT4 regulation at transcriptional level, as a result of *VHL* methylation and HIF-1α interplay;
- Test whether respective promoters are regulated by DNA methylation.

MATERIALS & METHODS

1. Patients and sample collection

In this study 241 cases of ccRCC primary tumors provenient from patients at the Portuguese Oncology Institute of Porto were used. A total of 25 normal kidney tissues were used as controls and were obtained from patients with upper urinary tract urothelial carcinoma that performed nephrectomy. For methylation studies, 25 normal samples were used, being 9 from fresh frozen tissues and 14 from formalin-fixed paraffin-embedded (FFPE) tissues. For transcript expression studies 12 normal samples from fresh frozen tissues were used. Additionaly, the clinical pathological data from the patients were collected. All the samples included in the study were previously evaluated by a pathologist to guarantee that tumor and normal tissue accessible did not including inflamatoty processes. The cohort characteristics are described in Table 3.

Table 3: Clinical data regarding ccRCC cases cohort.

	Positive (ccRCC) n=241
Age of diagnosis	
median	64
Gender	
Male	155
Female	86
Stage	
I	130
II	33
III	71
IV	6
Grade	
1	7
2	102
3	106
4	26
Recurrence	
Yes	44
No	196
Overall Survival	
Dead	94
Alive	147
Cancer specific survival	
Cancer related death	41
Alive or non-cancer related death	200

2. Cell lines and 5-AZA assay

In this study the 6 kidney cell lines were purchased from America type culture collection (ATCC®, USA). The kidney cell lines used encompass normal epethilial kidney cell line (HK2), 3 ccRCC cell lines (769-P, 786-O and A498), 1 metastatic ccRCC cell line (Caki-1) and 1 metastatic pRCC cell line (ACHN). Regarding cell culture conditions, Caki-1 grown in McCoy's 5A modified Liquid Medium (Biochrom, Merk, Germany), whereas 786-O, 769-P and HK-2 in RPMI 1640 Liquid Medium (Biochrom, Merk, Germany). Eagle's Minimum Essential Medium (Biochrom, Merk, Germany) was used for ACHN and A-498 development. All cell lines growth in specific culture medium suplemmented with 10% of Fetal Bovine Serum Superior (FBS, Biochrom, Merk, Germany) and 1% Penicillin/Streptomycin (GIBCO®, Carlsbad, CA, USA). Mycoplasma spp. contamination tests were done periodically.

3. Aza-2-deoxycytidine (5-Aca-CdR) treatment

The normal epithelial kidney cell line and ccRCC cell lines used in this study were treated with DNA methyltransferase inhibitor, 5-Aza-2-deoxycytidine. The 5-Aza was dissolved to 100mM stock solution in 50% ethanol: 50% acetic acid (v/v) and storage at -80ºC until use. Briefly, cells were seeded at $1.5x10^5$ cell/mL on $25cm^3$ flasks in complete culture medium and incubated at 37ºC, 5% CO_2 allowing to adhere. Then, the different cell lines were treated with 1µM of 5-Aza-CdR in complete culture medium during three consecutive days. The control condition were exposed to 1% ethanol: acid acetic solution during the same time. After that, cells were collected for DNA and RNA extraction. Three independent experiments were performed.

4. DNA and RNA extraction

4.1. Formalin-fixed embedded paraffin (FFEP) tissues

DNA and RNA extraction from FFEP tissues were performed by macrodissection of interested areas delimitated by a pathologist of a total of 10 serial 40µm thickness slides. The RNA and DNA were obtained from FFPE RNA/DNA Purification Plus Kit Norgen Biotek, Thorold, Canada, according to the manufacturer's instructions. Briefly, FFPE samples were deparaffinizated followed digestion with 20 mg/mL proteinase K (NZYTECH, Portugal) and incubated 15 min at 55ºC. Then, RNA-containing supernatant was seprated from DNA-containing pellet. Using specific buffers provided by the kit and 100% ethanol, the RNA and DNA were loaded in Purification Micro Column. The nucleic

cids bind to the columns were whased with provided wash solution and eluted in 30µL elution solution.

4.2. Fresh frozen tissues and cell lines

DNA extraction was performed according to the phenol-chloroform method. Firstly, tissues and cell pellets were digested with SE buffer (75 mM NaCl and 25 mM EDTA), 10% SDS and proteinase K at 20 mg/mL concentration (NZYTECH, Portugal) at 55ºC in agitation. Additional proteinase K [20mg/mL (NZYTECH, Portugal)] was added at twice a day until complete digestion was achieved. After complete digestion, phenol-chloroform solution at pH=8 (Sigma-Aldrich®, Germany) was added and samples centrifuged 20min at 4000 rpm. Then, aqueous phase containing DNA were collected and DNA precipitation were achived by adding 2 volumes (of original amount of aqueous phase) of absolute ethanol (Merck, Germany) and 1/3 volume of 7.5M Ammonium acetate (Sigma-Aldrich, Germany), and the samples were incubated overnight at -20°C. Following centrifugation at 13,000rpm for 20min, the DNA pellets were washed two times with 70% ethanol. After dry, the pellets were eluted in sterile distilled water (B.Braun, Melsungen, Germany).

RNA extraction was performed according to the Ribozol reagent method. Firstly, fresh frozen tissues and pellet cells were dissociated mechanically in TripleXtractor reagent (GRiSP®, Portugal) using disposable pestle (VWR international, USA) and a neddle, respectively. Followed 5 min incubation, chloroform (VWR Chemicals, USA) was added, samples were vortexed and incubated 3 min at room temperature before centrifugation 10,600 rpm during 15min at 4ºC. The aqueous phase was collected and samples were incubated with isopropanol (Millipore, Germany) during 10 min at room temperature followed centrifugation 13,000 rpm during 10 min, to RNA precipitation. The, RNA pellets were washed in 75% ethanol and when dry, the pellets were eluted in RNA storage solution.

Acid nucleics (DNA and RNA) concentration and purity were assessed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

5. cDNA synthesis and quantitative RT-PCR

For MCT1, MCT4, VHL and HIF-1α genes expression study, a reverse transcriptase from 250-1000 ng RNA was performed using RevertAid RT kit (ThermoScientific Inc., USA), according to the manufacturer's instructions. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed in 396-well plates LightCycler480II (Roche Diagnostics, Germany) using 4,5 µL cDNA with Xpert Fast SYBER Mastermix Blue (GE22.2501; GRiSP®, Portugal) and 0.3-0.5µL of specific primers described in

Table 4. All the samples were run in triplicates. For each gene, relative transcript levels were calculated as the ratio between the target gene mean quantity and β-GUS, housekeeping gene, mean quantity (Gene Expression Level = Gene Mean Quantity / β-GUS Mean Quantity).

6. Sodium bisulfite modification and quantitative methylation specific PRC

To study the promoter methylation, sodium bisulfite conversion was performed from 1000 ng of DNA using the EZ DNA Methylation-GoldTM Kit (Zymo Research, USA) according to the manufacturer's instructions.

Briefly, 1000 ng DNA was combined to CT Conversion Reagent for DNA modified synthesis in a Veriti 96 well thermal cycler (Applied Biosystems). After that, modified DNA were purified, eluted in distilled water and storage at -80ºC until use. DNA totally methylated CpGenomeTM Universal Methylated DNA (Merck Millipore, Germany) were used as a positive control. Quantitative methylation-specific PCR (qMSP) was performed to determine MCT1, MCT4 and VHL promoter genes methylation levels. In qMSP primers with CpGs designed specifically for bisulfite-converted DNA, that allows the specific amplification of methylated DNA, were used for each one of the target genes. The reactions were performed in 396-well plates LightCycler480II (Roche Diagnostics, Germany) using 2 µL of modified DNA, 5µL of Xpert Fast SYBER Mastermix Blue (GE22.2501; GRiSP®, Portugal) and 0.3-0.5 µL of specific primers described in Table 4, at a final volume of 10 µL per well. All the samples were run in triplicates. For each gene, relative methylation levels were calculated as the ratio between the target gene mean quantity and β-ACT, housekeeping gene, mean quantity (Gene Expression Level = Gene Mean Quantity / β-ACT Mean Quantity).

Table 4: RT-PCR and qMSP conditions for each gene investigated.

RT-PCR conditions			
Gene	**Sequence**	**Annealing temperature (°C)**	**Primers solution (10mM) quantity**
MCT1	F: 5'-CATGCCACCACCAGCGAAG-3'	60	0,3
	R: 5'-TGACAAGCAGCCACCAACAATC-3'		
MCT4	F: 5'-CCATGCTCTACGGGACAGG-3'	60	0,3
	R: 5'-GCTTGCTGAAGTAGCGGTT-3'		
HIF-1α	F: 5'-CAAGAACCTACTGCTAATGCCACC-3'	64	0,5
	R: 5'-GTATGTGGGTAGGAGATGGAGATG-3'		
qMSP conditions			
Gene	**Sequence**	**Annealing temperature (°C)**	**Primers solution (10mM) quantity**
MCT1	F: 5'-AAGTAAACGAGGTAGCGAGC-3'	62	0,4
	R: 5'-ACGAAACACCGTAAAATCC-3'		
MCT4	F: 5'-CGTTTTCGAGTTTCGAGTC-3'	64	0,5
	R: 5'-ACGCTACCGTAATTACCGAC-3'		
VHL	F: 5'-TGGAGGATTTTTTTGCGTACGCG-3'	62	0,5
	R: 5'-CGAACCGAACGCCGCGAA-3'		

7. Immnunohistochemistry

MCT1, MCT4, VHL and HIF-1α protein expressions were evaluated by immunohistochemistry (IHC). Representative 3 µm-thick tissues were used for immunohistochemical analysis. IHC for MCT1 and MCT4 were performed using UltraVision Detection System (Large Volume Anti-Polyvalent, HRP; Thermo Scientific Inc., USA) and VHL and HIF-1α through NovolinkTMMax Polymer Detection System (Leica Biosystems, Germany). After deparaffinization and rehydratation, antigen retrieval was performed in a heat water bath or a microwave, during 20min using a specific bufferd, according to the studied protein (see Table 5). Then, endogenous peroxidase activity inhibition with 3% hydrogen peroxide (H_2O_2, Millipore, Germany) in methanol during 10 min was done. Then, sections were blocked and incubated overnight with primary antibody at room temperature (Table 5). After, incubation with secondary antibody, polymer or streptavidin at specific times, the 3, 3′-diaminobenzidine (Sigma-Aldrich™, Germany) was used as a chromogen during 10min. The slides were

counterstained with haematoxylin and mounted with Entellan® (Merck-Millipore, Germany). For each immunoreaction a positive control was included (Table 5).

Immunoexpression was avaliated by a pathologist according a semi-quantitative method for both staining intensity (0 – no staining; 1 – weak intensity; 2 – moderate intensity; 3 – strong intensity) and extension of positive cells (0 – < 25%; 1 – 25-50%; 2 – 50-75%; 3 – >75%). These parameters scores were combined (staining intensity + extension of positive cells) and was considered as positive a final score ≥4.

Table 5: IHC conditions for each protein investigated.

<table>
<tr><th>Protein</th><th>Positive control</th><th>Antigen Retrieval</th><th>Dilution of Ab</th><th>Incubation</th></tr>
<tr><td>MCT1</td><td>colon carcinoma</td><td rowspan="2">• water bath
• citrate buffer</td><td>1:250</td><td rowspan="4">overnight at room temperature</td></tr>
<tr><td>MCT4</td><td>lung adenocarcinoma</td><td>1:500</td></tr>
<tr><td>HIF-1α</td><td>clear cell renal cell carcinoma</td><td rowspan="2">• microwave
• EDTA buffer</td><td>1:500</td></tr>
<tr><td>VHL</td><td>normal testicle</td><td>1:300</td></tr>
</table>

8. Statistical Analysis

For methylation and transcript levels studies in clinical samples and cell lines, the Wilcoxon-Mann-Whitney test was performed for comparison between two continuous variables. Spearman correlation test was done to determine the association between continuous variables in methylation a transcript expression assays. For IHC analysis, Fisher's test or chi-squared test were utilized for comparison between normal and ccRCC, as well as for association between studied proteins and also clinical pathological data.

For survival analysis, Kaplan-Meier with log rank test was used for prognostic value evaluation. Disease-free survival (DFS) was defined as the time between surgery date and recurrence date while disease-overall survival (DOS) was characterized as the time between surgery date and death due to cancer. In VHL methylation levels was defined a categoric variable as low or high methylation based on the cutoff value for the first quartile (p25).

RESULTS

1. MCT1 and MCT4 plasma membrane are upregulated in ccRCC

In order to understand the MCT1 and MCT4 regulation in ccRCC, first we characterized the transcriptional and protein levels of MCT1 and MCT4 in our cohort. At transcript levels, we observed *MCT1* (p<0.0001) and *MCT4* (p<0.0001) upregulation in ccRCC compared to normal kidney samples (Figure 6A). Since, cellular localization of MCTs is an important feature for their function as lactate transporters, the MCT1 and MCT4 expression was also assessed at protein level by IHC. First, we saw that MCT1 and MCT4 are expressed at the plasma membrane in ccRCC. MCT4 expression is absence in normal kidney tissues, whereas MCT1 present a prevalent cytoplasm expression (Figure 6B). Concerning only the global expression of MCT1 we observed that 63% [19/30] normal kidney tissues have a positive expression (Table 6). By other hand, MCT1 plasma membrane expression is negative in normal kidney tissues (0% [0/30], (Table 6). Therefore, considering only the plasma membrane positivity, MCT1 and MCT4 plasma membrane expression are upregulated in ccRCC compared to normal kidney tissues (23% [51/223], p=0.0001 and 57% [118/223], p<0.0001, respectively, Figure 6C, Table 7). Beyond the higher prevalence of MCT4 expression in ccRCC than MCT1, we observed that MCT4 presents a greater number of cases with +2 and +3 intensity of expression, than MCT1 which has more +1 intensity (Figure 6D). Additionally, MCT1 and MCT4 expression were associated with some clinical pathological parameters. We observed that MCT1 was significantly associated with ccRCC recurrence (p=0.009, Table 8), where we verify that 66.8% of ccRCC negative for MCT1 did not present recurrence. Further, MCT1 protein is also significantly associated with follow up (p=0.032, Table 8), being observed that 49.8% of negative MCT1 expression are ccRCC patients' which are alive without the desiase. Although not significantly association was founded, we observed a tendency to MCT1 correlates with stage (p=0.079, Table 8). For MCT4, we found a positive and significant association with the presence of metastases (p=0.04, Table 8). MCT1 expression was significantly associated with disease-free survival (p=0.016), but not with disease-overall survival (p=0.131) (Figure 7A). No statistical significance was observed between MCT4 expression and disease free and overall survival (Figure 7B).

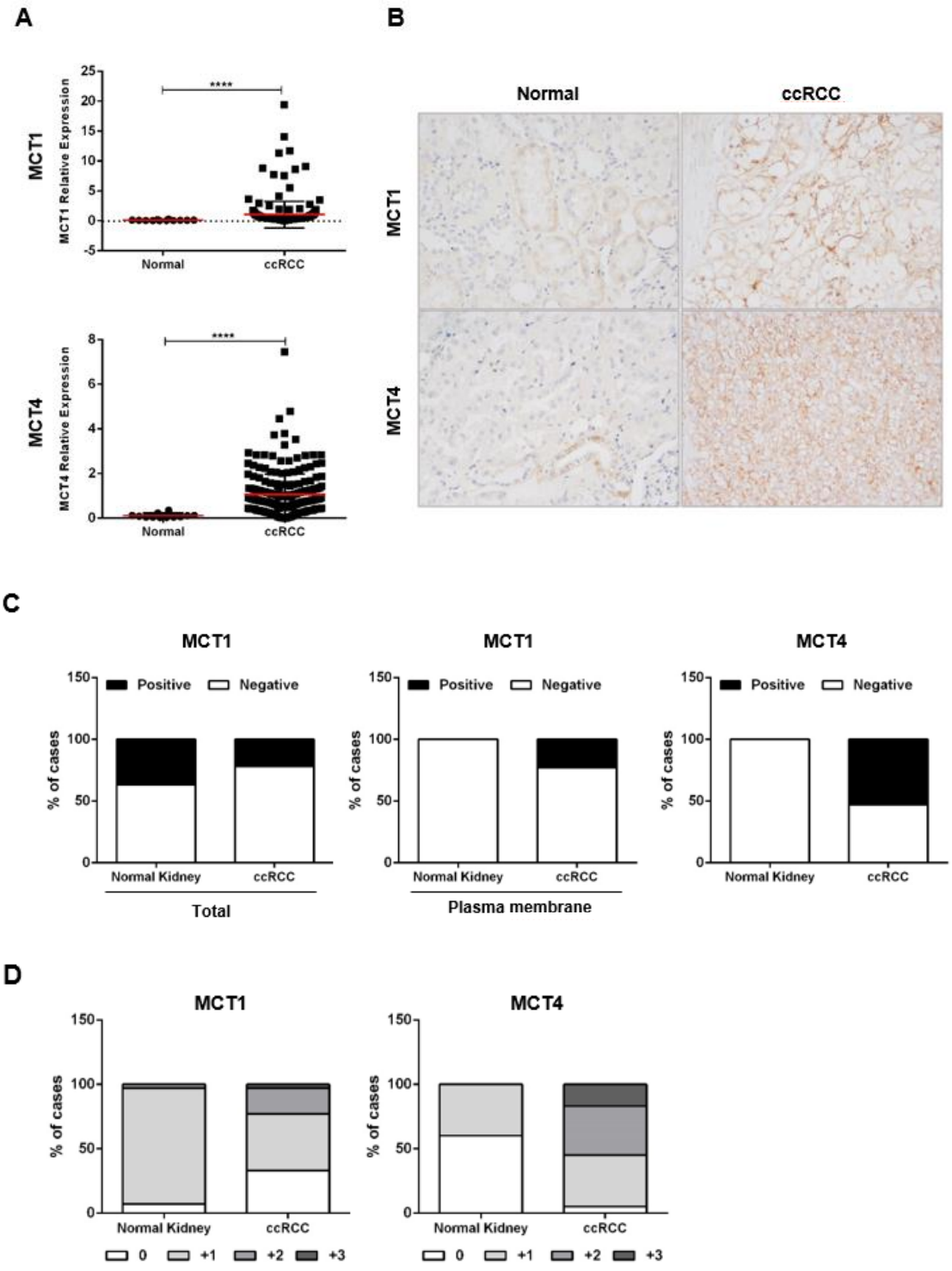


Figure 6: MCT1 and MCT4 upregulation in ccRCC. (A) Transcriptional MCT1 and MCT4 expression in ccRCC compared to normal kidney tissues; **(B)** Immunohistochemical pictures for MCT1 and MCT4 expression in ccRCC and normal kidney tissues; **(C)** Graphical representation of MCT1 and MCT4 negative vs positive cases in ccRCC and normal kidney tissues; **(D)** MCT1 and MCT4 intensity expression in ccRCC and normal kidney samples. **** p<0.0001 for ccRCC vs normal kidney.

Table 6: MCT1 global expression (cytoplasm + plasma membrane) in ccRCC and normal kidney samples

		Normal Kidney	ccRCC
MCT1	n	30	223
	Negative (%)	11 (37)	172 (77)
	Positive (%)	19 (63)	51 (23)
	***p* value**	<0.0001	

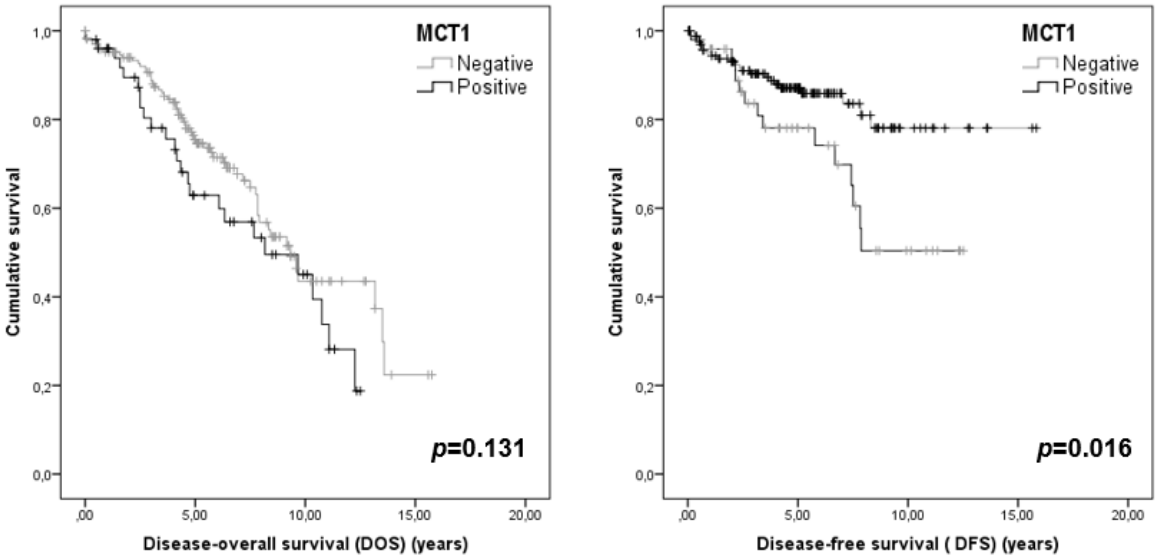
Table 7: MCTs plasma membrane, HIF-1α and VHL expression in ccRCC and normal kidney samples

		Normal Kidney	ccRCC
MCT1	n	30	223
	Negative (%)	30 (100)	172 (77)
	Positive (%)	0 (0)	51 (23)
	***p* value**	0.0001	
MCT4	n	30	223
	Negative (%)	30 (100)	105 (43)
	Positive (%)	0 (0)	118 (57)
	p value	<0.0001	
HIF-1α	n	30	223
	Negative (%)	30 (100)	59 (27)
	Positive (%)	0 (0)	163 (73)
	p value	<0.0001	
VHL	n	30	223
	Negative (%)	1 (3)	222 (99.6)
	Positive (%)	29 (97)	1 (0.4)
	p value	<0.0001	

Table 8: Clinical pathological associations with MCT1, MCT4, HIF-1α and VHL protein, as well as VHL promoter methylation

		MCT1			MCT4			HIF-1α			VHL			VHL methylation		
n	223	Negative (%)	Positive (%)	p	Negative (%)	Positive (%)	p	Negative (%)	Positive (%)	p	Negative (%)	Positive (%)	p	Low (%)	High (%)	p
Metastasis	Yes	4 (1.8)	1 (0.4)	0.678	0 (0)	5 (2.2)	0.04	2 (0.9)	3 (1.3)	0.401	5(2.2)	0 (0)	0.978	0 (0)	5 (2.3)	0.230
	No	168 (75.3)	50 (22.4)		105 (47.1)	113 (50.7)		57 (25.6)	160 (71.7)		217 (97.3)	1(0.4)		56 (25.2)	161 (72.5)	
pTNM stage	I	100 (44.8)	23 (10.3)	0.079	61 (27.4)	62 (27.8)	0.453	31 (13.9)	92 (41.3)	0.382	122 (54.7)	1 (0.4)	0.405	34 (15.3)	89 (40.1)	0.808
	II	23 (10.3)	6 (2.7)		12 (5.4)	17 (7.6)		7 (3.1)	22 (9.9)		29 (13)	0 (0)		6 (2.7)	22 (9.9)	
	III	44 (19.7)	21 (9.4)		30 (13.5)	35 (15.7)		18 (8.1)	46 (20.6)		65 (29.1)	0 (0)		15 (6.8)	50 (22.5)	
	IV	5 (2.2)	1 (0.4)		2 (0.9)	4 (1.8)		3 (1.3)	3 (1.3)		5 (2.2)	0 (0)		1 (0.5)	5 (2.3)	
Recurrence	Yes	23 (10.3)	15 (6.7)	0.009	17 (7.6)	21 (9.4)	0.445	17 (7.6)	21 (9.4)	0.006	38 (17)	0 (0)	0.830	10 (4.5)	28 (12.6)	0.505
	No	149 (66.8)	36 (16.1)		88 (39.5)	97 (43.5)		42 (18.8)	142 (63.7)		184 (82.5)	1 (0.4)		46 (20.7)	138 (62.2)	
Follow up *	I	111 (49.8)	24 (10.8)	0.032	66 (29.6)	69 (30.9)	0.398	32 (14.3)	102 (45.7)	0.065	134 (60.1)	1 (0.4)	0.440	36 (16.2)	98 (44.1)	0.908
	II	2 (0.9)	2 (0.9)		2 (0.9)	2 (0.9)		1 (0.4)	3 (1.3)		4 (1.8)	0 (0)		1 (0.5)	3 (1.4)	
	III	39 (17.5)	12 (5.4)		24 (10.8)	27 (12.1)		10 (4.5)	41 (18.4)		51 (22.9)	0 (0)		12 (5.4)	39 (17.6)	
	IV	20 (9)	13 (5.8)		13 (5.8)	20 (9)		16 (7.2)	17 (7.6)		33 (14.8)	0 (0)		7 (3.2)	26 (11.7)	

A



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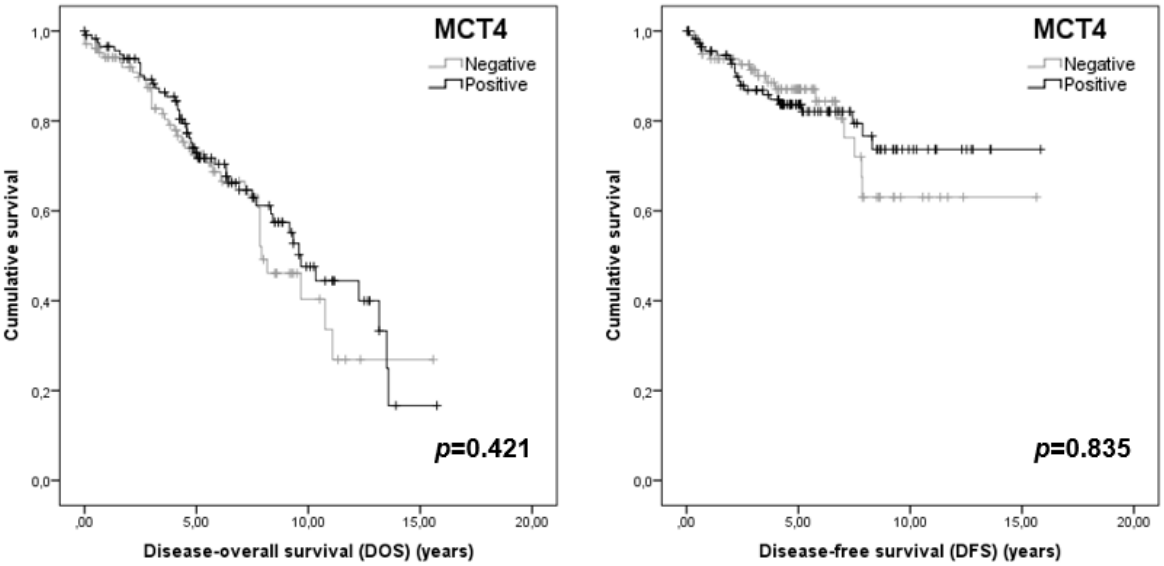

Figure 7: Kaplan-Meier estimated disease-overall survival and disease-free survival for MCT1 (A) and for MCT4 (B) expression of ccRCC patients.

2. HIF-1α is associated with MCT1 and MCT4 trasncriptional expression in ccRCC

The transcription factor HIF-1α is known to be important in tumor cellular metabolism reprogramming. In our study, we intend to study how HIF-1α is involved in MCTs expression, namely MCT1 and MCT4, the well known lactate transporters in cancer. First, we characterize HIF-1α expression in our cohort. Unlike expected, at transcriptional levels, we observed a significant decrease on HIF-1α expression at ccRCC samples compared to normal kidney samples (Figure 8A). However, the IHC analysis shown a significantly nuclear increase in HIF-1α expression for ccRCC tissues compared nomal kidney (p<0.0001, 73% [163/223] vs 0% [0/30], Figure 8C and 8D, Table 7). Additionally, we observed that the majority of ccRCC tissues presented a strong intensity expression, with +2 and+3 score compared with normal kidney tissues, where +1 intensity score is preferential (Figure 8E).

Furthermore, in our ccRCC samples, we observed that, *HIF-1α* mRNA levels have a positive and significant correlation with both *MCT1* (Spearman's r = 0.374 and p< 0.0001; Figure 8B) and *MCT4* (Spearman's r = 0.185 and p=0.005; Figure 8B) mRNA levels. However, a significant correlation of HIF-1α with MCT1 and MCT4 at protein level was not founded (p=0.239 and p=0.428, respectively, Table 9). Regarding the clinical pathological features, HIF-1α expression was only significantly associated with recurrence (p=0.006), showing a tendency to associated with follow up parameter (p=0.065). Disease-overalL survival and disease-free survival were not associated with HIF-1α expression (Figure 9).

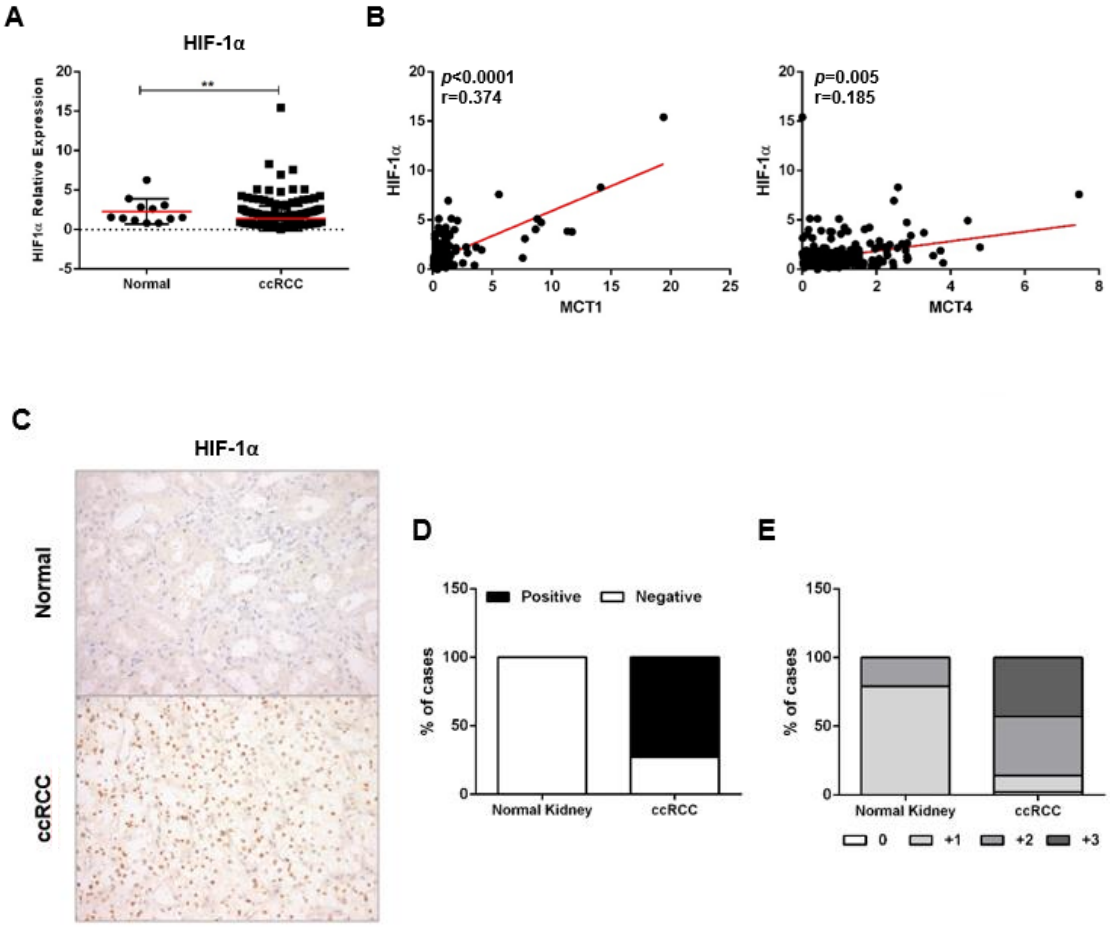

Figure 8: HIF-1α transciptional upregulation is associated positively with MCT1 and MCT4 mRNA levels. (A) Transcriptional HIF-1α expression in ccRCC compared to normal kidney tissues; **(B)** Sperman correlation between HIF-1α vs MCT1 and HIF-1α vs MCT4; **(C)** Immunohistochemical pictures for HIF-1α expression in ccRCC and normal kidney tissues; **(D)** Graphical representation of HIF-1α negative vs positive cases in ccRCC and normal kidney tissues; **(E)** HIF-1α intensity expression in ccRCC and normal kidney samples. ** $p<0.01$ for ccRCC vs normal kidney.

Table 9: HIF-1α and MCTs plasma membrane expression association in ccRCC samples

		HIF-1α	
		Negative (%)	Positive (%)
MCT1	Negative (%)	46 (21)	128 (57)
	Positive (%)	16 (7)	35 (15)
	***p* value**	0.239	
MCT4	Negative (%)	29 (13)	76 (34)
	Positive (%)	30 (14)	87 (39)
	***p* value**	0.428	

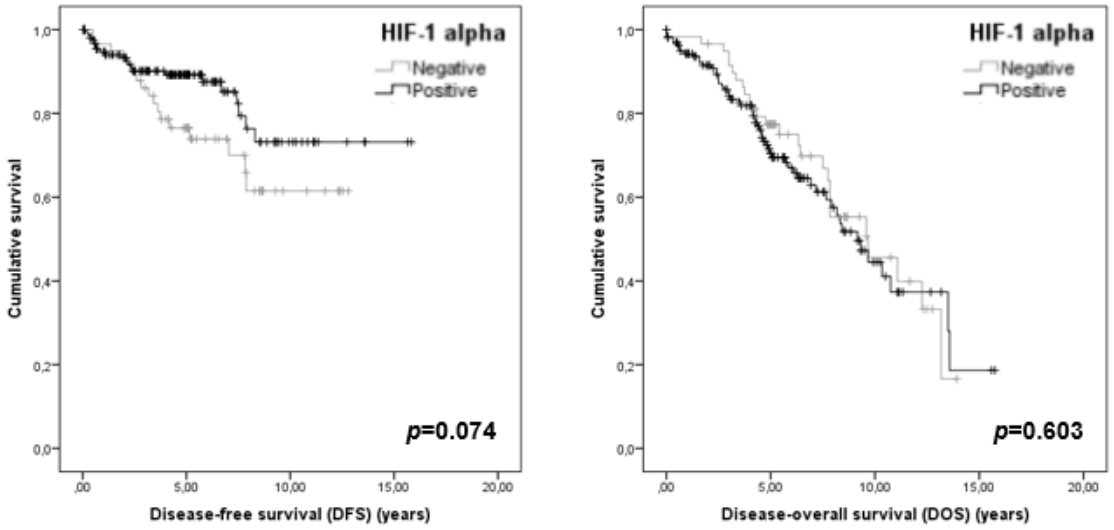

Figure 9: Kaplan-Meier estimated disease-free survival and disease-overall survival for HIF-1α expression of ccRCC patients.

3. VHL promoter methylation in ccRCC

Since VHL alteration is a characteristic of ccRCC, such as hypermethylation, leading to HIF-1α constitutive activation, we intend to understand how VHL could be involved in HIF-1α- MCTs association. For that we characterize the VHL mRNA, VHL promoter methylation and VHL protein expression in our ccRCC cohort. The *VHL* mRNA levels significantly descreases in ccRCC compared to normal kidney samples (Figure 10A). In accordance with that, we observed a significantly increases in VHL promoter methylation in ccRCC versus normal kidney samples (Figure 10B). Additionally, we have a tendency for a negative correlation between VHL mRNA levels and VHL methylation levels, however it is not significant statistically (r= -0.05; *p*=0.429, Figure 10C). Regarding *VHL* methylation levels and *MCT1* and *MCT4* transcript levels, we saw a significant positive correlation of VHL methylation and *MCT1* (r=0.155; p=0.02), but only a positive tendency was observed for *MCT4* (r=0.128; *p*=0.055) in ccRCC samples (Figure 10C). Further, we did not observe a significant correlation between VHL promoter methylation and HIF-1α in ccRCC samples (Figure 10C). Relative to VHL protein expression, we verified a cytoplasm expression at the normal kidney tissues (Figure 11A) being practically absence in ccRCC tissues (0.4% [1/223], *p*<0.0001, Table 8, Figure 11B). In Figure 10B, we show that VHL intensity expression is mostly +1 and +2 score in normal kidney, whereas in ccRCC it is absence. VHL expression at protein and promoter methylation levels was not significantly associated with MCT1, MCT4 and HIF-1α, as well as clinical pathological data (Table 8, 10 and 11). Moreover, we assessed the prognostic value of *VHL* methylation levels and discovered that, in univariable analysis, higher methylation status is associated with shorter time without recurrence for DFS (Figure 12).

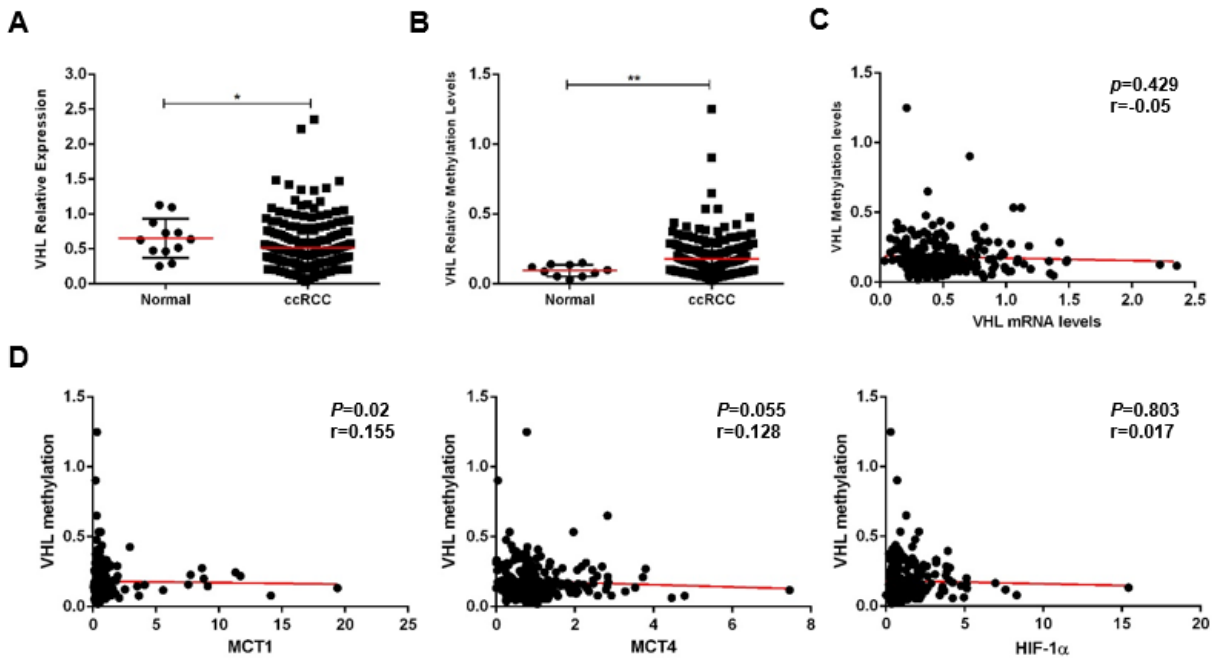

Figure 10: VHL promoter methylation and transcriptional expression in ccRCC. **(A**) Transcriptional VHL expression and **(B)** VHL promoter methylation in ccRCC compared to normal kidney tissues; **(C)** Sperman correlation between VHL expression vs VHL promoter methylation; (D) Sperman correlation between VHL promoter methylation and MCT1, MCT4 and HIF-1α. * p <0.05; ** p <0.01 for ccRCC vs normal kidney.

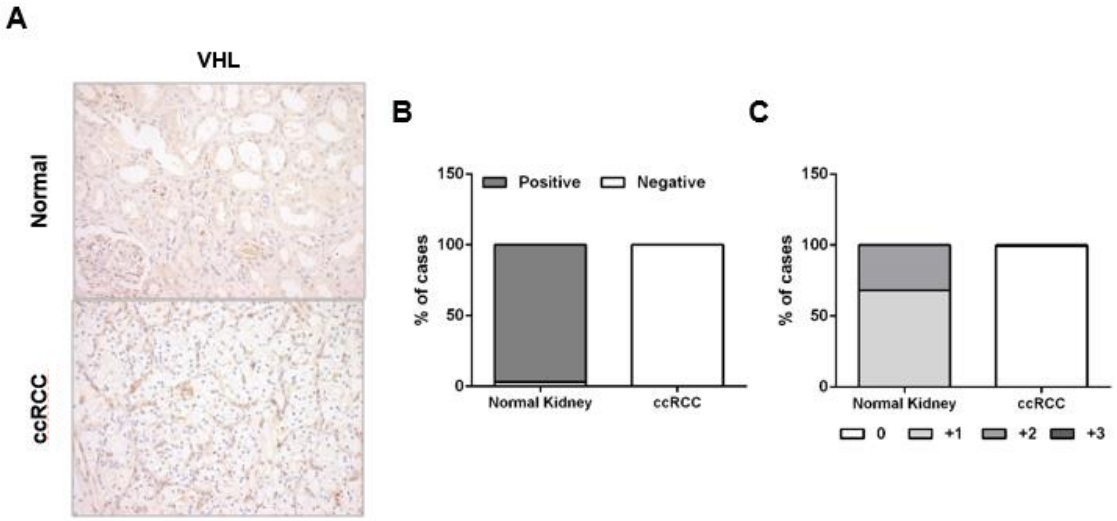

Figure 11: VHL protein is downregulated in ccRCC. **(A)** Immunohistochemical pictures for VHL expression in ccRCC and normal kidney tissues; **(B)** Graphical representation of VHL negative vs positive cases in ccRCC and normal kidney tissues; **(C)** VHL intensity expression in ccRCC and normal kidney samples.

Table 10: Association of VHL protein expression with MCTs plasma membrane andHIF-1α expression in ccRCC samples.

		VHL	
		Negative (%)	Positive (%)
MCT1	Negative (%)	171 (76.6)	1 (0.4)
	Positive (%)	51 (23)	0 (0)
	***p* value**	0.771	
MCT4	Negative (%)	104 (46.6)	1 (0.4)
	Positive (%)	118 (53)	0 (0)
	***p* value**	0.471	
HIF-1α	Negative (%)	58 (26)	1 (0.4)
	Positive (%)	164 (73.6)	0 (0)
	***p* value**	0.266	

Table 11: Association of VHL promoter methylation with MCTs plasma membrane and HIF-1α expression in ccRCC samples

		VHL methylation	
		Low (%)	High (%)
MCT1	Negative (%)	43 (19.4)	128 (57.6)
	Positive (%)	13 (5.8)	38 (17.2)
	***p* value**	0.547	
MCT4	Negative (%)	24 (10.8)	80 (36)
	Positive (%)	32 (14.4)	86 (38.8)
	***p* value**	0.296	
HIF-1α	Negative (%)	13 (5.8)	46 (20.4)
	Positive (%)	43 (19.4)	119 (53.4)
	***p* value**	0.310	

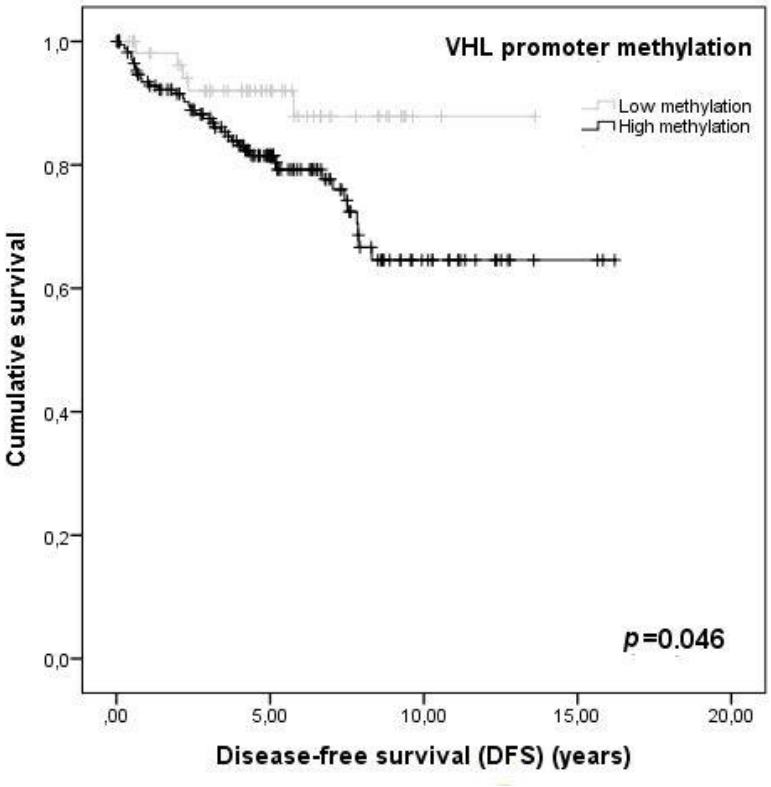

Figure 12: Kaplan-Meier estimated disease free survival of ccRCC patients based on *VHL* methylation levels.

4. Promoter methylation is not an essential mechanism in the regulation of MCT1 and MCT4 expression

We intended to see if MCT1 and MCT4 were regulated by DNA methylation in their own promoter region. First, we assessed in ccRCC and in normal kidney tissue samples, *MCT1* and *MCT4* methylation status. We verified that MCT1 and MCT4 did not appear to be regulated by this epigenetic mechanism, since amplification in qMSP did not occur for both genes. In order to confirm these results in fresh frozen tissue samples, we characterize MCT1 and MCT4 expression and methylation levels in normal kidney cell line and also ccRCC cell lines. We verify that *MCT1 and MCT4* are expressed in all ccRCC and normal kidney cell lines studied (Figure 13A). Additionally, we verify that *MCT1* is not methylated in any ccRCC or normal kidney cell lines, whereas *MCT4* showed some methylation in two of the primary ccRCC cell lines (A-498 and 769-P), but none in normal kidney cell line (HK-2) and in metastatic ccRCC cell line (Caki-1) (Figure 12B).Considering that *MCT4*, contrary to *MCT1*, showed methylation in its promoter in A-498 and 769-P cell lines, we decided to evaluate the impact of 5-aza-2-deoxycytidine (5-Aza) treatment, in order to completely understand if *MCT4* could be regulated by DNA methylation in ccRCC. ACHN cell line, a metastatic papillary renal carcinoma cell line, was used as positive control, since we know that have high levels of *MCT4* methylation. We observed that 5-Aza leads to a significant decrease of MCT4 methylation levels for both primary ccRCC cell lines (A-498, p= 0.004 and 769-P, p=0.002; Figure 13C), and at the same time, a significant increase in MCT4 mRNA levels in both cell lines (A-498, p=0.019 and 769-P, p=0.008; Figure 13D).

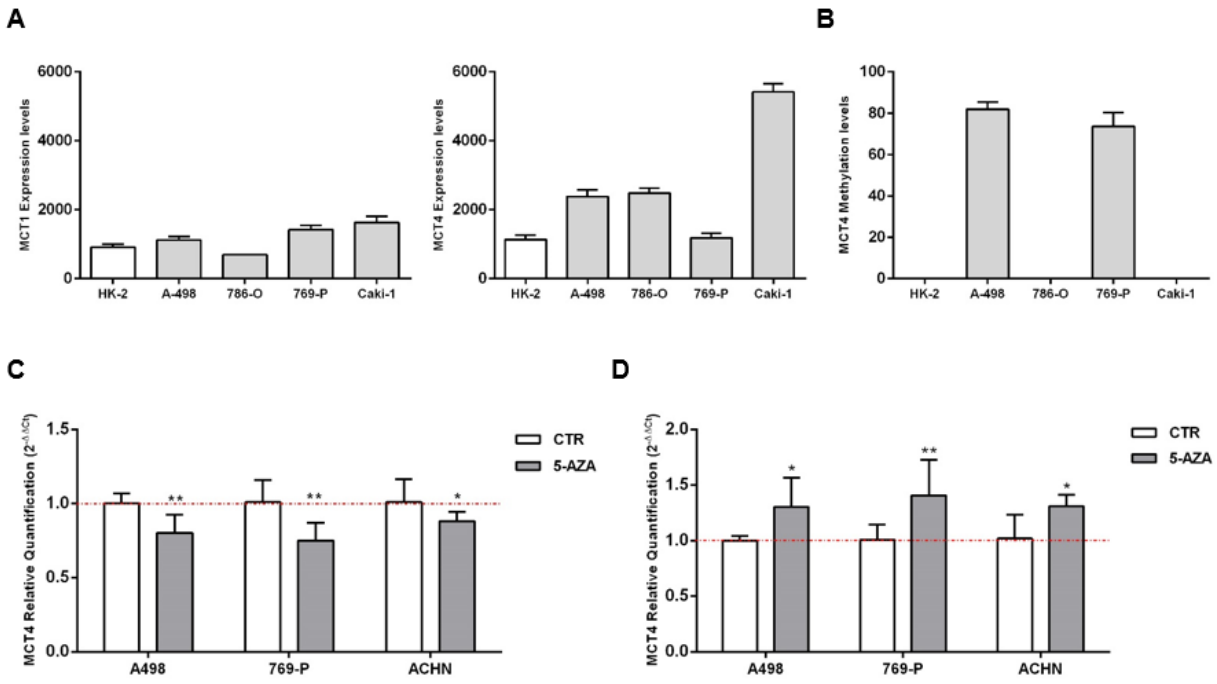

Figure 13: MCTs promoter methylation in ccRCC. (A) MCT1 and MCT4 expression in ccRCC cell lines and normal kidney cell line by RT-PCR; **(B)** MCT4 promoter methylation in ccRCC cell lines; **(C)** MCT4 promoter methylation and **(D)** MCT4 transcriptional expression after 5-Aza-2-deoxycytidine (5-Aza) treatment. * p <0.05; ** p <0.01 for 5-Aza vs CTR.

DISCUSSION

The ccRCC is characterized by a worse prognosis, being evident the presence of mutation in *VHL* at 60-90% of cases, which constitute the major driver of this malignancy[129]. *VHL* gene mutation or promoter gene methylation are also implicated in ccRCC development[130], by promoting constitutive activation of HIF-1α[131]. HIF-1α activation leads to a glycolytic phenotype even in normoxic conditions[132]. Thus, the glycolytic metabolism described in RCC appears to be a very appealing strategy for the treatment of these tumors.

Metabolic reprograming in cancer cells constitute hallmark of cancer[133]. Actually, it is recognized, that even in normoxia, tumor cells opt for aerobic glycolysis, defined as "Warburg effect", instead of oxidative phosphorylation[134]. Increased glycolytic activity generates considerative amounts of lactate that need to be exported from neoplastic cells in order to maintain their proliferative rates and intracellular acidification prevention[77]. For that, monocarboxylate transporters (MCTs) performed the lactate transport in tumor cells[77]. Up-regulation of MCTs has been descrived in some solid tumors[79–81,135]. However, MCTs regulation is poorly understood and little is known about the involvement of epigenetic mechanism in their regulation, particularly in kidney cancer.

For that we initially evaluated MCTs expression levels in 223 ccRCC cases. In our study, we observed that MCT1 and MCT4, both transcript and protein levels, were higher in the ccRCC compared to the normal series. In fact, it has drescribed previously the MCT1 and MCT4 overexpression in ccRCC[81,135]. In all ccRCC analyzed, MCT1 and MCT4 present exclusively a plasma membrane expression. However in normal kidney tissues we observed a cytoplasm expression, particularly for MCT1 protein. According their role as lactate transporters, functional MCTs are localized at the plasma membrane in order to support the high glycolytic rates of tumor cells. Their cytoplasm expression is not associated with lactate transport to microenvironment, but MCT1 could be associated to intracellular organelles. The MCT expression has been describe in mitochondrias and peroxisomes[136], where it have some function in accordance with their localization. Furthermore, in *in vitro* approach ccRCC cell lines presented higher *MCT4* expression in comparison to normal kidney cell line. Additionally, the metastatic ccRCC cell line (Caki1) has high *MCT4* mRNA values, in comparison to the primary tumor cell lines, suggesting that *MCT4,* is important for tumor aggressiveness and progression[135]*.* In accordance, in our ccRCC samples we observed that MCT4 is associated with presence of metastasis.

Since HIF-1α is a transcription factor that promotes upregulation of glycolitic pathway, and its deregulation is responsable for the pseudohypoxia condition, a characteristic of ccRCC, we decided to evaluate its expression and association with MCTs. At protein level, as expected, we observed an increase in nuclear HIF-1α expression for ccRCC compared to normal kidney tissues, where its expression was absence. However, at transcriptional level HIF-1α gene expression decreases significantly compared to normal kidney samples studied. These results point out the post-translational modification associated to this protein at normoxia situation, leading to its proteasomal degradation[137,138], namely in normal samples. Additionally, the mRNA normal samples derived from upper unirary tract is composed by a pool of different cells and depending on their localization we can have some of them with HIF-1α positivity, which is not reflected in IHC analysis. To understand if HIF-1α is involved in MCTs regulation, we correlate HIF-1α and MCTs expression at transcriptional and protein level in our ccRCC samples. Previous studies reported HIF-1α upregulation in ccRCC[139], which is in accordance with our results. However, the association between HIF-1α and MCTs in ccRCC was not performed previously. We observed, in ccRCC samples a positive and significant association between HIF-1α and MCTs, only at mRNA levels, but not protein. This indicates that HIF-1α is involved in MCT1 and MCT4 regulation at transcriptional level. Thus, in hypoxic microenvironment, a characteristic of solid tumors, HIF-1α activation leads to increased MCT1 and MCT4 expresion at transcriptional levels. In fact, on literature MCTs regulation in hypoxia, through HIF-1α, is once against, particularly for MCT1. Contrary what is decribed for MCT4, some studies observed a downregulation of MCT1 in hypoxia conditions[107,110]. Nevertheless, in other studies, both MCT1 and MCT4, showed an increase in transcript and in protein levels, in different cell lines[109,117]. Unlike MCT4, which directly is regulated by HIF-1α through hypoxia-responsive elements (HRE) at their promoter[110], MCT1 expression is affected by hypoxia not directly by HIF-1α binding but likely due to downstream targets induced through HIF-1α pathway activation[110,116,118]. The absence of association at protein level could be explained by the pos-translational modifications on MCTs. Concerning IHC analysis, HIF-1α expression is present in 73% (163/223) of ccRCC, whereas MCT4 in 57% (118/223) and MCT1 only in 23% (51/223). So, taking in account the results of transcript, the lower number of MCT1 and MCT4 posititivy in ccRCC can be explained by post-translational mechanisms. In fact, some studies reported the post-translational regulation of MCTs, particularly MCT1, by miRNA. Downregulation of MCT1 in medulloblastomas through miRNA124 has been described[128]. Additionally, miRNA29a has been involved in MCT1 downregulation in pancreatic β cells [127] and leukemia[140].

It is established that HIF-1α permanent activity, in normoxia conditions, is due to VHL inactivation, a tumor supressor gene, which mutations and promoter hypermethylation are reported in more than 90% and 30%, of ccRCC cases[15,16], respectively. Taking this into consideration, the fact that methylation is a reversible mechanism, it is interesting to determine, in our cohort, *VHL* methylation status and evaluate its association with *HIF-1α* and both MCTs transcripts. It was shown that *VHL* promoter was hypermethylated significantly in ccRCC. Furthermore, to best of our knownledge, for the first time we demonstrated that *VHL* methylation presents a significant and a positive correlation with *MCT1* and *MCT4* mRNA levels. The observed VHL promoter methylation is in accordance with the absence of VHL protein expression in ccRCC.This results support the already defined idea that VHL regulates HIF-1α, since we observed higher protein levels of the transcription factor in the tumor tissue, where VHL was obviously not present, in opposite to the normal tissue where lack of HIF-1α expression was accompanied of VHL presence. Despite this, no statistical association between VHL methylation and HIF-1α proteins expression was found. Although not studied here, the HIF-1α expression could be explained by the frequence of VHL mutations in our cohort. The overall lack of correlation can be explained by HIF-1α alternative regulation pathways that can probably mask the significant correlation with VHL. Indeed, this transcription factor expression can be affected by other mechanisms, both at post-transcriptional and post-translational levels. As examples, it is recognized that the RBM38, a RNA-binding protein, affects *HIF-1α* mRNA translation, in different types of cancer[141]. Additionaly,miR-182 blocks both PHD2 and FIH1 activities which are responsable for HIF-1α downregulation, thereby enhancing its expression levels in prostate cancer[142]. Besides this, the *Myc* oncogene, in breast cancer[143], and the mitogen-activated protein kinase (MAPK), in HeLa cells[144], are also notorious for HIF-1α stabilization, provoking its accumulation in the nucleus with consequent activity promotion. Since none of this is described in ccRCC, we can not ignore the extension of this mechanisms in HIF-1α expression levels in our cases. As for MCTs, their proteins levels were no correlated with those of VHL.

Furthermore, since the epigenetic field is growing and little is known about MCTs regulation in this context, we decided to investigate their promoter DNA methylation status. The lack of amplification, both in ccRCC cases and in normal kidney tissue samples, for *MCT1* and *MCT4*, shows that these genes promoters are not methylated, indicating that MCT1 and MCT4 expression are not dependent on methylation in their promoters. These results were confirmed by in *in vitro* studies by 5-Aza treatment in A-498 and 769-P methylated cells. Here we observed that 5-Aza leads to an increase on

MCT4 expression, however promoter methylation is not a determinant factor in MCT4 expression regulation, since all cell lines expressed MCT4 at basal conditions.

Our results do not corroborate the only study so far published regarding MCT4 methylation[125]. Like us, they noticed overexpression of MCT4 in the ccRCC series, but, in opposition to our results, founded higher methylation in the normal kidney in relation to the tumor, in a TCGA cohort and also in 64 ccRCC with matched normal tissue. Effectively, our normal series is smaller (25) and perhaps is not representative enough which draws a limitation. Nevertheless, our results were confirmed in cell lines studies, where no amplification was found in HK-2 cell line and also by the 5-AZA experiment. Actually, the *in vitro* studies proved that methylation is not an epigenetic mechanism relevant for regulation of *MCT4* expression. Concerning *MCT1*, Asada et al.[123] afirmed that it is hypermethylated in 4 of 20 breast cancers, using 5-Aza-2-deoxycytidine demethylating agent to comprove the regulation by methylation in *in vitro* studies. The absence of MCT1 and MCT4 promoter methylation in our ccRCC cohort suggest that this epigenetic mechanism can be tumor dependent.

Overall, our results show, first of all, that MCTs indeed are overexpressed in ccRCC, which points out their important role in the tumor metabolism. Additionally, the significant association between *MCTs* transcript levels and both *VHL* methylation and *HIF-1α* transcript, suggests a crosstalk of VHL methylation and HIF-1α mRNA levels with the MCT1 and MCT4 transcriptional regulation. The loss of this correlation at proteins levels, points out a hypothesis of a post-translational modification in MCTs regulation. Moreover, our findings indicate that MCT1 and MCT4 are not methylated in our cohort, suggesting that DNA promoter methylation is not a determinant epigenetic mechanism on MCTs regulation.

CONCLUSIONS

Our results demonstrate that MCTs are relevant in glycolytic metabolism, since they are overexpressed in ccRCC, a tumor that is highly known for its pseudohypoxic behavior. Until now, we are the first group that explored how VHL absence, by methylation, can affect MCTs regulation through HIF-1α action. The information available regarding this subject is rather limited. Indeed, it is important to understand ccRCC metabolism in order to check the potential paths or proteins that are capable of compromising HIF-1α activity. In this way we will be able to stablish more accertainly the impact of VHL role in this transcription factor, as a proteasome degradation indicator and how its inactivation truly alter HIF-1α expression. All studies that report MCTs regulation by hypoxia were performed in other types of tumors and remain a controversial topic. MCT1 and MCT4 can suffer alterations throughout their journey to the cell membrane that are independent of HIF-1α activity that disturb their relation. Indeed, MCTs are a world to be discovered and so we can not disregard that this transporters may be target of many unknown processes. In order to completely understand the effect of VHL action in MCTs regulation by HIF-1α interplay, is essential that our results are confirmed in *in vitro* studies and studied by other groups with different approaches and techniques.

Moreover, *MCT1* is not regulated by DNA methylation, in opposition to *MCT4*. Nevertheless, our resuts explain that this epigenetic mechanism is not crucial for *MCT4* regulation in ccRCC. Studies concerning *MCT4* methylation in pRCC would be interesting to assess wheather DNA methylation is a mechanism responsible for tumor aggressiveness with consequent metastization.

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