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Characterization of glycolytic metabolism of oesophageal carcinomas and evaluation of its potential as therapeutic target

Dissertação de Mestrado
Mestrado em Ciências da Saúde

Trabalho efetuado sob a orientação de
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Cofinanciado por:

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ABSTRACT

Tumour cells exhibit a preference for glycolytic metabolism for energy production, even in the presence of oxygen, a phenomenon denominated "Warburg effect". Tumours have high glycolytic rates leading to an increase in lactate produced, which is transported to the tumour microenvironment via monocarboxylate transporters (MCTs), thus contributing to increased proliferation, migration and survival of tumour cells. The role of MCTs is poorly understood in esophageal cancer. The main objectives of this work are to explore the role of MCTs in this type of cancer, by characterizing their expression in oesophageal carcinoma tissues and correlate their expression with metabolic markers and assess the clinical-pathological impact; and to characterize the expression of MCTs and metabolic markers in oesophageal squamous cell carcinoma cell lines and the effects of MCT down-regulation evaluating cell viability and glucose consumption and lactate efflux. To achieve our objectives, we performed a detailed characterization of the expression of MCTs and other metabolic markers in a clinical series of two histological types of esophageal cancer, oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC). Expression of MCTs was subsequently correlated with CD147 and with clinical data. Then a metabolic characterization of squamous cell carcinoma cell lines of the esophagus was performed and the effect of inhibition of MCTs in these cell lines was evaluated by levels of biomass and cellular metabolism.

This study showed that tumour location and age are important factors for low survival in adenocarcinoma. Gender is a factor of low survival for esophageal carcinoma. It also showed that the expression of MCT1 and MCT4 in the plasma membrane is more evident in squamous cell carcinoma than in adenocarcinoma and that for squamous cell carcinoma, MCT4 and CA IX can be considered prognostic factors for low survival. In vitro studies have shown that the dual silencing of MCT1 and MCT4 leads to a decrease in cell viability but had little effect on glucose consumption and lactate production. However, further studies will be needed to observe what this dual inhibition may cause in proliferation and migration. Only in this way will it be possible to know if MCTs could be rational therapeutic targets for use in this type of cancer.

The results obtained in this study intend to contribute to a better understanding of role of MCTs, and to open new therapeutic possibilities for esophageal cancer.

KEYWORDS: MONOCARBOXYLATE TRANSPORTERS – "WARBURG EFFECT" – OESOPHAGEAL CANCER – OESOPHAGEAL ADENOCARCINOMA – OESOPHAGEAL SQUAMOUS CELL CARCINOMA

RESUMO

As células tumorais recorrem ao metabolismo glicolítico para a produção de energia, um fenómeno conhecido como "Efeito de Warburg". Os tumores apresentam elevadas taxas glicolíticas levando a um aumento do lactato produzido, que é transportado para o microambiente tumoral através dos transportadores de monocarboxilatos (MCTs), contribuindo assim para um aumento da proliferação, migração e sobrevivência das células tumorais. O papel dos MCTs é pouco esclarecido em cancro de esófago. Os objetivos deste trabalho consistem em explorar os MCTs neste tipo tumoral, caracterizando a sua expressão em tecidos e correlacionar a sua expressão com marcadores metabólicos e avaliar o impacto clinico-patológico, caracterizar também a expressão dos MCTs e de marcadores metabólicos em linhas de carcinoma escamoso do esófago e ver os efeitos da inibição dos MCTs avaliando a viabilidade celular, o consumo de glicose e produção de lactato. De forma a alcançar os nossos objetivos, primeiro foi realizada uma caracterização detalhada da expressão dos MCTs e outros marcadores metabólicos numa série clínica de dois tipos histológicos de cancro de esófago, o carcinoma escamoso do esófago (OSCC) e o adenocarcinoma do esófago (OAC). A expressão dos MCTs foi posteriormente correlacionada com a CD147 e com os dados clínicos. Foi feita a caracterização metabólica de linhas celulares de OSCC e observado o efeito da inibição dos MCTs nessas linhas celulares ao nível da biomassa e metabolismo celular.

Este trabalho evidência que a localização tumoral e a idade são fatores importantes para a baixa sobrevivência no OAC, já o gênero, é um fator de baixa sobrevivência para o OSCC. Também demonstrou que a expressão do MCT1 e do MCT4 na membrana plasmática é mais evidente no OSCC do que no OAC e que para este mesmo subtipo histológico o MCT4 e o CAIX podem ser considerados fatores de prognostico para uma baixa sobrevivência. Estudos *in vitro*, demonstram que o silenciamento duplo do MCT1 e do MCT4 levam a uma diminuição na viabilidade celular, no entanto pouco afetam o seu consumo de glucose e produção de lactato. No entanto, serão necessários mais estudos para observar o que esta dupla inibição poderá provocar a nível da proliferação e migração, só assim se poderá saber se os MCTs poderão ser um possível alvo terapêutico para ser usado neste tipo de cancro.

Os resultados obtidos neste estudo pretendem contribuir para uma melhor compreensão dos mecanismos de ação dos MCTs, e abrir novas possibilidades terapêuticas para o cancro do esófago.

PALAVRAS-CHAVE: TRANSPORTADORES DE MONOCARBOXILATOS – "EFEITO DE WARBURG" – CANCRO DO ESÓFAGO – ADENOCARCINOMA DO ESÓFAGO – CARCINOMA DE CÉLULAS ESCAMOSAS DO ESÓFAGO

TABLE OF CONTENDS

FIGURES

TABLES

Abbreviation List

5-FU – 5-fluorouracil

αKG – α-ketoglutarate

ACLY – ATP citrate lyase

ATP – Adenosine Triphosphate

CA IX – Carbonic Anydrase IX

ccRCC – clear cell Renal Cell Carcinoma

CHC – α-cyano-4-hydroxycinnamate

Co-A – Acetyl Coenzyme-A

DCA – Dichloroacetate

DIDS – 4,4'-diisothiocyanostilbene-2,2'-disulphonate

$FADH_2$ – Flavin Adenine Dinucleotide

FASN – Fatty Acid Synthase

FBS – Fetal Bovine Serum

FDG-PET – FluoroDeoxygGucose Positron Emission Tomography

G6P – Glucose-6-phosphate

GLUT 1 – Glucose Transporter 1

GLS – Glutaminase

HIF – Hypoxia Inducible Factor

HK II – Hexokinase II

HRQoL – Health-related quality of life

IGF1 – Insulin-like growth receptor 1

IGF1R – IGF1 receptor

IHC – Immunohistochemistry

IF – Immunofluorescence

kDa – Kilodalton

LDH – Lactate Dehydrogenase

Mal – malate

MCT – Monocarboxylate Transporter

NADH – Nicotinamide Adenine Dinucleotide

NHE1 – Na+/H+ exchanger 1

NPPB – 5-nitro-2-(3-phenylpropylamino)-benzonate

OAA – oxaloacetate

OAC – Oesophageal Adenocarcinoma

OC – Oesophageal Cancer

OSCC – Oesophageal Squamous Cell Carcinoma

OXPHOS – Oxidative Phosphorylation

PBS – Phosphate Buffer Saline

PC – Pyruvate Carboxylase

pCMBS – p-chloromercuribanzene sulphonate

PDH – Pyruvate Dehydrogenase

PDK – Pyruvate Dehydrogenase Kinase

PEP – phosphoenol pyruvate

PFK-1 – phospho-fructokinase-1

pH_i – intracellular pH

PK – pyruvate kinase

R5P – ribose 5-phosphate

SCL16 – Solute Carrier Family 16

siRNA – small/short interference RNA

SRB – Sulforhodamine B

TBS-T – Tris-Buffer Saline – TWEEN 20

TCA – TriCarboxylic Acid

TMD – Transmembrane Domain

TNM – Tumour – Node – Metastasis Classification

WHO – World Health Organization

1. INTRODUCTION

1.1 Cell metabolism

Metabolism is described as a series of cellular activities in which many metabolic pathways cooperate to convert nutrient molecules into other macromolecules required for specialized functions and to obtain chemical energy for vital processes. In mammalian cells, the metabolic response requires a permanent coordination of cell activity, including cell proliferation with nutrient availability, hormonal and stress signalling and with regulation of energy homeostasis. In normoxic condictions, carbohydrates are transformed into glucose that is converted into pyruvate in the processes called glycolysis. Pyruvate is then converted into acetyl coenzyme-A (CoA) that is metabolized in the tricarboxylic acid (TCA) pathway producing most of the ATP (adenosine triphosphate) by the oxidative phosphorylation (OXPHOS). On the other hand, under hypoxia, the glucose is converted into lactate, producing less ATP but in a faster way (1,2). Increased cell proliferation is characteristic of tumour cells, thus, proliferating tumour cells present an altered metabolism, contrasting to normal cells that rely primarily on mitochondrial oxidative phosphorylation for energy production (1).

1.2 Tumour cell metabolism

Carcinogenesis is a complex, multifactorial and a multistep process, described as 'somatic evolution', since it requires the acquisition of many biological capabilities in evolution of a normal cell to a malignant state (3,4). The somatic evolution from early carcinogenesis to invasive carcinoma arises as an adaptive mechanism, generating a characteristic phenotype which confers a selective growth that is an advantage to tumour cells. Most of the alterations observed by Hanahan and Weinberg during carcinogenesis have been postulated as hallmarks of cancer and comprise alterations at molecular, biochemical and functional level (4). As a consequence of these alterations cancer cells have a behaviour that allow them to have a limitless replicative potential, in which result in the following features: sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumour-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death, and deregulating cellular energetics (Figure 1).

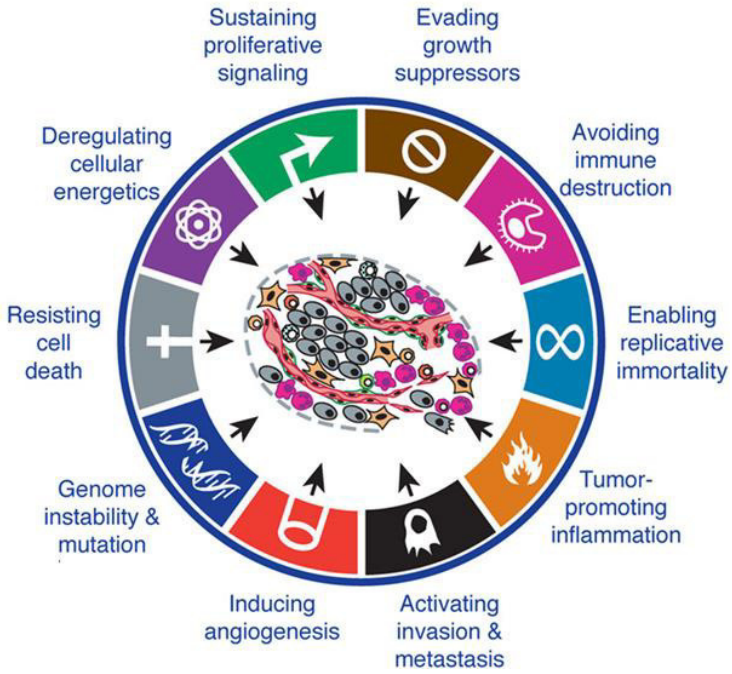

Figure 1 – Integrative view of the ten hallmarks of cancer. Although through various mechanistic strategies, tumour cells acquire the same set of functional capabilities during the cancer development which constitute the hallmarks of cancer (4).

The chronic and uncontrolled cell proliferation that represents the cancer cells involves deregulated control of cell proliferation and adjustments of energy metabolism. These adjustments represent important problems in cellular metabolism since proliferating cells must balance the divergent catabolic and anabolic requirements of sustaining cellular homeostasis while duplicating cell mass, and thus engage in a metabolic program distinct from that of the normal tissue from which they arise (1,4). Proliferating cells have ability to acquire necessary nutrients due to the excess of bioenergetic needs, from a frequently nutrient-poor environment and utilize these nutrients to both maintain viability and build new biomass. These needs result from alterations in intracellular and extracellular metabolites that accompany cancer-associated metabolic reprogramming. Altering intracellular and extracellular metabolites will have profound effects on gene expression, cellular differentiation and the tumour microenvironment. In view of the changes in the metabolism of the carcinogenic cells, Pavlova and Thompson (5), organized the known cancer-associated metabolic changes into six hallmarks: deregulated uptake of glucose and amino acids, use of opportunistic modes of nutrient acquisition, use of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production, increased demand for nitrogen, alterations in metabolite-driven gene regulation, and metabolic interactions with the microenvironment (Figure 2).

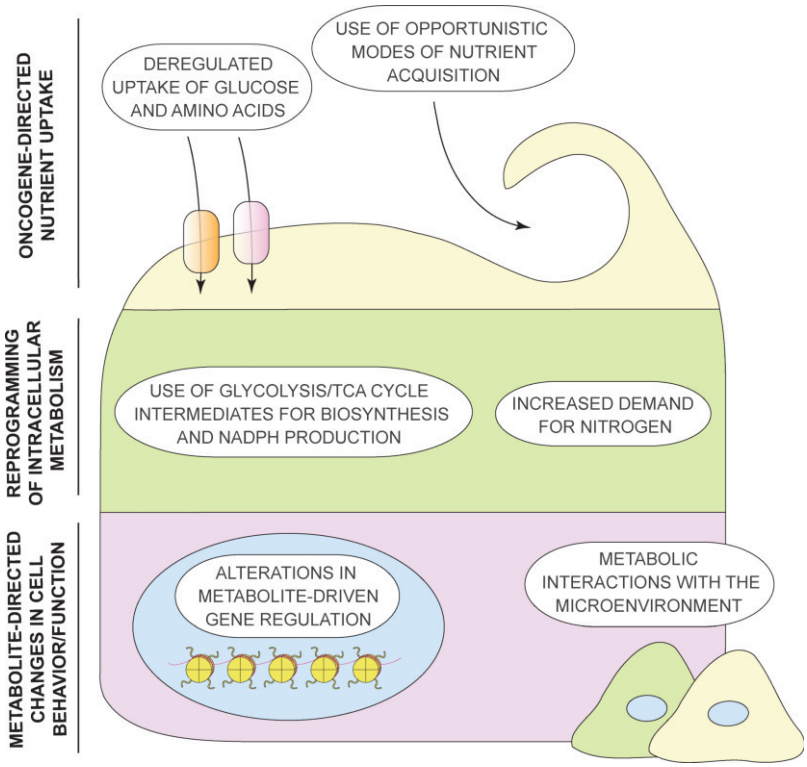

Figure 2 – The emerging hallmarks of cancer metabolism. Cancer cells accumulate alterations that allow them to gain access to nutrient sources. They utilize these nutrients towards the formation of new biomass to sustain deregulated proliferation, and take advantage of the ability of select metabolites to affect the fate of cancer cells themselves as well as a variety of normal cell types within the tumour microenvironment (5).

The metabolic alterations and adaptations of cancer cells have been studied over the past century. These alterations create a phenotype that is capable to fulfil the bioenergetics demands of cancer cells that are associated with proliferation (5,6). The two principal nutrients that cells use for growth and survival are glucose and glutamine. The catabolism of glucose (glycolysis) and glutamine (glutaminolysis) helps sustaining the function of TCA uninterruptedly (5,7) (Figure 3). In glycolysis, glucose is converted into pyruvate, that is oxidized via pyruvate dehydrogenase (PDH) to the two-carbon unit acetyl-CoA, and subsequently combined with the four-carbon oxaloacetate to generate citrate or can serve as an anaplerotic substrate when converted to oxaloacetate by the enzyme pyruvate carboxylase (PC). In glutaminolysis, the conversion of glutamine to glutamate via glutaminase (GLS), can also support anaplerosis via production of α-ketoglutarate (αKG) (7,8). So, the controlled oxidation of carbon skeletons of glucose and glutamine allows the cell to capture their reducing power either in the form of NADH and $FADH_2$ transferring the electrons to the electron transport chain to feed ATP generation (1,8,9).

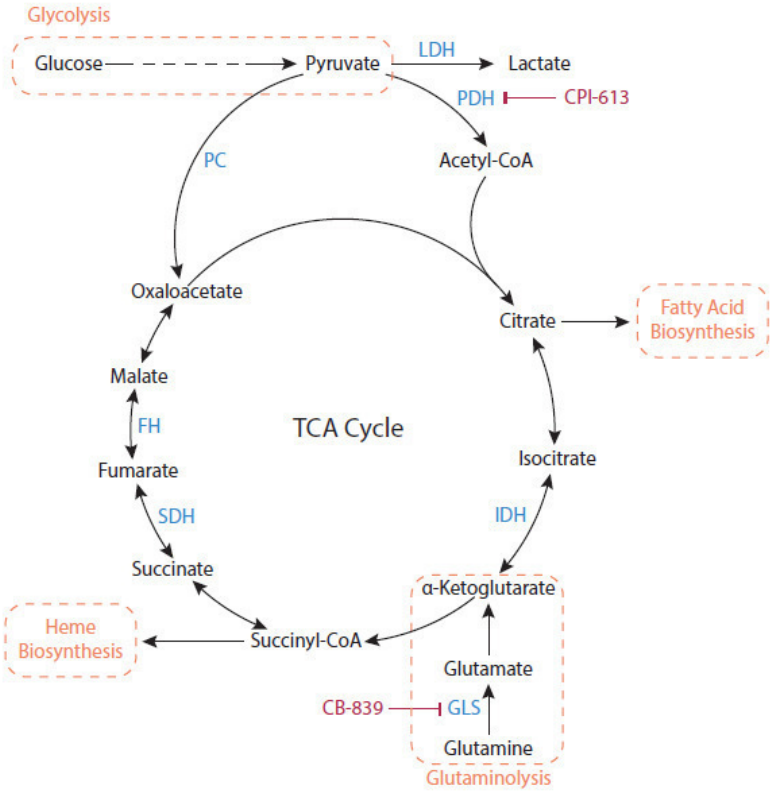

Figure 3 – TCA Cycle. Pyruvate produced by glycolysis and glutamate produced by glutaminolysis can be metabolized by the TCA cycle (8).

In contrast to normal differentiated tissues, most cancer cells rely more on glycolysis for ATP production independently on the oxygen availability in the extracellular microenvironment (Figure 4). Reprogramming of energy metabolism by cancer cells elucidate part of the explanation for Warburg's observations. Otto Warburg (10) stated that tumour cells, even in the presence of oxygen, have a high rate of glycolysis and do not take advantage of the bioenergetic benefits offered by the coupling of glycolysis to the TCA cycle. This phenomenon is designated by "Warburg effect". As a result, tumour cells convert most of the incoming glucose into lactate (around 85%) rather than metabolizing pyruvate, produced by glycolysis, in the mitochondria through oxidative phosphorylation (around 5%) (11,12). As a result of misinterpretation, Warburg and other investigators, thought that this phenomenon was caused by an "irreversible injury of respiration" and subsequently were less efficient in producing ATP from the phosphorylation of glycolysis (10). Nevertheless, recent investigations have shown that tumour cells have a functional mitochondria and therefore are capable to preform OXPHOS, since the depletion of mitochondrial DNA leads to a decrease in tumorigenic potential of cancer cell lines (13,14). So, it is possible to say, that the Warburg effect is a regulated metabolic feature and it is beneficial during a time of high proliferation.

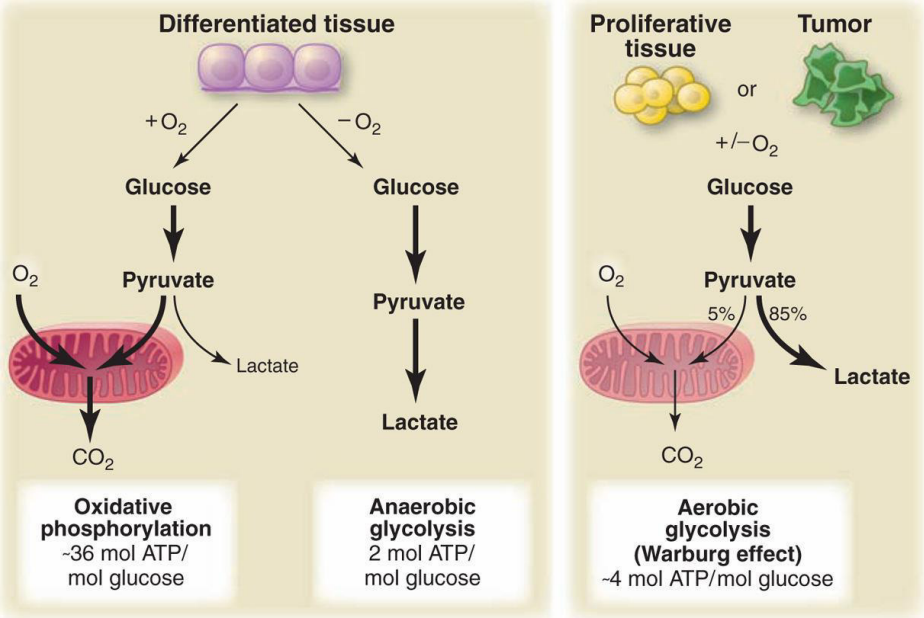

Figure 4 – Schematic representation of glucose metabolism in differentiated tissues (left panel), normal proliferative tissues and tumour cells (right panel) (11).

Tumour cells present a metabolic flexibility in which aerobic glycolysis and mitochondria can cooperate during cancer progression (15,16). Recent studies demonstrate that there is not only a direct link between the Warburg effect and mitochondrial defects, but also there is oncogenic activation and mutation in signalling pathways that regulate glucose uptake, namely PI3K/Akt/mTOR pathway (11,17). Additionally, selection of the glycolytic phenotype in tumour cells can be mediated by the action of transcription factors (12,17,18), like the hypoxia-inducible factor (HIF-1α) (19–21), MYC (19), Ras and p53 (21–23).

The dependence of tumour cells on the increased glucose uptake has proved useful for tumour detection and monitoring, with this phenotype serving as the basis for the clinical [^{18}F] fluorodeoxyglucose positron emission tomography (FDG-PET) imaging technique (Figure 5). FDG injected into the bloodstream is taken up by glucose transporters (GLUT) on the cell surface and then phosphorylated by hexokinases (HK) to form FDG-phosphate where is no further metabolized and becomes trapped within the cells. Accumulation of the radioactive glucose analogue is then detected by PET, which is able to detect tumour cells and distinguish them from normal tissue (24).

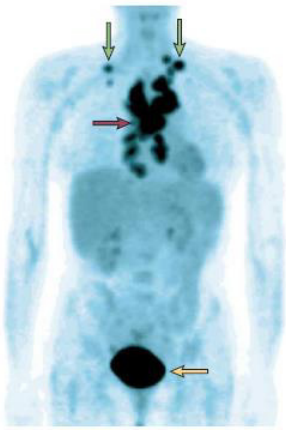

Figure 5 – Positron-emission tomography imaging with ^{18}fluorodeoxyglucose of a patient with lymphoma. The mediastinal nodes (purple arrow) and supraclavicular nodes (green arrows) show high uptake of 18fluorodeoxyglucose (FdG), showing that tumours in these nodes have high levels of FdG uptake. The bladder (yellow arrow) also has high activity, because of excretion of the radionuclide (24).

The proliferation state of cancer cells leads to a development from a normal epithelium to an interstitial neoplasia that is characterized by an uncontrolled cell proliferation that progresses to a carcinoma *in situ.* The glycolytic phenotype that characterizes tumour cells results from the hypoxia that exerts a selective pressure for up-regulation of glycolytic pathway even in the presence of oxygen (24). Tumour cell population that possesses this glycolytic phenotype, have a powerful proliferative advantage, which it is able to alter the intracellular pH in a way that is harmless to itself, but toxic to other phenotypes. This environmental change leads to cellular acidosis, through increased concentration of lactate, contributing therefore to an invasive phenotype through (Figure 6) (11,12,17,24).

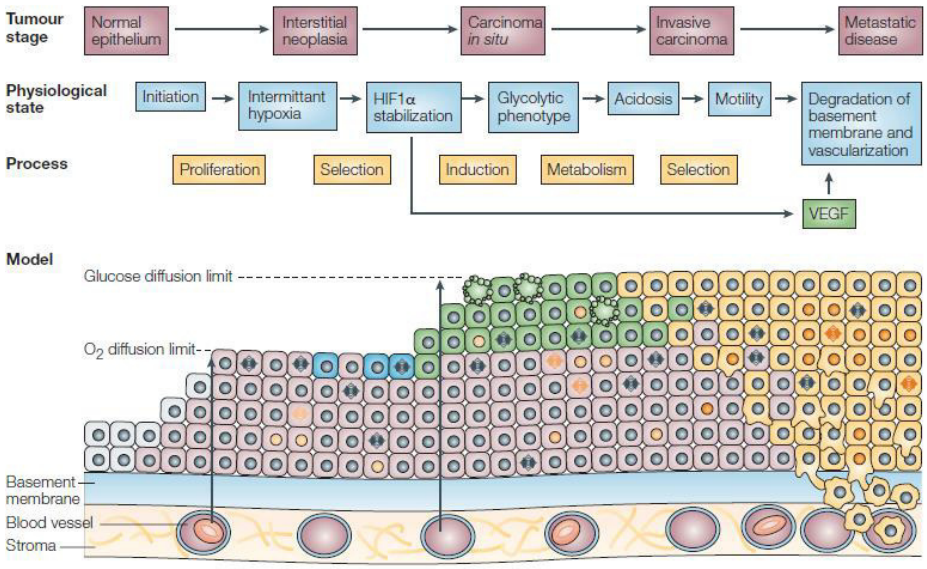

Figure 6 - Schematic representation of the cell-microenvironment interactions associated with the carcinogenesis process. Normal epithelial (grey), hyperproliferative (pink), hypoxic (blue), glycolytic (green) and motile cells (yellow) (24).

In normoxic conditions, cells utilize oxygen for energy production through OXPHOS. However, in conditions of hypoxia, i.e., presence of low oxygen levels, cells have the capacity to adapt. This adaptation consists in an adaptive response including metabolic alteration, angiogenesis and erythropoiesis. Several studies demonstrate that hypoxia-inducible factors (HIFs) mediate this adaption to the hypoxic microenvironment (1,20). Under these conditions, the activation of HIF1-α leads to increasing upregulation of genes that are involved in glycolysis, including glucose transporter 1 (GLUT 1) in order to increase the glucose uptake, hexokinase II (HK II) which catalyse the initial step of glycolysis and lactate dehydrogenase A (LDH-A) that converts the pyruvate into lactate. HIF1 also promotes induction of pyruvate dehydrogenase kinase (PDK), a negative regulator of pyruvate dehydrogenase (PDH) that catalyses the conversion of pyruvate to acetyl-CoA (6–8,25,26). The increase in lactate production amongst the diminished vascular dispersion of CO_2 contributes to hypoxic acidosis, namely to the acid-resistant phenotype. In order to ensure the regulation of the pH, HIF1-α induces the expression of specific genes that have the task of regulating the pH. These regulators can be carbonic anhydrase IX (CAIX) and monocarboxylate transporters (MCTs), among others (27,28). CA IX is a hypoxic marker and a prognostic indicator which performs the reversible conversion of CO_2 to bicarbonate and proton, contributing to acidification of tumour microenvironment and consequently to control of intracellular pH (29,30). This upregulation reduces electron flux through OXPHOS and subsequently reduces oxidative stress resulted from mitochondrial metabolism (31). MCT4 is up-regulated in hypoxia and cooperates with MCT1 in the efflux of generated lactate. In fact, MCT4 will not only be important for the acid-resistant phenotype, but also for the hyper-glycolytic phenotype in the way that, by exporting newly formed lactate, will allow continuous conversion of pyruvate to lactate and, therefore, continuous aerobic glycolysis (32,33).

The increasing in the glycolytic phenotype of cancer cells leads to acute and chronic acidification of the tumour microenvironment. The principal culprits for this acidification of the extracellular environment are the monocarboxylate transporters, that co-transport H+ and lactate. It is known that cellular acidosis is associated with the aggressive behaviour of cancer cells, i.e., proliferation, increased survival, migration, invasion, angiogenesis and radioresistant (34), making lactate a key player in cancer (24,35,36). Since lactate produced by glycolytic tumour cells has an important role in tumour microenvironment, being associated to poor prognosis, disease-free and overall survival in human cancer, it is possible to say that it plays an active role in progression of malignant diseases. Lactate concentrations can be associated with radioresistance, this feature could be due to the antioxidant properties of lactate, making it a suitable candidate as a diagnostic and prognostic indicator for a wide variety of tumours (37,38).

Additionally, hypoxia inducible gene expression can be regulated by lactate and pyruvate, and this is completely independent from hypoxia conditions, since it occur by stimulating the accumulation of HIF1-α (39). This indicates that the hyper-glycolytic phenotype is stimulated by lactate, providing a positive feedback.

Although glucose is the major source of lactate in most solid tumours, it is important to note that other cancer pathways rather than glycolysis can contribute to lactate production, such as glutaminolysis (7,25).

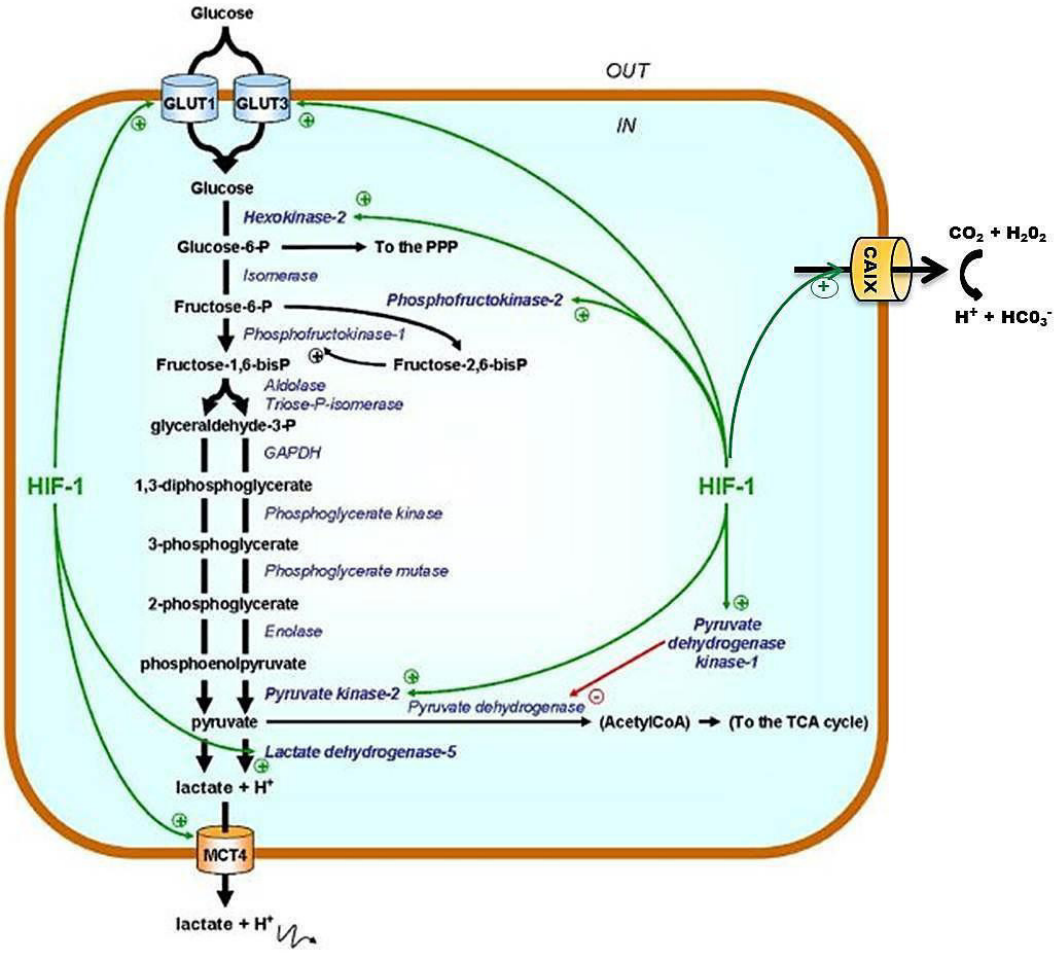

Figure 7 – HIF-1 promotes the expression of glycolytic enzymes and transporters (adapted from (40)). Green arrows point at HIF-1 target gene products directly involved in increased glycolytic flux.

1.3 Role of MCTs in cancer metabolism

Lactate is the monocarboxylic acid that can be formed as end-product of glycolysis and its intracellular accumulation results in acidification of the cytosol and inhibition of phospho-fructokinase-1 (PFK-1) and consequently glycolysis (41). In order to stop this cellular acidification, the monocarboxylate transporters (MCTs) are needed, since they are essential for the maintenance of the pH balance (42). The efflux of lactate is particularly important for high glycolytic cells such as most cancer cells, white and red blood cells, and tissue skeletal muscle (43).

1.3.1 The MCT family

Solute Carrier Family 16 (SLC16) is the name of the monocarboxylate transporters family and it is composed by 14 members and encoded by the SLC16 gene family. As shown in Figure 8 the identification of the 14 members was identified by a sequence homology whose predicted phylogeny and the analysis provides valuable information regarding the functional clustering of the human MCT family (44). It is possible to see that MCT1-MCT4 are associated in the same cluster, presenting high homology, and they are the only isoforms that have been demonstrating to transport monocarboxylic acids.

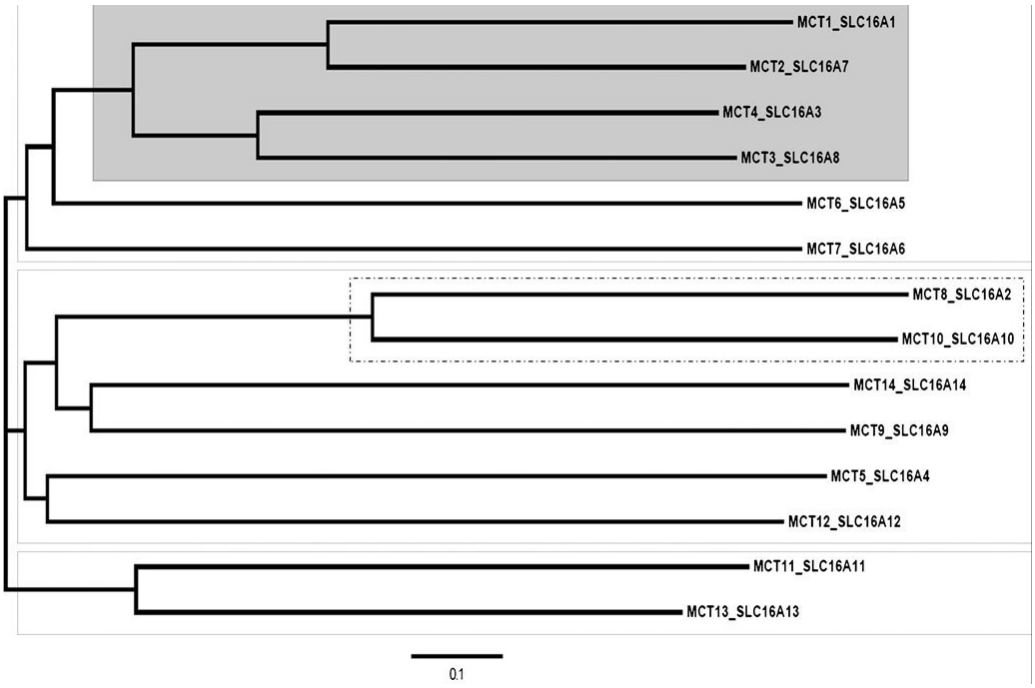

Figure 8 – Human MCT family members phylogram (44).

MCTs have 12 transmembrane domains with the N- and C-termini located in the cytoplasm (45). The transmembrane domains (TMDs) are highly conserved between isoforms with the greatest sequence variations observed in the C-terminus and the large intracellular loop between TMDs 6 and 7, which has a range of 29–105 amino acid residues (46). This observed variability is common to transporters with 12 TMDs and it is thought that these sequence variations are related to substrate specificity or regulation of transport activity (45,46). Topological prediction has been experimentally confirmed only for MCT1 in erythrocytes (Figure 9), nevertheless, analysis of the deduced amino acid sequences of the other MCT orthologs reveals a similar predicted topology (45).

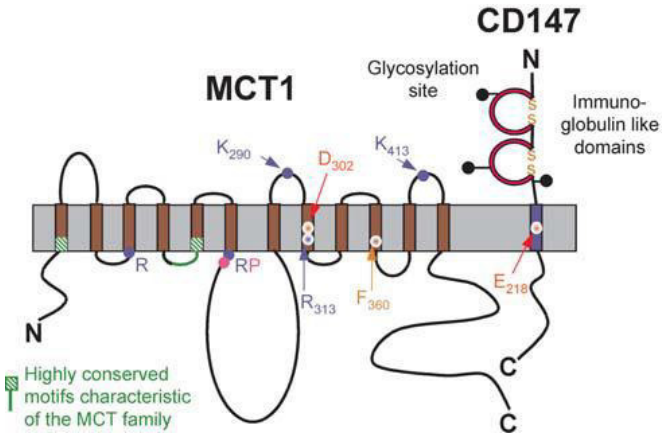

Figure 9 – The proposed topology of the monocarboxylate transporter (MCT) family members (45).

1.3.2 MCT isoforms

MCT1 is the most well-studied and functionally characterized member of the MCT family. This is due to the fact that MCT1 is the only monocarboxylate transporter expressed in human erythrocytes and it has the broader tissue distribution, it also has a wider range of substrates when compared to the other family members (44,47). MCT1 has an ubiquitous distribution and catalyses the cellular uptake and efflux of monocarboxylates such as pyruvate (Km 0.6-1 mM), L-lactate (Km 2.2-4.5 mM), propionate (Km 1.5 mM), acetate (Km 3.7 mM), acetoacetate (Km 5.5 mM), L-β-hydroxybutyrate (Km 8.1-11.4 mM) and D-β-hydroxybutyrate (Km 8.1-10.1 mM) and is mostly responsible for lactate uptake for oxidation in red skeletal muscles and heart or efflux in glycolytic cells, such as erythrocytes, according to the substrate concentration and pH gradient (44,45,47). This transporter functions as a proton-dependent cotransporter/exchanger and transport across the membrane occurs by ordered sequential binding with association of a proton followed by lactate binding (48).

MCT2 isoform has higher affinity for lactate and pyruvate than MCT1. So, when expressed in the same tissue MCT1 and MCT2 are located in distinct cells, as they have been suggested to play different

roles in metabolic shuttles (49,50). This MCT is located principally in tissues that have high requirements of lactic acid for use as a respiration fuel, like neurons, or for gluconeogenesis, like the liver parenchymal cells and the kidney proximal convoluted tubules (47).

MCT3 is the only MCT that have a confined expression, being expressed in the RPE and choroid plexus epithelia where it is located on the basal membrane in contrast to MCT1 which is found on the apical membrane (47).

MCT4 is mostly expressed in tissues that rely on glycolysis such as white skeletal muscle fibers, astrocytes, white blood cells, chondrocytes, and some mammalian cell lines (44). It was characterized by heterologous expression in *Xenopus laevis* oocytes, exhibiting the highest Km values for most substrates and inhibitors when compared to MCT1 and MCT2 (45). As mentioned above, hypoxia is known to be a regulator of MCT expression, and MCT4 was described to be regulated by the hypoxia inducible factor-1α (HIF-1α)(51).

Membrane expression of MCTs is necessary for their activity (monocarboxylates efflux/uptake), however they do not present glycosylation, a characteristic phenomenon of membrane spanning proteins. This is why MCTs have the need of being associated with other glycosylated membrane proteins in the plasma membrane, namely CD147 also known as basigin or EMMPRIN, which belongs to the immunoglobulin superfamily and is a broadly distributed plasma membrane protein (52,53). MCT1, 3 and 4 require association with the mature glycosylated form of CD147 protein, while MCT2 isoform requires the association with integral membrane glycoprotein gp70 or EMBIGIN (52). CD147 is important for MCT membrane location and activity and it was already demonstrated that MCT1 and MCT4 regulates the maturation and trafficking to the plasma membrane of CD147. This contributes to the malignant phenotype since MCT contribution is not limited to the transport of monocarboxylates (lactate) and the regulation of pH, they may have an indirect role in tumour growth and angiogenesis (45,54–56). Some studies indicate the existence of a cofactor that is responsible for the localization of MCTs on the plasma membrane and that they are active, in tissues that do not possess CD147. CD44 may be another chaperone for MCT expression at the plasma membrane, leading to an important role in cancer progression and tumour cell chemoresistance (57).

1.3.3 MCT in cellular homeostasis

Monocarboxylate transporters passive, bidirectional symporters that are associated to energy metabolism and intracellular pH (pH_i) regulation, since they assure the continuous transporting of lactate, avoiding cellular acidosis and guarantee the flux of glycolysis (Figure 10) (58,59). As already known,

MCTs are more involved in the export of lactic acid than pH_i regulation, since they are not activated by the pH change. Nevertheless, several studies have demonstrated that inhibition of MCT1 leads to a decrease in intracellular pH in several melanoma and neuroblastoma cell lines at a low tumour-like extracellular pH, this induced a decrease in cell viability (60,61). Additionally, down-regulation by short interference RNA (siRNA) of MCT1 and MCT2 in malignant human glioma cells caused a decrease of 0.6 units in the intracellular pH and rapid cell death (62). Gerlinger et al. (63) reported that de down-regulation of MCT4 decreased pH_i, cellular ATP and survival of two clear cell renal cell carcinoma (ccRCC) cell lines. Almost all cancer cells are known to express both MCT1 and MCT4, so it is not a surprise that when MCT1 or MCT4 are inhibited, there may not be a marked decrease on the intracellular pH. Still, the combination of silencing both MCTs resulted in an increase in the intracellular proton concentration, leading to a decrease in the intracellular pH (64). Carbonic anhydrases (CA), are proteins that have an important role in the H^+/lactate transport facilitation (29). Probably, CAs in close region of MCTs are able to function as "H^+ -distributing antennas" that help in the dissipation of local protons and facilitate the import or export of lactate (30,65,66).

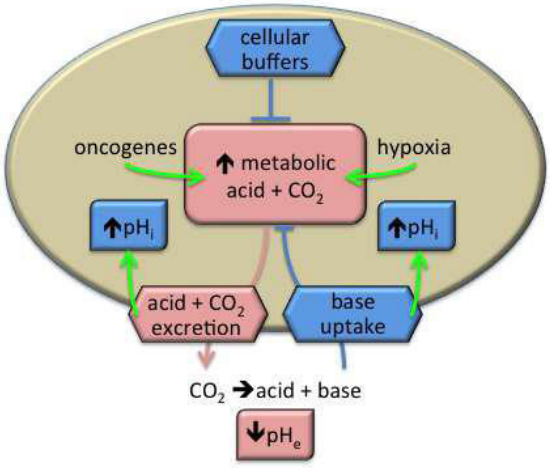

Figure 10 – Generalised model of tumour generated acidity and pH regulation (67).

1.3.4 MCT regulation

Little is known about the regulation of MCT expression in different tissues. MCT1 is the most widely expressed member of its family in normal and cancer tissues, including head and neck and lung (68), breast (35,69), osteosarcomas (70), glioma (71), gastrointestinal stromal tumours (GIST) (72), cervix (73) and colorectal (74) cancers. Depending on the physiological and pathological conditions MCTs expression can vary. Several studies report that altered MCT expression is a result of changes in MCT substrate concentration and/or signals arising from changes in cellular metabolism (75). MCTs are

regulated at various points up to the functional protein, including both transcriptional and post-transcriptional level which affects protein amounts, as well as, by regulators of transporter activity, like chaperone proteins. MCT expression appears to be regulated in a tissue specific manner (46,75).

Hypoxia is known to induce the glycolytic phenotype it is also associated with the alteration of the expression of MCTs (32,68,76–79). Studies suggest that the increase in neuronal, astrocytic and endothelial MCT1 expression, observed after permanent occlusion of the left middle cerebral artery, is mediated by HIF-1α, the major transcriptional regulator of adaptation to hypoxic stress (80). Nevertheless, this view was questioned by Ullah and collaborators who, after performing functional studies with MCT1, MCT2 and MCT4 promoters, demonstrated that only MCT4 promoter was activated by hypoxia and that this response was mediated by HIF-1α (32). Additionally, MCT4, but not MCT1, was shown to be up-regulated by hypoxia in trophoblast cells (76), in human bladder cancer cells (81) and in C6 glioma cells (82). However, there is evidence that describes a hypoxia mediated increase in both MCT1 and MCT4 and decrease in MCT2, with MCT1 and MCT4 change being HIF-1α-dependent (78). A recent study demonstrated that there is an up-regulation of MCT1 expression in presence of hypoxia, still, this regulation was independent of HIF-1α, but dependent of p53 status (79).

1.3.5 MCT inhibitors

Due to their association with specific glycosylated molecular chaperones, MCTs can be targeted at the plasma membrane, in order to stop their transport activity. Thus, they are attractive targets for systemic therapy. Identification of MCT inhibitors was achieved during characterization of MCT1, as the most studied MCT isoform (45). A great number of MCT1 inhibitors are known and can be divided into three categories:

1. Bulky or aromatic monocarboxylates which are competitive inhibitors, including 2-oxo-4-methylpentanoate, phenyl-pyruvate and derivatives of α-cyanocinnamate, as α-cyano-4-hydroxycinnamate (CHC) (45).
2. A range of amphiphilic compounds with different structure including bioflavonoids (e.g. quercetin and phloretin) and anion transport inhibitors such as 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and niflumic acid (45).
3. Stilbenedisulphonates (e.g. 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS)) (45).
4. Irreversible inhibitors, such as p-chloromercuribanzene sulphonate (pCMBS) and amino reagents (e.g. pyridoxal phosphate and phenyl-glyoxal) (45).

Though these inhibitors have a good capacity to inhibit MCTs, there are not specific to the MCT isoforms, in other words these inhibitors have different affinities for the different MCT isoforms (Table 1).

Table 1 - Inhibitors of monocarboxylate transporters.

MCT Isoform	Inhibitor	References
MCT1	CHC	(42,83)
	pCMBS	(84)
	DIDS	(42,85)
MCT2	CHC	(42,83)
MCT4	CHC	(42,83)
	pCMBS	(84)
	Fluvastatin	(86)
	Atorvastatin	(86)
	Lovastatin	(86)
	Simvastatin	(86)

As already mentioned, none of the MCT classical inhibitors is either MCT specific or MCT isoform specific. Therefore, to investigate the role of MCT in cellular function, MCT specific inhibitors should be used. The pharmaceutical company AstraZeneca, developed a new set of immunodulatory compounds as potent and selective inhibitors of MCT1 activity in human activated and rat T cells (87,88).

1.3.6 MCTs as a therapeutic target in cancer

Since the metabolic phenotype of cancer cells is the basis for several mechanisms of tumour resistance to traditional therapy several anticancer strategies targeting tumour metabolism have been explored (Figure 11). Metabolic strategies include indirect targets that consist in signalling pathways that regulates cellular metabolism altered in cancer, and direct targets such as metabolic enzymes (6).

The acid-resistant phenotype that cancer cell present is essential for their survival. However this phenotype gives some problems to the cell when it concerns the pH regulation, therefore it is essential that pH regulators, like Na+/H+ exchanger (NHE1) and CAs, exist (30). Even though MCTs do not regulate the intracellular pH in a direct way, as it was mentioned above, they are considered pH regulators, since they regulate acidic environment of cancer cell in an indirect way. MCTs perform a double role in the

adaptation to hypoxia, they export lactate, that is important for the maintenance of the glycolytic phenotype, and they regulate the pH, that is essential for the acidic-resistant phenotype. So, if MCTs were inhibited there will be a direct effect on cell pH regulation and glycolytic rates, therefore having an important effect on cell viability. Thus, targeting these transporters will comprise the perfect phenotype that cancer cells have acquired (30).

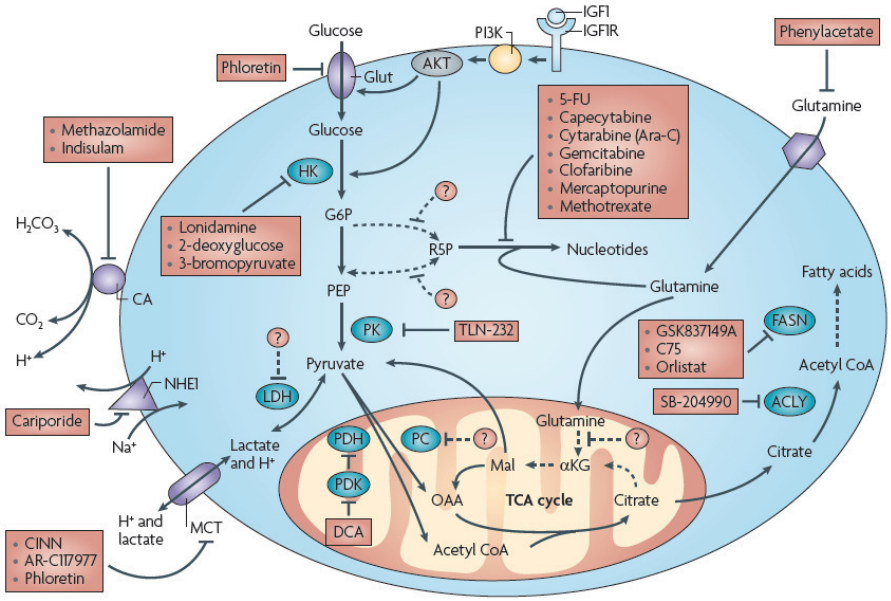

Figure 11 – Schematic representation of metabolic targeting tumour strategies (6). 5-FU – 5-fluorouracil; αKG – α-ketoglutarate; ACLY – ATP citrate lyase; CA – carbonic anhydrase; CINN – α-cyano-4-hydroxycinnamate; DCA – dichloroacetate; FASN – fatty acid synthase; G6P – glucose-6-phosphate; Glut – glucose transporter; HK – hexokinase; IGF1 – insulin-like growth receptor 1; IGF1R – IGF1 receptor; LDH – lactate dehydrogenase; Mal – malate; MCT – monocarboxylate transporter; NHE1 – Na+/H+ exchanger 1; OAA – oxaloacetate; PDH – pyruvate dehydrogenase; PDK – pyruvate dehydrogenase kinase; PEP – phosphoenol pyruvate; PK – pyruvate kinase; R5P – ribose 5-phosphate; TCA – tricarboxylic acid cycle.

1.4 Oesophageal cancer

1.4.1 Epidemiology and pathology

Oesophageal cancer (OC) is the eight most common cancer worldwide (composing 3% of all cancers) and the sixth most common cause of cancer-related death with 400 000 deaths in 2012 (4.9% of all cancers deaths) (89). This neoplasia is comprised principally by two main subtypes: the oesophageal adenocarcinoma (OAC) and the oesophageal squamous cell carcinoma (OSCC). Other subtypes are mainly carcinomas, such as leiomyosarcomas, lymphomas and small cell carcinoma, and

represent less than 10% of all cases (90–92). Benign tumours of the oesophagus are extremely rare (about 1% of all oesophageal tumours) and normally they are asymptomatic (93). This malignancy has a poor prognosis, since the 5-year survival is around 15%-25% (94). This dreadful prognosis appears to be more related to the features of the esophagus and due to the fact that the tumour is usually identified in its last stages (95).

The main subtypes of this neoplasia have different incident rates. Oesophageal squamous cell carcinoma represents almost 87% of all cases of cancer of the oesophagus, with 398 000 diagnosis of the total number of oesophageal cancer (89,96) and has a high-incidence in eastern Asia, the "Asian Oesophageal Cancer Belt" (Fig. 12 a), in eastern and southern Africa and in south America (96,97), however its incidence has been decreasing over the years. On the other hand, oesophageal adenocarcinoma has a higher prevalence in western populations, like America, Europe and Australia (Fig. 12 b) (96). These geographical divergencies are due to several human behaviours and comorbidities that may lead to the formation of this malignancy.

Oesophageal cancer continues to be a male dominant disease, being two to three times more common in SCC, generally, this male predominance is even more marked in AC subtype (98–100). This male predominance is still a mystery since there is no consensus regarding this huge gender difference in this type of cancer, nevertheless, recent studies have demonstrated that severe reflux in men may be a contributing factor (100). Another hypothesis is the influence of sex hormones. Studies suggest that there is an androgenic effect and a estrogenic effect regarding the ratio male-to-female of 4.4 (101). This theory is supported by the by sex steroid hormone involvement in the inflammatory process, since there are associations between testosterone and inflammatory markers, sex steroid hormone receptor protein expression in esophageal cancer tissue, and lower rates of AC among men with prostate cancer, who are likely to receive anti-androgen therapies (100,101). Supporting this data, a small hospital-based study reported high levels of testosterone in adenocarcinoma cases than in controls (102). There is also been reported positive associations for free androgens in Barret's metaplasia, in two studies of oesophageal adenocarcinoma (103).

Squamous cell carcinoma of the upper and middle thirds of the esophagus arise from the stratified squamous epithelial lining of the oesophagus (92) this occurs when oesophageal mucosa is exposed to repeated damage, which result in changes to the squamous oesophageal mucosa leading to a squamous hyperplasia that precedes to low-grade and high-grade squamous dysplasia, which then develops into invasive cancer (96). Adenocarcinoma of the distal esophagus develops from exposition to repeated damage resulting in a metaplastic epithelium (Barrett oesophagus – were the squamous

epithelium is replaced by columnar glandular cells) that is transformed through low-grade and high-grade dysplasia to invasive cancer (96).

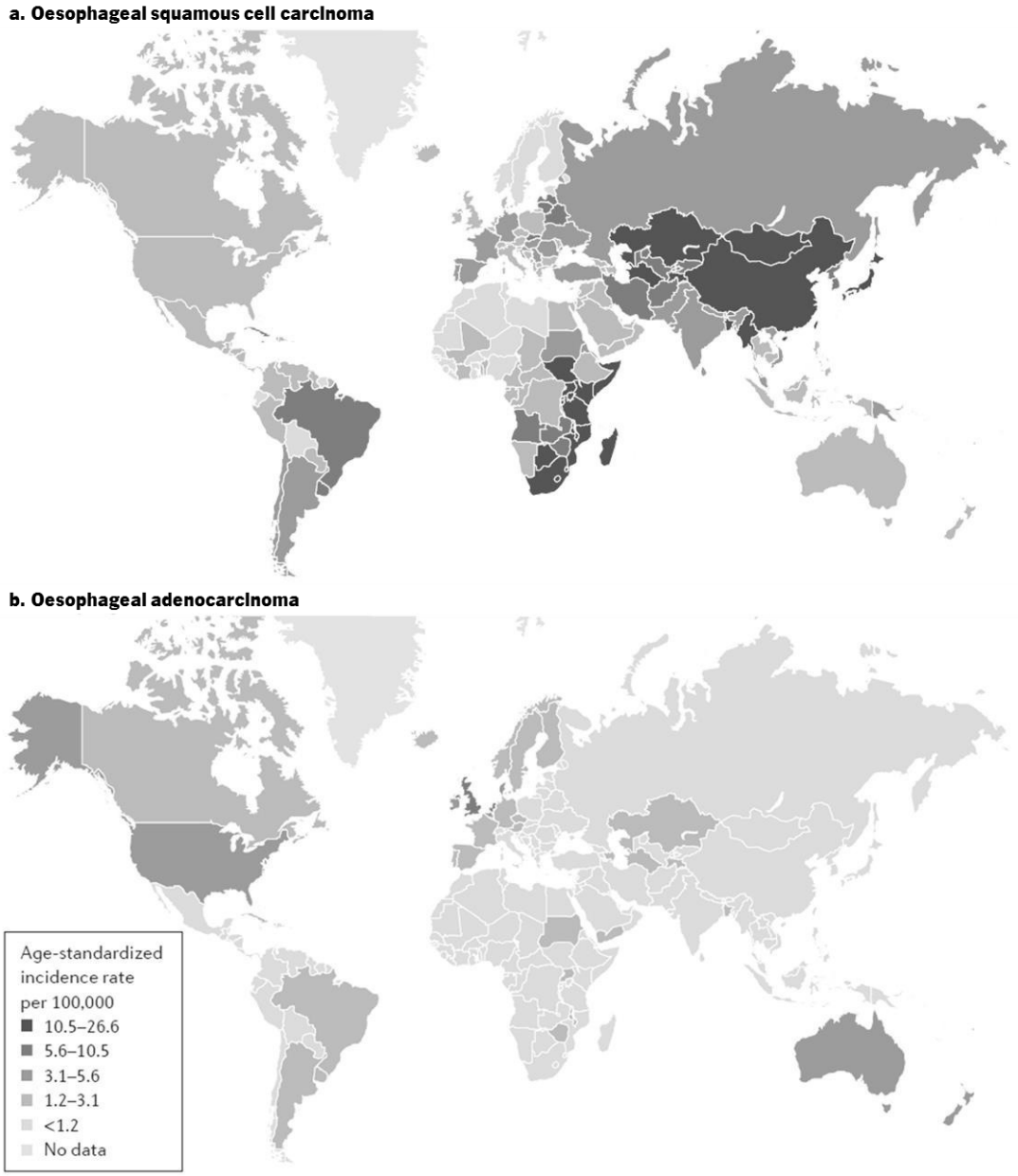

Figure 12 – The global annual incidence of OSCC and OAC (96).

1.4.2 Risk factors

The patterns of oesophageal cancer are very different at a geographical level. Although the mechanism of oesophageal tumorigenesis is not fully understood, there is several factors that are known to increase the probability of developing this neoplasia. Some of these factors are shared by the two main

subtypes and others are specific for each histological type. Table 2 summarizes the main risk factors that contribute to the development of oesophageal cancer.

Table 2 – Risk Factores that affect the development of oesophageal malignancies (98).

Risk factors	ESCC	EAC
Tobacco smoking	+++	++
Excess alcohol consumption	++	++
Barrett's esophagus	NS	+++
Reflux (GERD) symptoms	NS	+++
Obesity	NS	++
Excess energy consumption	NS	+
Excess fat consumption	NS	++
Poverty	++	NS
Low education level	+	NS
Excess intake of hot beverage (thermal injury)	+	NS
H. pylori infection*	Protective[3]	Protective[4]

+++, Very strong effect; ++ moderate effect; +, some effect. EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; GERD, gastroesophageal reflux disease; NS, not significant. *Role of *Helicobacter pylori* infection as a risk factor affecting esophageal carcinoma was cited from sources other than [2].

1.4.3 Diagnosis and treatments

Due to the muscular and expansive features of the oesophagus, the symptoms associated to developing malignancy are mistaken with symptoms of a cold or a sore throat and normally patients tend to ignore until it is too late and when the tumour is detected it has reached a relatively locally advanced or metastatic stage. The normal warning signals that something is not right include difficulty swallowing (dysphagia) or pain when swallowing (odynophagia), involuntary and progressive weight loss, and hoarseness or cough; cough can signify laryngeal nerve involvement or aspiration (96).

The most used diagnosis tool for oesophageal cancer is endoscopy, although it is not the best examination. Endoscopy can be improved with the use of Lugol's iodine dye (where it can be used in a chromoendoscopy). This dye will help in the identification of early OSCC or by using narrow-band imaging, in which light of specific wavelengths is used to improve the resolution of the surface mucosa (92,96). During the examination if the endoscopist find erosions, ulcers, strictures, or metaplasia, he has to decide if the origin of these changes is nonneoplastic or neoplastic (104). The endoscopist will perform a biopsy, if there is a suspicion of a neoplastic origin. A biopsy can reach a level of confidence of 96% if a minimum of 8 biopsy are taken from the margins and the centre of the lesion (96,104).

Staging of a neoplasia is essential in order to establish the appropriate treatment choices for oesophageal cancer. The staging for oesophageal cancer should be performed according the tumour-node-metastasis (TNM) classification (Figure 13), apart from the TNM classification there's three other

classifications that depend on the mode of staging: clinical or radiological staging (c stage), pathological staging (p stage) determined after primary surgery or endoscopy for localized disease or pathological staging after neoadjuvant therapy (yp stage) (96).

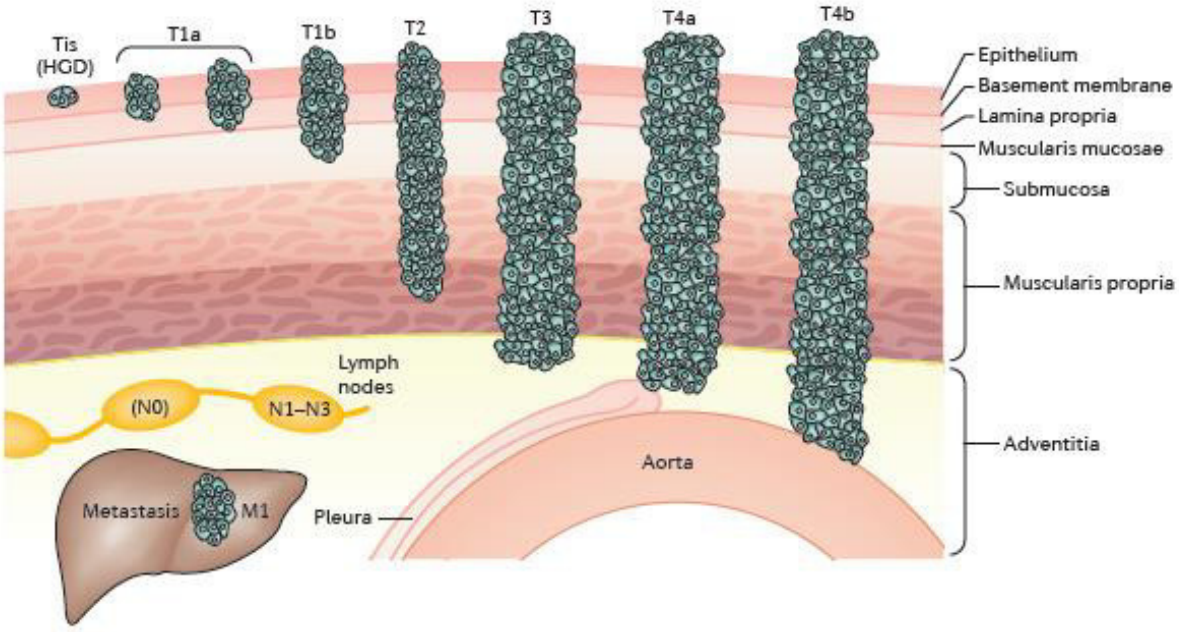

Figure 13 – Tumour-node-metastasis categories. Tumour classification according to the tumour–node–metastasis (TNM) categories. T refers to the size of the primary tumour and whether it invades the nascent tissue as shown. N refers to lymph node involvement: N0 describes no regional lymph node metastasis; N1 describes regional lymph node metastases involving one or two nodes; N2 describes regional lymph node metastases involving from three to six nodes; and N3 describes regional lymph node metastases involving seven or more nodes. M refers to distant metastasis and is categorized as M0 (no distant metastasis) or M1 (distant metastasis). HGD, high-grade dysplasia; Tis, cancer in situ.

After the TNM stage is defined, the patient will be presented with a multidisciplinary tumour conference, were the best treatment will be decided. This decision is based on the localization of the disease, if the patient gave locoregional (stage 1-3) disease or if he has metastasis (stage 4). When the neoplasia is locoregional, patients are classified as: (1) medically fit and receptive to surgery, chemotherapy, or chemoradiation; (2) medically unfit or unreceptive for surgery but medically fit for chemotherapy or chemoradiation; or (3) unfit for either surgery, chemotherapy, or chemoradiation (Figure 14) (96,105).

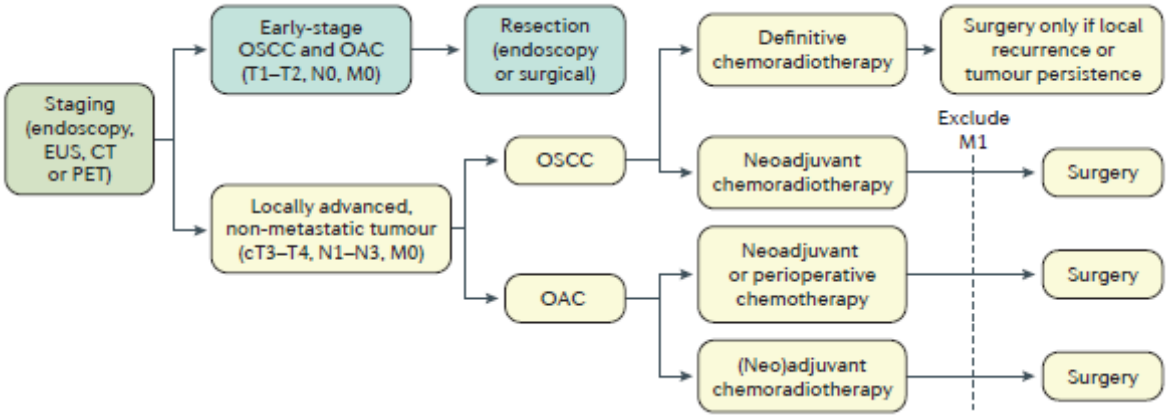

Figure 14 – An algorithm for the management of localized oesophageal cancer. The algorithm depicts the treatment options for early-stage and locally advanced oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC), in the absence of metastasis. EUS, endoscopic ultrasonography (96).

1.4.1 Oesophageal cancer metabolism

The presence of metabolic remodelling has been described in oesophageal carcinomas (106,107). Several studies have been drawing attention to the role of key metabolic enzymes and their association with aerobic glycolysis in OC, trying to provide a new molecular therapeutic targeting in adenocarcinoma and squamous cell carcinoma of the oesophagus (108–110). The studies focus on the importance of GLUT1 (108,111), LDH-A (112), pyruvate kinase (PKM2) (108,113), CA IX (109,114) and HIF1-α (110,115) in the metabolic remodelling of oesophageal cancer . However, there are few studies that characterized the role of MCTs on the survival and progression of oesophageal tumours.

1.5 Rationale and Aims

Since the expression and activity of MCTs is unknown in oesophageal carcinomas, the main aim of this work was to explore the role of MCTs in this type of cancer, by characterizing their expression in oesophageal carcinoma tissues and correlate their expression with metabolic markers studied and assess the clinical-pathological impact. The second aim was to characterize the expression of MCTs, CD147 and metabolic markers in oesophageal squamous cell carcinoma cell lines and the effects of MCT down-regulation evaluating cell viability and glucose consumption and lactate efflux.

Thus, the specific aims were:

– Characterization of the expression of MCTs and other metabolism-related markers in a series of human oesophageal carcinomas tissue by Immunohistochemistry and cell lines by immunofluorescence and Western Blot.

– Evaluation of the effects (cell viability and metabolism) of MCT inhibition in oesophageal squamous cell carcinoma cells. Cell viability was assessed by sulforhodamine-B assay and cell metabolism (glucose consumed and extracellular lactate) was measured using colorimetric kits.

2. MATERIALS AND METHODS

2.1 Protein expression assessment in human samples

2.1.1 Tissue samples

A series of paraffin-embedded oesophageal carcinomas tissue samples were obtained from Hospital de Câncer de Barretos, São Paulo, Brasil. This series was composed by 45 cases of oesophageal adenocarcinoma and 50 cases of oesophageal squamous cell carcinoma. This study was approved by the Local Ethical Committee and the samples were unlinked and unidentified from their donors.

2.1.2 Immunohistochemistry

Immunohistochemistry is a technique based on the principle of antibody-antigen interaction that allows the detection of target proteins in biological tissues. The antibody-antigen interaction is visualized using a chromogen, in which the enzyme conjugated to the antibody cleaves a substrate to produce a colour precipitate at the location of the interest protein.

Protein expression of MCT1, MCT2, MCT4, CD147, GLUT-1, HKII and LDH-A was evaluated by immunohistochemistry (IHC) in representative 4μm-thick tissue sections of human oesophageal samples. Each slide was dewaxed in an oven at 80°C for 10 minutes, washed for 5 minutes in three consecutive xylol baths, hydrated with decreasing concentrations of ethanol and finally placed in water. Subsequently, the antigenic recovery was carried out in a water bath at 98°C for 20 minutes in specific buffer, followed by a cooling-off period of 20 minutes. Each slide was washed twice in TBS 1x buffer for 5 minutes, and then the inactivation of endogenous peroxidases was carried out with 3% H_2O_2 solution, diluted in methanol. The immunohistochemical reactions of MCT1 and CD147 were performed with the Lab Vision UltraVision ONE Detection System: HRP Polymer (Thermo Fisher Scientific) polymer system, according to the manufacturer's instructions. The reactions for MCT2, MCT4 and GLUT1 were performed with the Lab Vision UltraVision Large Volume Detection System: anti-Polyvalent, HRP (Thermo Fisher Scientific), based on the streptavidin-biotin peroxidase principle, following the manufacturer's recommendations. Reactions of HKII and LDH-A followed the avidin-biotin peroxidase principle, using R.T.U. Vectastain Elite ABC Reagent (Vector Laboratories), according to the manufacturer's instructions. To visualize the reactions, the Liquid DAB + Substrate Chromogen System (Dako) was used as the chromogen, following the manufacturer's instructions. Finally, the slides were stained with Mayer's Hematoxylin (Dako) and

mounted with Entellan mounting medium (Merck). As a negative control, the primary antibodies were replaced with an appropriate universal negative control antibody (N1698 and N1699, Dako). Squamous cell carcinoma tissue of the oral cavity was used as a positive control for MCT1, MCT4, HKII and LHH-A. Normal kidney tissue as a positive control of MCT2, normal colon tissue as a positive control for CD147, placenta as a positive control for GLUT1, normal stomach tissue for CAIX, tumoral colon tissue for PDK and glioblastoma for HIF1-α. Details of the immunoreaction are summarised in the table 3.

Table 3 – Details about the immunohistochemical procedure used to visualize the different proteins

Protein	**Positive Control**	**Antigen Retrieval**	**Primary antibody**	
			Dilution	Company
MCT1	Oral Squamous Cell Carcinoma	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min	1:300, overnight, room temperature	AB3538P Chemicon International
MCT2	Kidney	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min	1:200, 2 hours, room temperature	sc-50322 Santa Cruz Biotechnology
MCT4	Oral Squamous Cell Carcinoma	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min	1:500, 2 hours, room temperature	sc-50329 Santa Cruz Biotechnology
CD147	Colon	EDTA (1 mM, pH=8.0), 98°C, 20 min	1:500, overnight, room temperature	sc-71038 Santa Cruz Biotechnology
GLUT1	Placenta	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min	1:500, 2 hours, room temperature	ab15309 Abcam
HKII	Oral Squamous Cell Carcinoma	EDTA (1 mM, pH=8.0), 98°C, 20 min	1:1000, 2 hours, room temperature	ab104836 Abcam
LDH-A	Oral Squamous Cell Carcinoma	EDTA (1 mM, pH=8.0), 98°C, 20 min	1:500, 2 hours, room temperature	ab101562 Abcam
CA IX	Stomach	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min	1:2000, 2 hours, room temperature	ab15086 Abcam
PDK	Tumor tissue of colon	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min	1:250, 2 hours, room temperature	sc-28783 Santa Cruz Biotechnology
HIF1-α	Glioblastoma	EDTA (1 mM, pH=8.0), 98°C, 20 min	1:200, overnight, room temperature	610958 BD BIOSCIENCES

2.1.3 Evaluation of the immunoreactions

Immunostained tissue was evaluated semi-quantitatively, by a pathologist, considering the intensity of staining for each region determined above. The score for immunoreactive extension was as follows: score 0 (0% of immunoreactive cells); 1 (<5% of immunoreactive cells); score 2 (5-50% of immunoreactive cells) and 3 (>50% of immunoreactive cells). Cellular localization of staining (cytoplasm / plasma membrane) of the studied markers was also evaluated. Staining intensity was scored as: 0: negative; 1: weak; 2: intermediate; and 3: strong. The final score was defined by the sum of these semi-quantitative parameters and grouped as negative (score 0-2) and positive (score 3-6).

2.2 Cell lines and cell culture

In the present study, the human oesophageal squamous cell carcinoma cell lines KYSE-30 and KYSE-410 were used.

Cells were cultured in RPMI-1640 medium supplemented with 10% of Fetal Bovine Serum (FBS), 1% of antibiotic solution (Penicillin-Streptomycin solution). They were incubated at 37°C under a humified atmosphere with 5% of CO_2, in an incubator. The maintenance of cell culture was done in 25 cm^2 culture flasks (T25 flasks). The exponential growth phase was achieved by subculture, when 80% confluence was reached, by treatment with 0.05% TrypLE Express solution.

2.3 Protein expression in oesophageal carcinoma lines

2.3.1 Total protein extraction

For the expression analysis of proteins in basal conditions, cells were grown in 25 cm^2 culture flask at a confluence of 80%. Later, cells were harvested with lysis buffer (1% Triton-X100; 1% NP-40; 0.1mM EDTA; 50mM Tris-HCl, pH=7.5; 150mM NaCl), afterwards, protease inhibitors were added, and cells were homogenized and put in ice for 10 minutes. After that, homogenized samples were centrifuged (13000 rpm, 15 minutes, 4°C) and the supernatants were stored at -80°C until quantification and analysis.

2.3.2 Protein quantification

Protein concentration was determined using the Bradford assay and a protein standard, the Bovine Serum Albumin (BSA) protein. Diluted BSA standards (0.1 µL; 0.25 µL; 0.5 µL; 1 µL; 5 µL) and Bradford reagent were prepared according to the standard protocol. In a 96-well plate, 200 µL of Bradford reagent was added to the standards of BSA standards or 2 µL of samples of total protein extracts and finally, 98 µL of PBS was added. After incubation at room temperature for 5 minutes, absorbance was measured at 590 nm using the SkanItTM software. The protein concentrations of total protein extracts were obtained using the standard curve with the absorbance of BSA standards versus its concentration in µg/mL.

2.3.3 Western Blot assay

After total protein extraction and protein quantification described above, samples were analysed by a Western Blot assay. For that purpose, 20 µg of total protein samples were mixed with Laemmli Sample Buffer (final volume 40 µL) and denatured at 95°C for 5 minutes, followed by a short spin. After protein denaturation, samples were separated by electrophoresis on 10% polyacrylamide gel (resolving gel solution: 375 mM Tris-HCl pH 8.8 SDS 0.4%, 15% glycerol, 0.1% TEMED, 0.05% APS; stacking gel solution: 125 mM Tris-HCl pH 6.8 SDS 0.4%, 0.1% TEMED, 0.05% APS). Molecular weight marker (Thermo Scientific) was used in all the blots to access protein size. The electrophoresis was performed in a Mini-Protean Tetra System (Bio-Rad) electrophoresis system at 100 V per gel, using the running buffer (0.025 M Tris base, 0.192 M Glycine, 0.1% SDS). The proteins separated in the polyacrylamide gel were then transferred to a nitrocellulose membrane previously hydrated with transfer buffer (0.025 M Tris base, 0.192 M Glycine, 0.1% SDS, 20% methanol). Electrotransfer occurred in a Mini-Protean Tetra System (BioRad) at 54 mA for half an hour. Membranes were rinsed with PBS and blocked in TBS-T (TBS with 0.1% Tween-20) containing 5% non-fat dry milk (Molico) for 1 hour at room temperature to block non-specific binding sites. Then, membranes were washed with TBS-T and incubated with the specific primary antibodies diluted in TBS-T with 5% non-fat dry milk or 5% BSA, overnight at 4°C, with agitation in a Roller Mix. After overnight, membranes were washed two times for 5 minutes and once for 15 minutes with TBS-T to remove excess of unbound antibody followed by incubation with appropriate secondary antibodies diluted in TBS-T during 1 hour at room temperature, with agitation in a Roller Mix. Membranes were washed two times for 5 minutes and once for 15 minutes with TBS-T and protein immunodetection was revealed by chemiluminescence detection kit (BioRad), using the ChemiDoc XRS (BioRad). The intensity of bands was quantified using the ImageJ software and β-actin was used as internal control. The

results of three independent experiments were quantified, and correspondent values were divided by the β-actin value. All primary antibodies and secondary antibodies and their dilutions used in this study are listed in Table 4.

Table 4 – Details of primary and secondary antibodies used in Western blot analysis

Protein	Size (kDa)	Primary antibody		Secondary antibody (dilution 1:5000)
		Company (reference)	dilution	
MCT1	40-48	sc-365501	1:500 (5% BSA)	Anti-mouse
MCT2	40-43	SC-166925	1:200 (5% milk)	Anti-mouse
MCT4	43	sc-50329	1:500 (5% milk)	Anti-rabbit
CD147	45-65	Sc-71038	1:500 (5% milk)	Anti-rabbit
GLUT1	45-55	ABCAM 15309	1:500 (5% BSA)	Anti-rabbit
HKII	102	ABCAM 104836	1:2000 (5% milk)	Anti-mouse
LDH-A	35	Sc-137243	1:200 (5% milk)	Anti-mouse
CAIX	50	ABCAM 15086	1:2000 (5% BSA)	Anti-rabbit
PDK	43-55	sc-28783	1:200 (5% milk)	Anti-rabbit
HIF1-α	120	BD BIOSCIENCES 610958	1:500 (5% milk)	Anti-mouse
β - actin	43	sc-1616	1:300 (5% milk)	Anti-mouse

2.3.4 Immunofluorescence

Protein expression of MCT1, MCT2, MCT4, CD147, GLUT-1, HKII, LDH-A, CAIX, PDK and HIF1-α in cell lines was evaluated by immunofluorescence. IF is a laboratory technique that uses fluorochromes to identify the presence of antibodies that are bound to specific antigens. These fluorochromes allow the visualization of the target distribution in the sample under a fluorescent microscope.

For the expression analysis of proteins at basal conditions, cells were grown in glass coverslips (4×10^4 cells/well cells/coverslip). Twenty-four hours post cell seeding, the cell culture medium was removed, and the cells were rinsed with PBS 1x. Then, the cells were fixed and permeabilized with cold methanol for 20 minutes and each coverslip was washed with PBS-TWEEN 0.05% 2 times, 5 minutes each. After that, the cells were blocked with 5% BSA for 30 minutes and finally the cells were ready for the immunostaining. The primary antibodies were diluted in 5% BSA (according to manufacturers' instructions) and then added to the coverslips and incubated overnight at 4°C. After incubation, the cells were washed with PBS-TWEEN 0.05% 2 times, 5 minutes each, in order to remove the primary antibody. From this point on, the coverslips must be covered from light. Next, the secondary antibody (also diluted

in 5% BSA) was added to the cells and incubated for 1h at room temperature. Subsequently, the cells were washed again with PBS-TWEEN 0.05% 2 times, 5 minutes each. Finally, a drop of mounting medium (containing antifade agent and DAPI) was placed on each coverslip and the cells were ready to be visualized on a fluorescence microscope. All primary antibodies and secondary antibodies and their dilutions used in this study are listed in Table 5.

Table 5 – Details of primary and secondary antibodies used in Immunofluorescence analysis

Protein	Primary antibody		Secondary antibody (dilution 1:500)
	Company (reference)	dilution	
MCT1	CHEMICON AB35388	1:200 (5% BSA)	Anti-rabbit
MCT2	Sc-166925	1:200 (5% BSA)	Anti-mouse
MCT4	sc-50329	1:500 (5% BSA)	Anti-rabbit
CD147	sc-71038	1:500 (5% BSA)	Anti-mouse
GLUT1	ABCAM 15309	1:500 (5% BSA)	Anti-rabbit
HKII	ABCAM 104836	1:750 (5% BSA)	Anti-mouse
LDH-A	sc-137243	1:200 (5% BSA)	Anti-mouse
CAIX	ABCAM 15086	1:2000 (5% BSA)	Anti-rabbit
PDK	sc-28783	1:200 (5% BSA)	Anti-rabbit
HIF1-α	BD BIOSCIENCES 610958	1:500 (5% BSA)	Anti-mouse

2.4 Silencing of MCT1 and MCT4 in oesophageal cell lines

Short (or small) interfering RNA is a tool for inducing a short-term (2-4 days) silencing of gene expression. It targets a specific mRNA and damages it.

Downregulation of MCT1 and MCT4 was performed in both oesophageal squamous cell carcinoma cells, using short interfering RNA (siRNA) (Invitrogen). A control group was also performed, using scrambled siRNA (Invitrogen) and another one without any siRNA or scrambled siRNA. For that, different silencing mixes were prepared in Opti-MEM medium (GIBCO, Invitrogen), including specific siRNA for MCT1 and MCT4 and lipofectamine RNAiMAX reagent (Invitrogen), according to manufacturers' instructions. Ten minutes later, the previous mixtures were distributed in different wells (6-well plate) and

cells were seeded in antibiotic-free medium (KYSE-30: 5 x 10^5 cells/well; KYSE-410: 4 x 10^5 cells/well). Twenty-four hours after transfection, the medium was replaced by a fresh complete medium.

2.5 Cell viability assay

Cell viability was assessed using the Sulforhodamine B (SRB)-based assay. The Sulforhodamine-B has the ability to bind to basic amino-acid residues under acidic conditions. The binding of SRB is stoichiometric, so the amount of dye extracted from the strained cells is directly proportional to the cell mass.

Non-silenced and silenced KYSE-30 and KYSE-410 were plated in 48-well plate, at a cellular density of 5,3×10^4 cells/well for KYSE-30 and 4,67×10^4 cells/well for KYSE-410 and allowed to adhere overnight in antibiotic-free RPMI 1640 medium. After that, cells were incubated for an additional period of 24 hours. At 48h post-silencing, cells were washed with PBS 1X and, then, incubated with 10% of trichloroacetic acid (TCA) (Sigma-Aldrich), for 1 hour at 4°C. Wells were rinsed, three to four times, with tap water, and allowed to dry overnight. On the next day, cells were incubated with Sulforhodamine B (SRB) (Sigma-Aldrich) for 30 minutes, at room temperature, and, then, wells were rinsed, four times, with acetic acid (1%). When wells were completely dried, cells were incubated with 10mM of Tris-base (Sigma-Aldrich) for 5 minutes, at room temperature, under shacking. The absorbance was measured, at 490nm using the SkanItTM software. The range of silencing used was between 48h-96h, being 48h of silencing the time 0h for the first SRB assay, 72h of silencing the 24h the second SRB assay and 96h of silencing the 48h of the last SRB assay.

2.6 Metabolism assay (glucose consumption and lactate production)

Metabolic activity of OSCC cells was evaluated through quantification of extracellular glucose and lactate, using different colorimetric assays. Non-silenced and silenced KYSE-30 cells (5.3×10^4 cells/well) and KYSE-410 (4.67×10^4 cells/well) were seeded into 48-well plates and incubated in antibiotic-free RPMI 1640 medium, for 24 hours. At 48h post-silencing, aliquots of supernatants (20 µl) were harvested for glucose and lactate (Spinreact SA) quantification. 200µl of working solution was mixed with 2µl of each sample. Blank contained 100µl of working solution and 2µl of PBS 1x. The mixture was homogenized and incubated for 10min at room temperature and then measured at 490nm using the

SkanItTM software. All results were normalized for total cell biomass, using the SRB assay described above.

2.7 Statistical analysis

Data from human tissues was analysed using the SPSS statistical software (version 25, SPSS Inc). Comparison of expression of the different markers of oesophageal squamous cell carcinoma and oesophageal adenocarcinoma tissues was evaluated for statistical significance using the Pearson's chi square ($\chi 2$) test with the threshold for significance being $p \leq 0.05$. Analysis of associations between the expression of different markers in both subtypes of oesophageal cancer and the clinical pathological data were assessed using a Pearson's chi square ($\chi 2$) test. For association with survival a Kaplan Meier curve with Cox regression was performed. For the in vitro studies, the GraphPad prism 6 software was used, with the Student's t test, considering significant values $p \leq 0.05$.

3. RESULTS

3.1 Analysis of MCTs, CD147 and metabolic markers in samples of oesophageal carcinomas

3.1.1 Characterization of expression of MCT1, MCT4, CD147 and other metabolic markers in oesophageal tissue

A total of 95 oesophageal carcinoma samples and 20 normal tissue samples were assessed for different metabolic markers (MCT2, HKII, LDH-A, GLUT-1), MCT1, MCT4 and their chaperone CD147 (Figures 15, 16 and 17).

Normal tissue was negative for MCT2, MCT4, PDK, CA IX and HIF1-α. The rest of the markers expression was present in at least one normal sample (Table 6).

Regarding the expression in the carcinoma samples, it was possible to observe that only MCT2 was negative in all samples of carcinomas. PDK obtained a negative expression in squamous cell carcinoma but not for the adenocarcinoma tissues (p=0.456). On the other hand, HIF1-α was negative for the adenocarcinoma tissues but not for the squamous cell carcinoma tissues (p=0.266). Samples were positive for all the other markers for the SCC tissues and for the AC tissues (MCT1 (p<0.008), MCT4 (p<0.001), CD147 (p<0.001), HK II (p<0.001), LDH-A (p<0.001), GLUT1 (p<0.001) and CA IX (0.085)) (Table 6).

Table 6 – Expression of MCTs, CD147 and key metabolic markers in SCC and AC tissues.

	Total n	MCT1	MCT4	CD147	HK II	LDH-A	GLUT 1	PDK	CA IX	HIF1-α
		Positive (%)	Positive (%)	Positive (%)	Positive (%)	Positive (%)	Positive (%)	Positive (%)	Positive (%)	Positive (%)
Non-neoplastic	**20**	6 (30)	0	3 (15)	1 (5)	1 (5)	10 (50)	0	0	0
SCC	**50**	25 (50)	23 (46)	41 (82)	40 (80)	33 (66)	40 (80)	0	10 (20.4)	2 (4)
AC	**45**	9 (20)	8 (17.8)	25 (55.6)	41 (91.1)	31 (68.9)	14 (31.1)	1 (2.2)	6 (13.3)	0
p		0.008	0.000	0.000	0.000	0.000	0.000	0.456	0.085	0.266

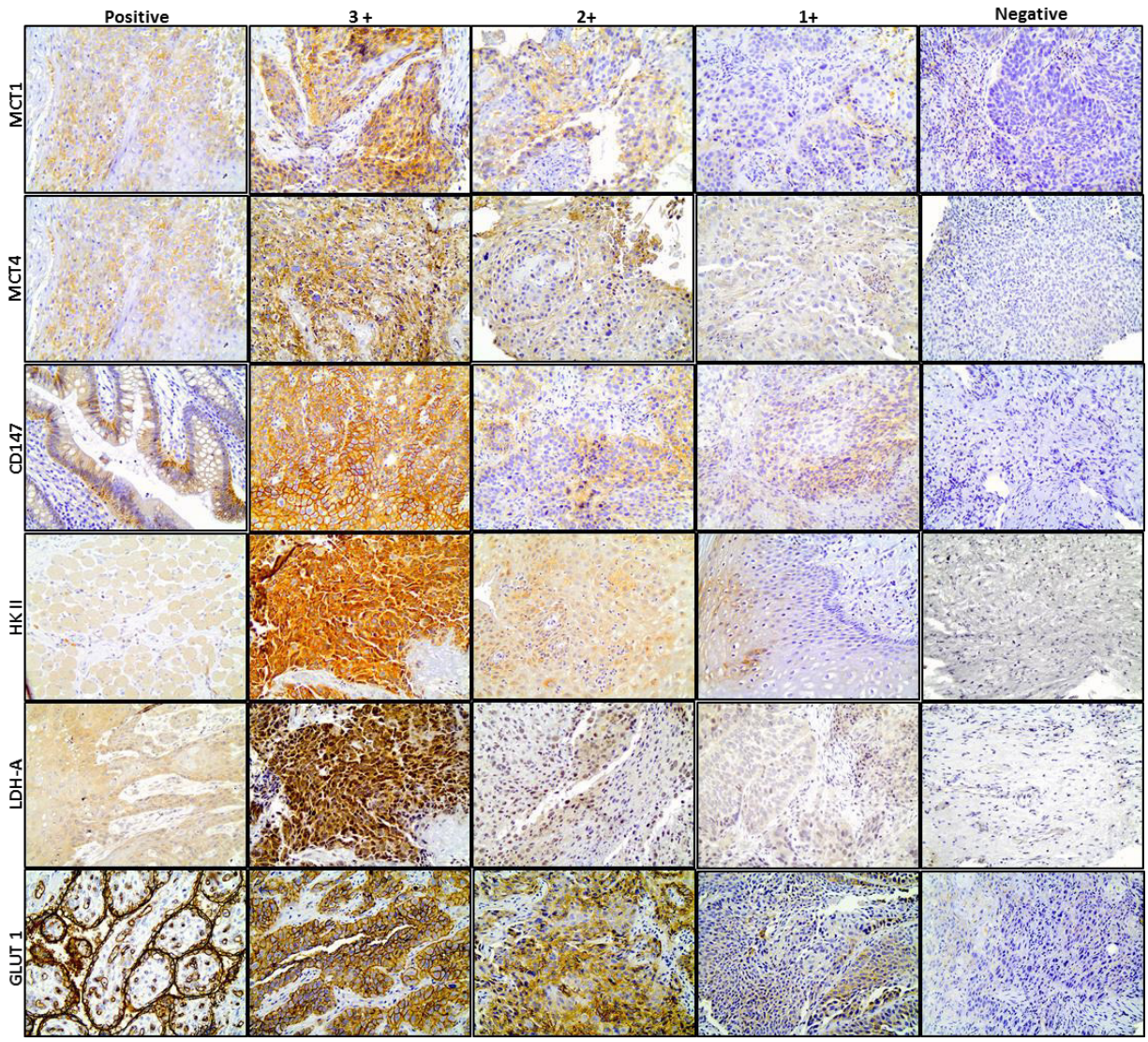

Figure 15 – Immunohistochemical expression of MCT1, MCT4, CD147, HK II, LDH-A and GLUT1 in oesophageal squamous cell carcinoma tissues (representative of the different scores). Pictures were taken at 400x magnification

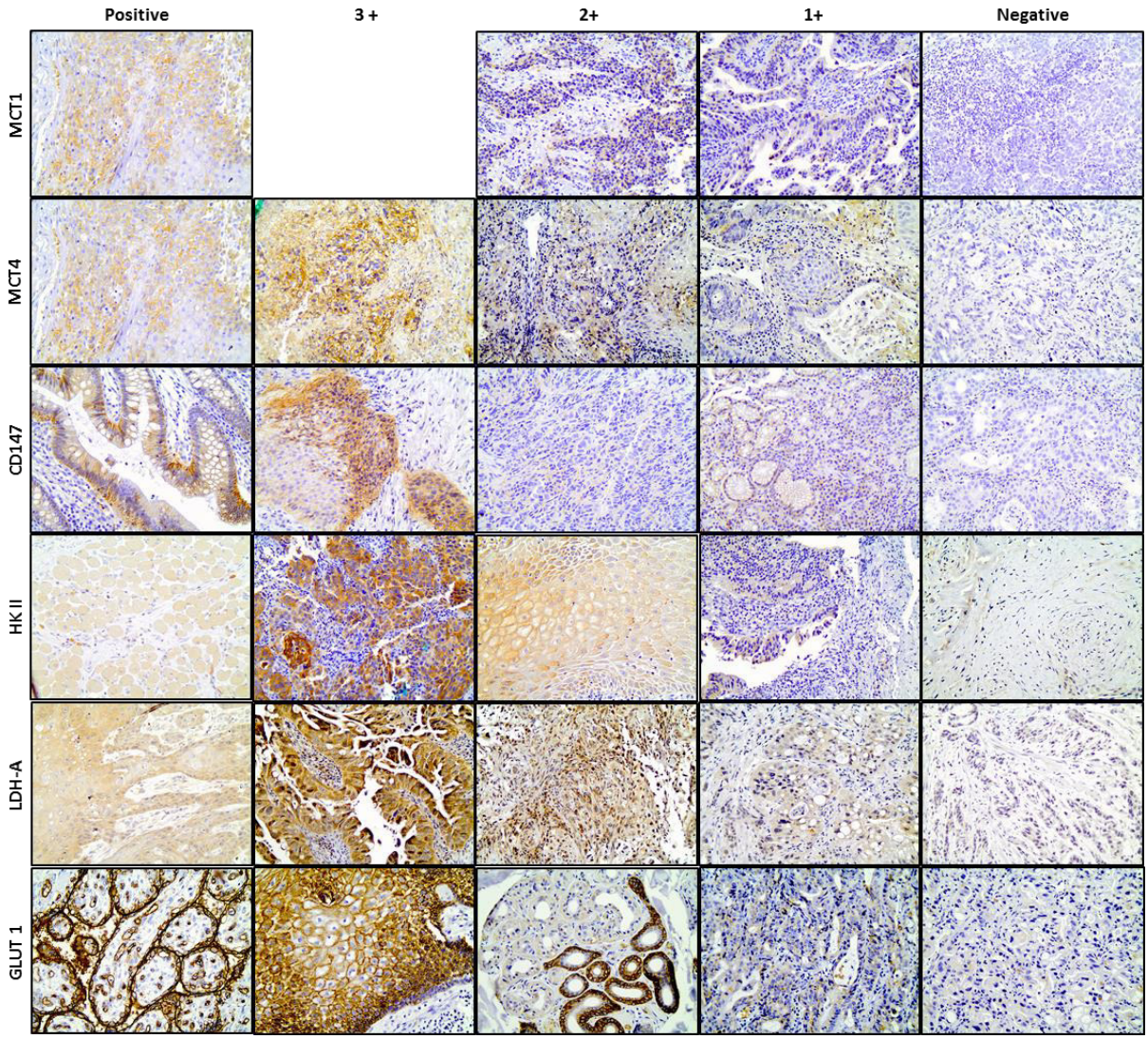

Figure 16 – Immunohistochemical expression of MCT1, MCT4, CD147, HK II, LDH-A and GLUT1 in oesophageal adenocarcinoma tissues (representative of the different scores). There was no 3+ case for MCT1. Pictures were taken at 400x magnification.

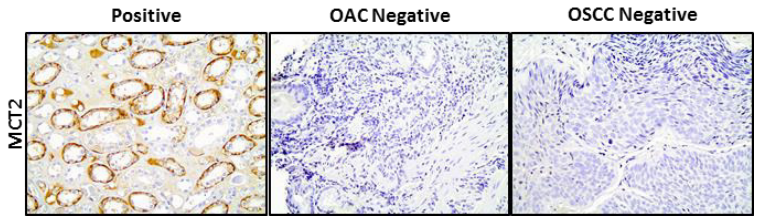

Figure 17 – Immunohistochemical expression of MCT2 in oesophageal adenocarcinoma (OAC) tissues and oesophageal squamous cell carcinoma (OSCC) tissues (representative of the different scores). There were only negative cases for this marker. Pictures were taken at 400x magnification.

3.1.2 Evaluation of associations between MCTs and metabolic markers in oesophageal tissue

Concerning MCTs and their chaperone CD147, there was an association between MCT1 and CD147 ($p < 0.023$) for squamous cell carcinoma but not for the adenocarcinoma samples (Table 7). For MCT4 and CD147 there were no correlation between the two proteins in any of the samples (Table 8).

Table 7 – Association between monocarboxylate transporter (MCT) 1 and CD147 expressions in oesophageal carcinoma samples

SCC	Total N	CD147	
		Positive (%)	*p*
MCT1			0.023
Negative	25	17 (68)	
Positive	25	24 (96)	

AC	Total N	CD147	
		Positive (%)	*p*
MCT1			0.260
Negative	36	18 (50)	
Positive	9	7 (78)	

Table 8 – Association between monocarboxylate transporter (MCT) 4 and CD147 expressions in oesophageal carcinoma samples

SCC	Total N	CD147	
		Positive (%)	*p*
MCT4			0.112
Negative	27	20 (74.1)	
Positive	23	21 (91.3)	

AC	Total N	CD147	
		Positive (%)	*p*
MCT4			0.206
Negative	37	19 (51.4)	
Positive	8	6 (75)	

3.1.3 Association between clinic-pathological data and MCTs and metabolic markers in oesophageal tissue

To understand if the MCTs and their chaperone can be a relevant clinical marker, the association of the expression of MCT1, MCT4 and CD147 with the clinical pathological data (Tables 9 and 10) was performed using a statistical analysis. Only MCT4 ($p = 0.043$) and CA IX ($p = 0.006$) had a significant association with patient survival for squamous cell carcinoma (Figure 18). For MCT1, CD147 and the other markers no significant associations were observed between their expression and overall survival of the patient's (Appendix I).

In order to know if any of the parameters of the clinical data could be a prognostic factor for each subtype, a statistical analysis was performed. Within all parameters, only tumour localization ($p = 0.000$) and age ($p = 0.022$) were relevant for adenocarcinoma (Figure 19), gender was the only parameter that had significance for squamous cell carcinoma ($p = 0.001$) (Figure 20). Even though lesion size ($p = 0.055$)

did not present any significant association, the value of p was very close to the significant value ($p < 0.05$) (Figure 20).

Table 9 – Clinical pathological data of patients with oesophageal squamous cell carcinoma and their associations with MCT1, MCT4 and CD147

Clinico-pathological Data For Squamous Cell Carcinoma	Total N	MCT1		MCT4		CD147	
		Positive (%)	*p*	Positive (%)	*p*	Positive (%)	*p*
Squamous Cell Carcinoma	50	25 (50)		23 (46)		41 (82)	
Age			1		0.152		0.452
>61	31	16 (51.6)		12 (38.7)		24 (77.4)	
≤61	19	9 (47.4)		11 (57.9)		17 (89.5)	
Gender			0.349		0.578		0.216
Male	45	24 (53.3)		21 (46.7)		38 (84.4)	
Female	5	1 (20)		2 (40)		3 (60)	
Tumour size			0.252		0.536		0.716
>4	29	17 (58.6)		13 (44.8)		23 (79.3)	
≤4	21	8 (38.1)		10 (47.6)		18 (85.7)	
Tumour localization			0.156		0.901		0.481
Proximal and/or middle	12	4 (33.3)		5 (41.7)		11 (91.7)	
Middle	15	6 (40)		7 (46.7)		11 (73.3)	
Distal and/or middle	20	13 (65)		10 (50)		16 (80)	
Metastasis	8	7 (87.5)	0.410	4 (50)	0.620	7 (87.5)	1

Table 10 – Clinical pathological data of patients with oesophageal adenocarcinoma and their associations with MCT1, MCT4 and CD147

Clinico-pathological Data For Adenocarcinoma	Total N	MCT1		MCT4		CD147	
		Positive (%)	*p*	Positive (%)	*p*	Positive (%)	*p*
Adenocarcinoma	45	9 (20)		8 (17.8)		25 (55.6)	
Age			0.461		0.456		0.545
>61	26	4 (15.4)		4 (15.4)		13 (50)	
≤61	19	5 (26.3)		4 (21.1)		12 (6.2)	
Gender			1		0.357		0.642
Male	40	8 (20)		8 (20)		23 (57.5)	
Female	5	1 (20)		0		2 (40)	
Tumour size			1		0.600		0.760
>4	18	4 (22.2)		3 (16.7)		11 (61.1)	
≤4	27	5 (18.5)		5 (18.5)		14 (51.9)	
Tumour localization			0.436		0.855		0.481
Proximal and/or middle	1	0		0		1 (100)	
Middle	4	0		1 (25)		3 (75)	
Distal and/or middle	35	9 (25.7)		7 (20)		20 (57.1)	
Metastasis	6	0	0.298	2 (33.3)	0.327	4 (66.7)	1

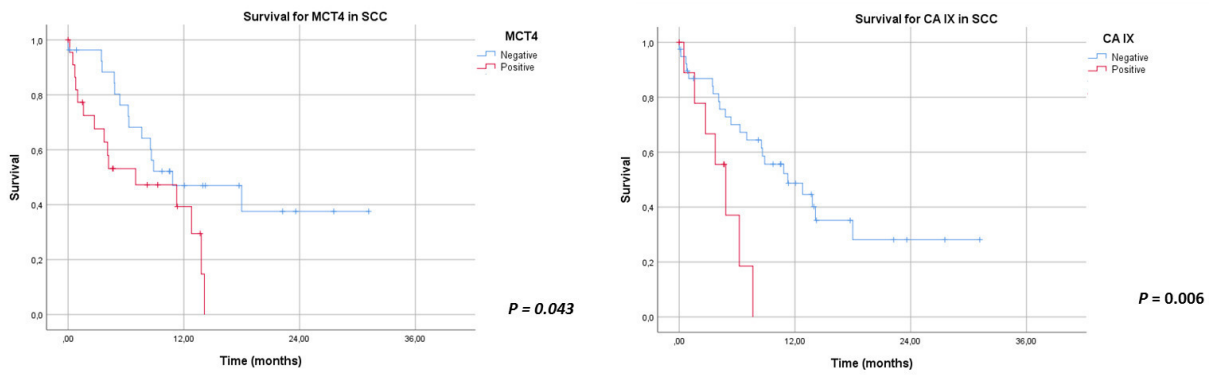

Figure 18 – Overall survival for MCT4 and CA IX in patients with oesophageal squamous cell carcinoma. ($p < 0.05$)

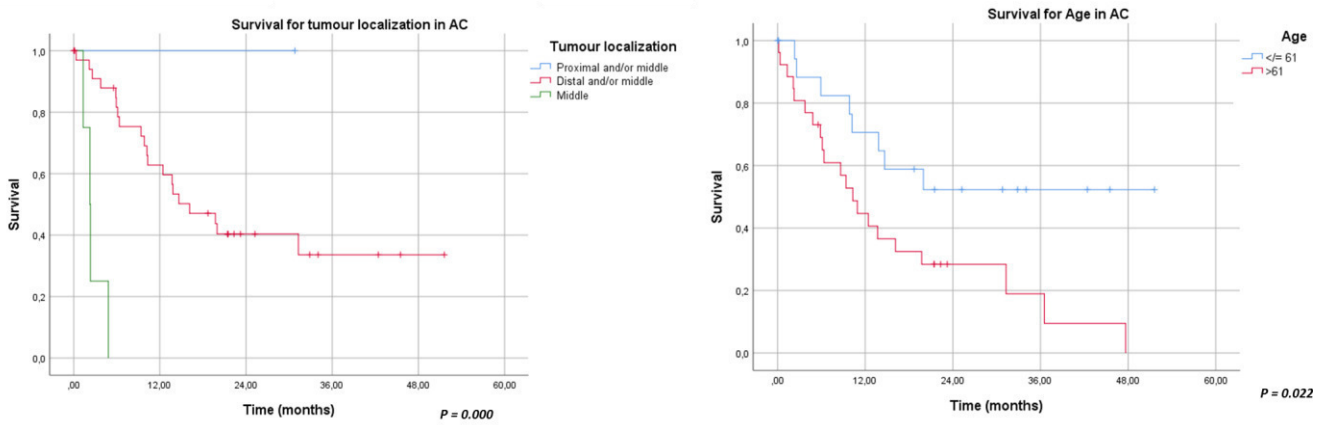

Figure 19 – Overall survival for tumour localization and age in adenocarcinoma. ($p < 0.05$)

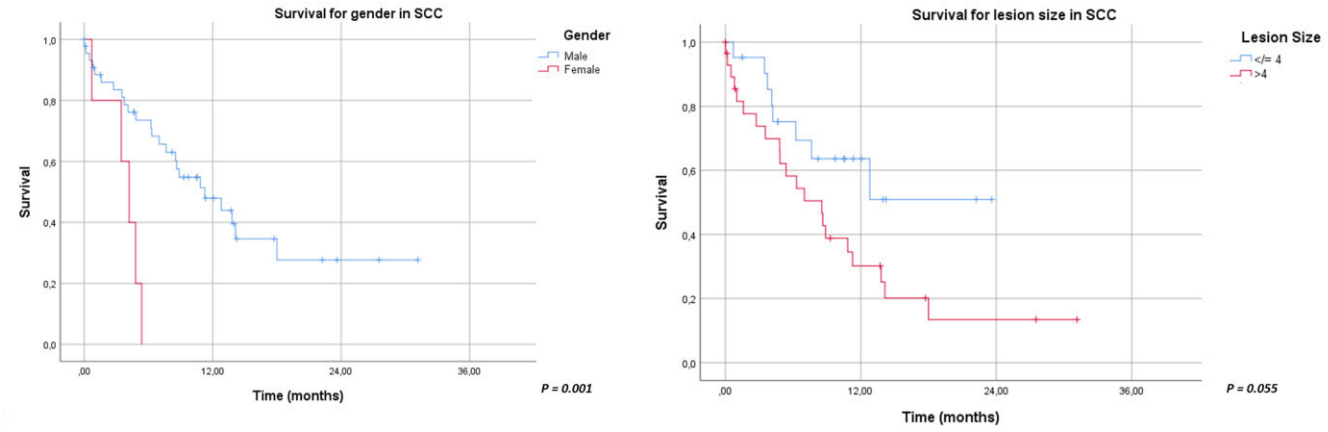

Figure 20 – Overall survival for gender and lesion size in squamous cell carcinoma. ($p < 0.05$)

3.2 Characterization of metabolic behaviour of squamous cell carcinoma cells

3.2.1 Characterization of expression of MCT1, MCT2, MCT4, CD147 and other metabolic markers in Squamous Cell Carcinoma cell lines

Western blotting and immunofluorescence were performed to assess the expression of MCTs, CD147 and other metabolism-related markers (GLUT1, HK II, PDK, LDH-A, CA IX and HIF1-α) in KYSE-30 and KYSE-410 oesophageal squamous cell carcinoma cell lines. Total protein extracts from KYSE-30 and KYSE-410 oesophageal squamous cell carcinoma cell lines were profiled, and Western blot analysis demonstrated that KYSE-30 and KYSE-410 cells expressed both MCT1 and MCT4 (Figure 21), however, KYSE-30 expressed more MCT1, MCT4 and CD147 then KYSE-410. Functional MCT transporter complexes require association with ancillary proteins. As stated above, it was reported that CD147 forms complexes with MCT1 and MCT4, which is essential for MCT membrane expression and catalytic activity (53,116). Thus, expression of CD147 was also evaluated. Western blot analysis demonstrated that both KYSE-30 and KYSE-410 cells expressed CD147 (Figure 21). Both high glycosylated (HG) CD147 and low glycosylated (LG) CD147 were detected in the blots. Both cell lines express all the metabolic markers that were analysed (Figure 22).

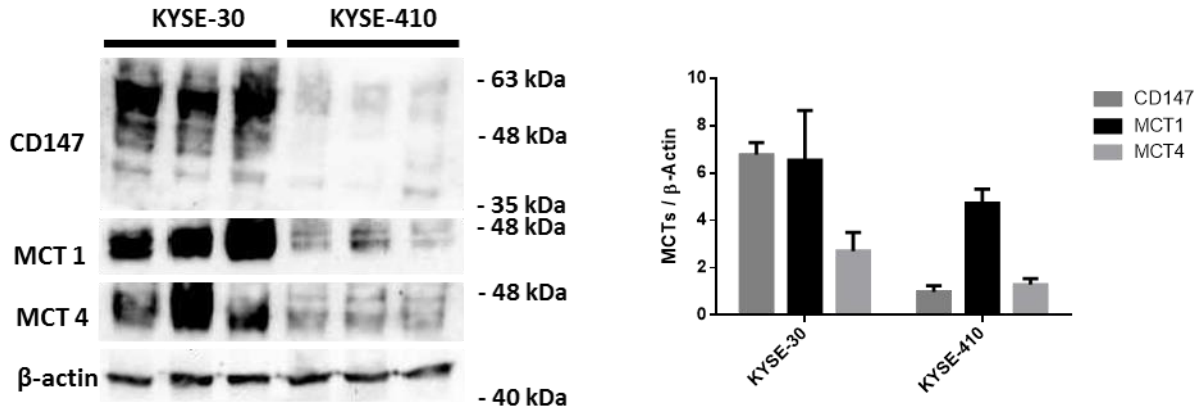

Figure 21 – Western blot analysis of MCT1, MCT4 and CD147 in SCC cell lines. ß-actin was used as internal loading control. Results of the Western blot are representative of at least three independent extractions, each one in triplicate.

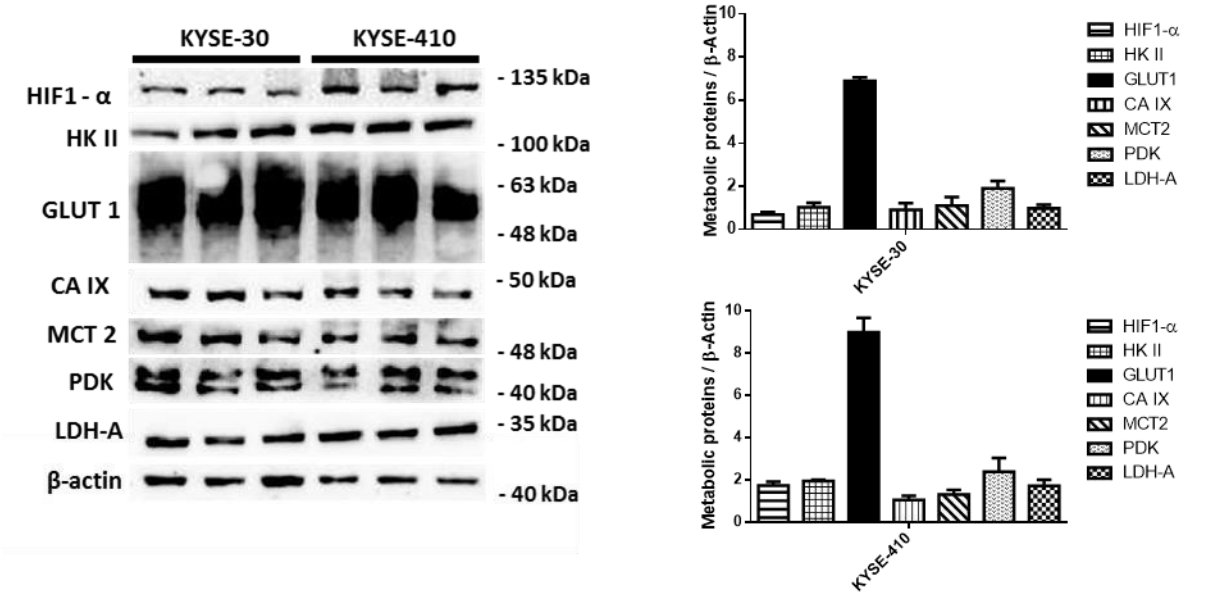

Figure 22 – Western blot analysis of metabolic markers in SCC cell lines. ß-actin was used as internal loading control. Results of the Western blot are representative of at least three independent extractions, each one in triplicate.

KYSE-30 and KYSE-410 cells were also analysed for MCT1, MCT2 MCT4, CD147, and metabolic markers, like GLUT 1, HK II, PDK, LDH-A, CA IX and HIF1-α by immunofluorescence. All the proteins were present in both cell lines (Figures 23, 24, 25 and 26). MCT1 and MCT4 were expressed in the cytoplasm of both cell lines, however, a plasma membrane expression of MCT4 was only observed in KYSE-30 and KYSE-410 cells (Figure 23 and 24). CD147 was markedly expressed in the plasma membrane of both cell lines and it was possible to see the merge of MCT1 with CD147 and MCT4 with CD147, which is in accordance to the literature (Figure 23 and 24). Regarding the glycolytic marker, GLUT 1, HK II and LDH-A, they were all expressed on both cell lines. GLUT 1 is highly express in the plasma membrane and in accordance with the Western blot. HK II, LDH-A and PDK are expressed in the cytoplasm, as expected (Figure 25 and 26). The pH regulator, CA IX, was expressed in both cell lines (Figure 25 and 26), with a more marked expression on the plasma membrane of KYSE-410 (Figure 26). Concerning HIF1-α, since the cells were not exposed to conditions of hypoxia, this marker appears on the cytoplasm and not on the nucleus (Figure 25 and 26).

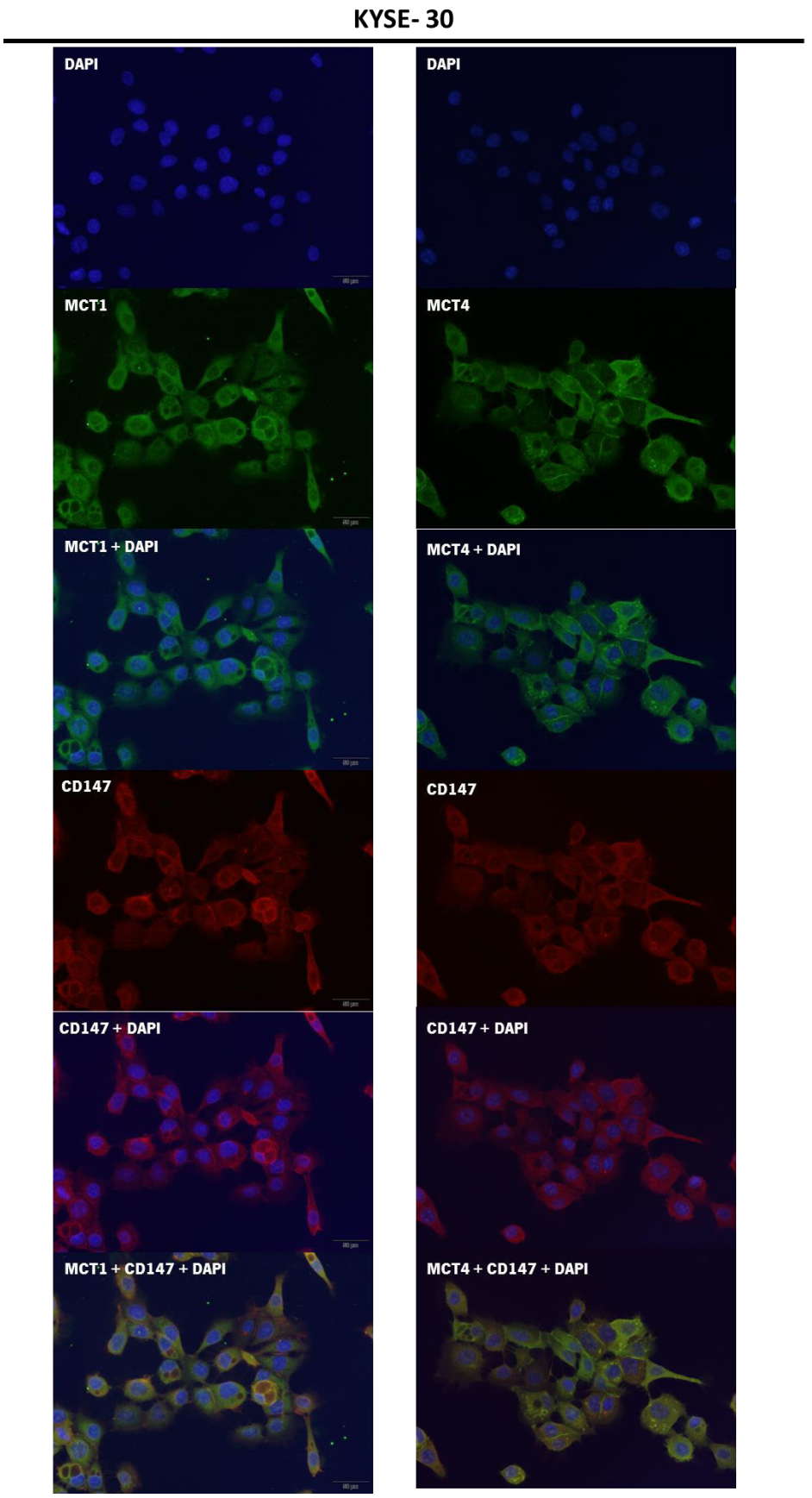

Figure 23 – Expression of MCT1, MCT4 and CD147 in KYSE-30 cell line by immunofluorescence. Plasma membrane staining was observed. MCT1 merged with CD147 and the same occur for MCT4 and CD147.

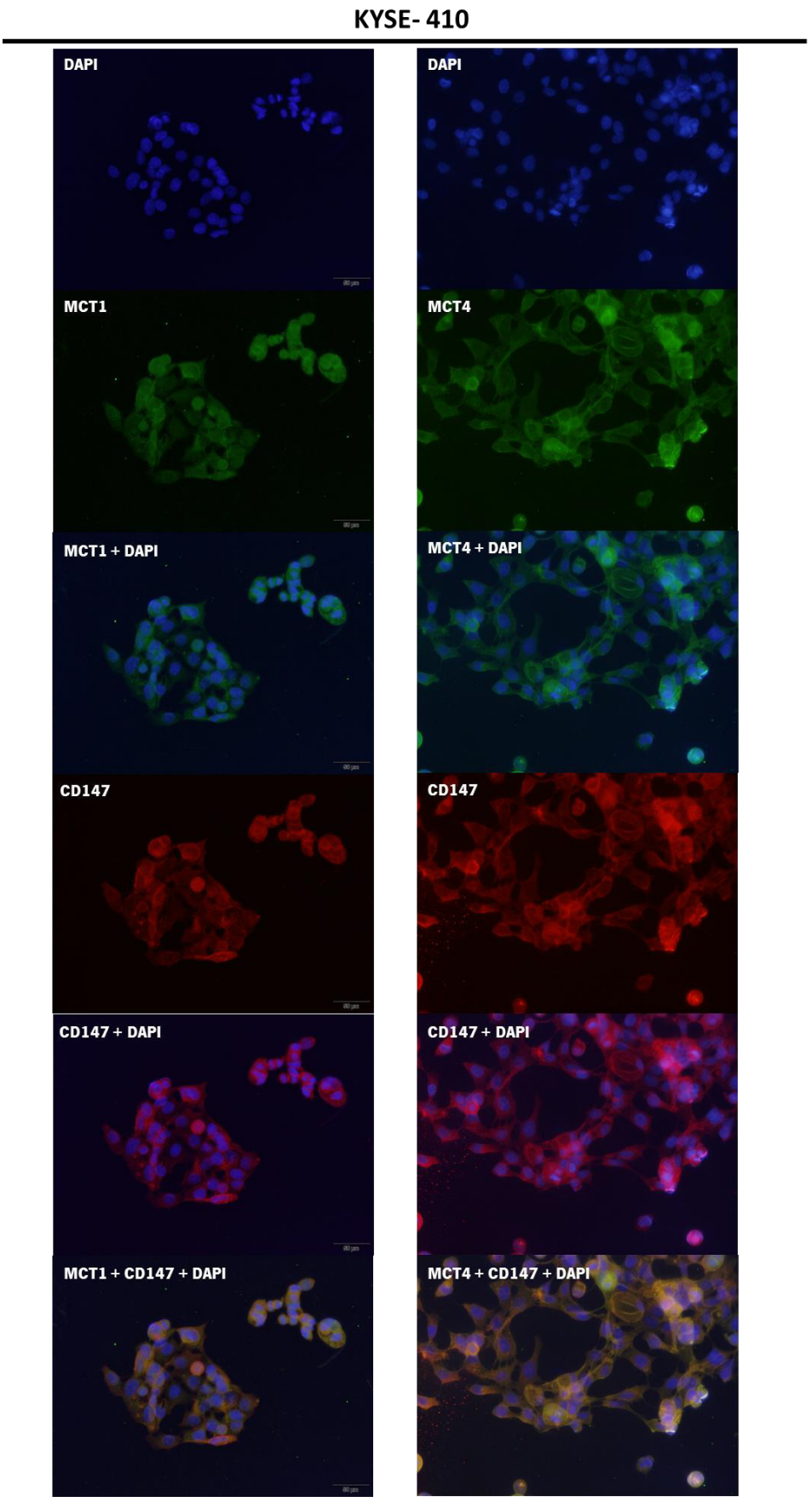

Figure 24 – Expression of MCT1, MCT4 and CD147 in KYSE-410 cell line by immunofluorescence. Plasma membrane staining was observed. MCT1 merged with CD147 and the same occur for MCT4 and CD147.

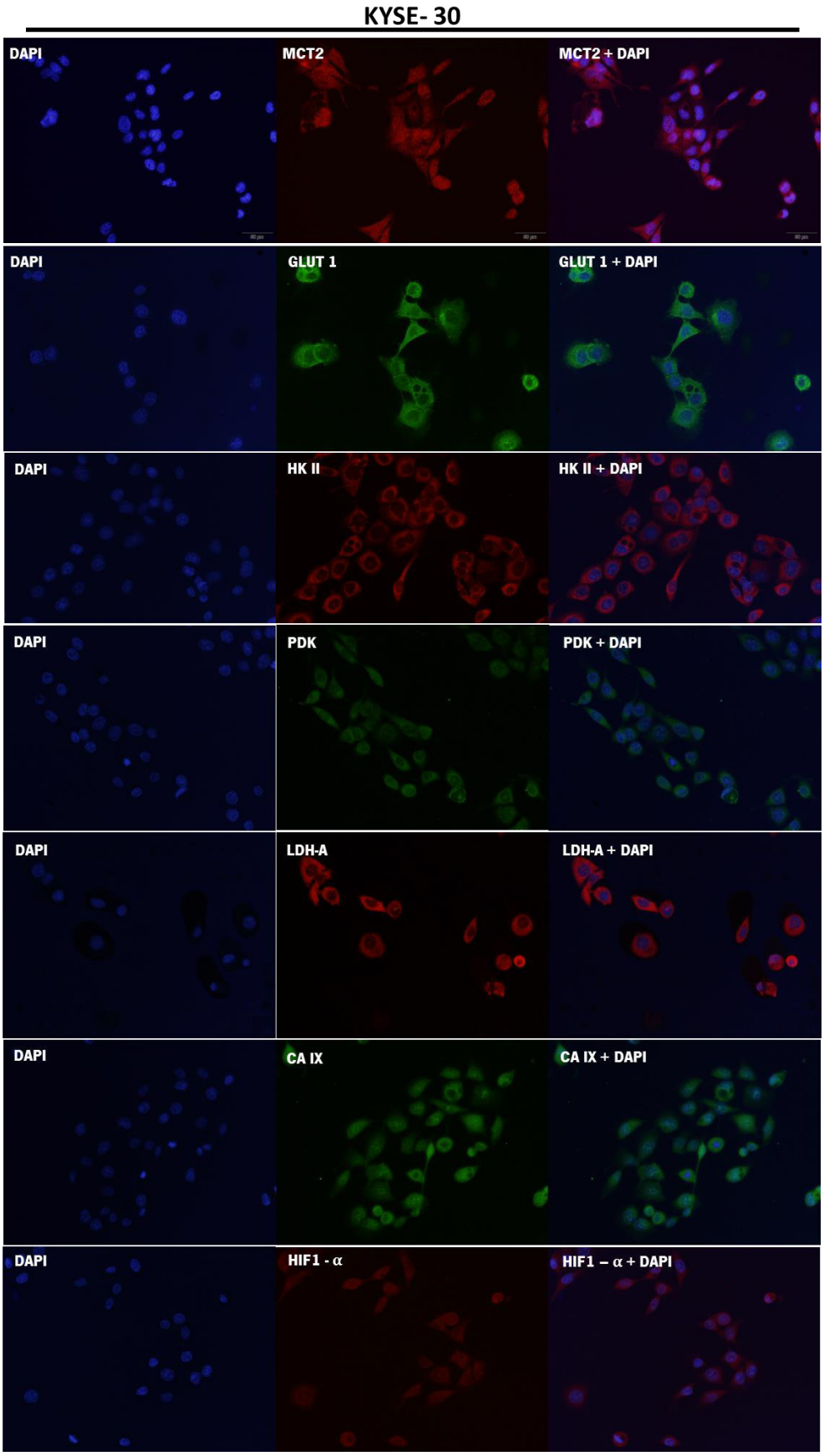

Figure 25 – Immunofluorescence expression of metabolic markers in KYSE-30 cell line.

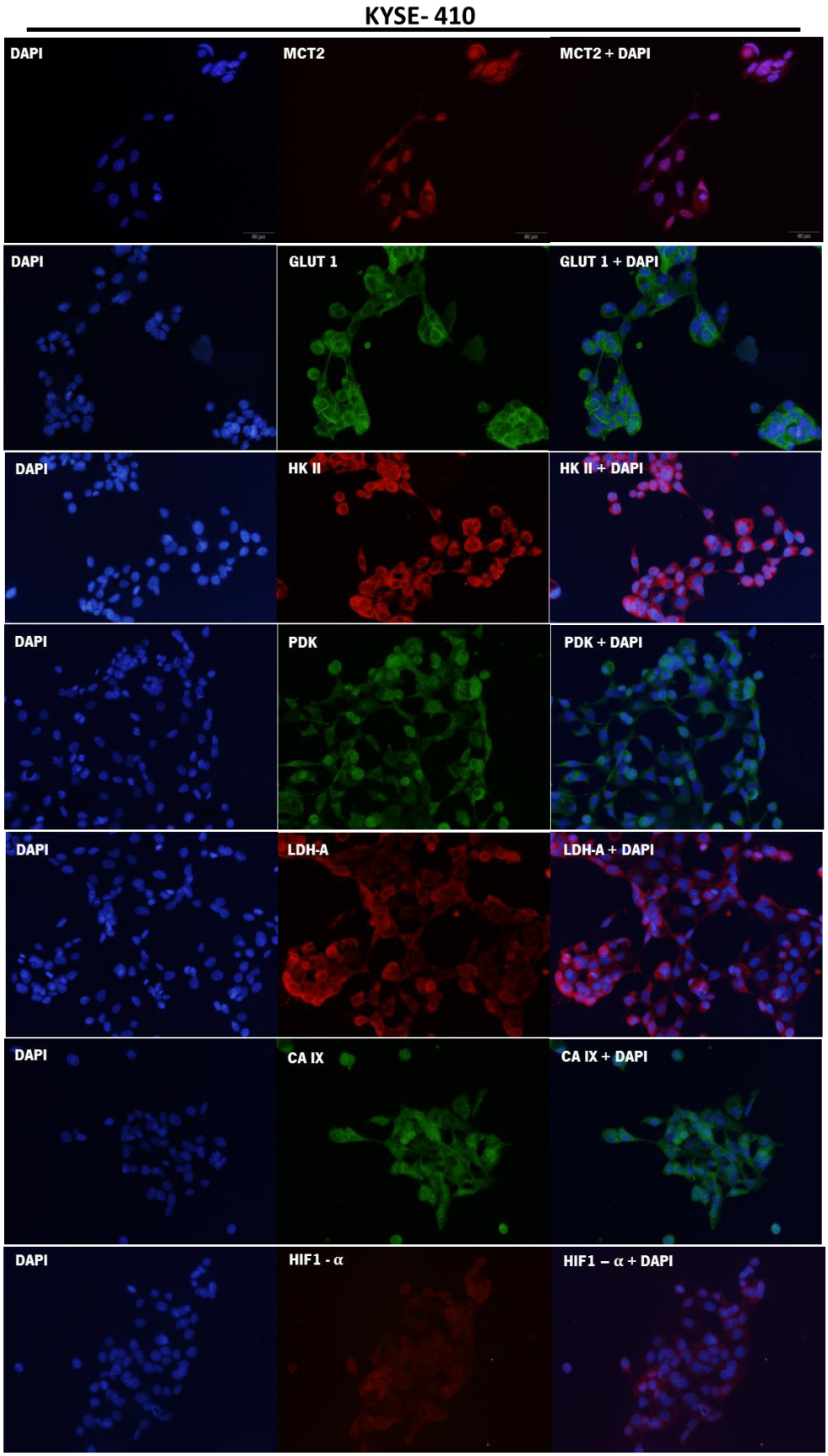

Figure 26 – Immunofluorescence expression of metabolic markers in KYSE-410 cell line.

3.3 Effect of MCT1 down regulation in cellular metabolism behaviour of glycolytic squamous cell carcinoma cells

In order to demonstrate the importance of the role of MCT1 expression in squamous cell carcinomas, as a mediator of lactate efflux in glycolytic tumour cells, down-regulation of MCT1 was performed using a small interference RNA (siRNA) for MCT1. Down-regulation of MCT1 in both cells was confirmed by Western blot (Figure 27).

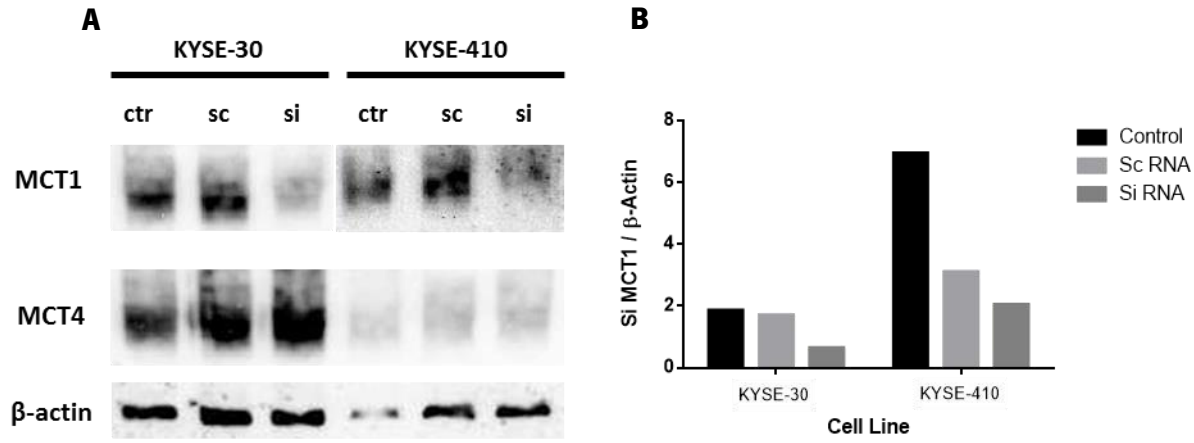

Figure 27 – Down-regulation of MCT1 expression in KYSE-30 and KYSE-410 cells with siRNA. A) Representative blot for siMCT1, MCT4 and the loading control β – Actin. B) Levels of MCT1 protein expression in KYSE-30 and KYSE-410 cells transfected with siMCT1. Ctr: control, Scr: scramble RNA; si: short interference RNA for MCT1.

3.3.1 Effect of MCT1 down regulation on cell viability and cell metabolism of SCC cells

Cellular viability and cellular metabolism were assessed in order to demonstrate the role of MCT1 in performing the lactate efflux in cells and to see if inhibition of this protein had any effect on cell viability. For that, scramble (scr) and siMCT1 were stimulated for 48 hours and the cell biomass, the extracellular amounts of glucose and lactate were quantified. As we can see in Figure 28, down-regulation of MCT1 did not lead to a significant decrease on cell viability. The lactate efflux was not affected, since there was not a decrease when in the siMCT1 is compared with the scramble (Figure 29 and 30). The most probable explanation is there is a mechanism of compensation, since as it was demonstrated in the Western blot, even though MCT1 is down-regulated, MCT4 is expressed in the silenced cells.

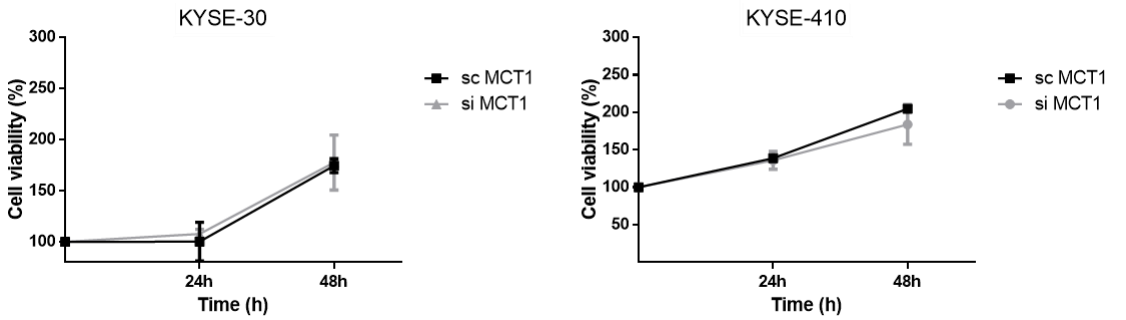

Figure 28 – Effect of MCT1 down-regulation on total cell biomass of KYSE-30 and KYSE-410 cells after 48 hours. Results are the mean ± SEM of at least three independent experiments, each one in triplicate.

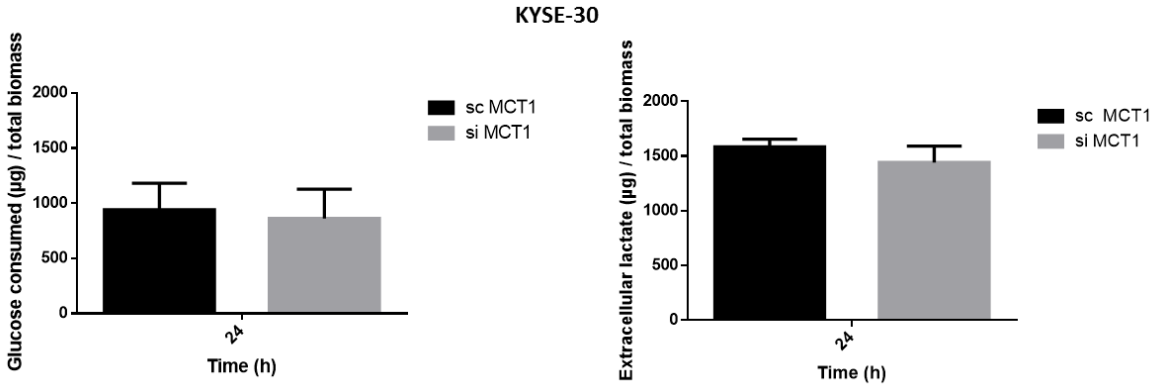

Figure 29 – Cellular metabolism of KYSE-30 siMCT1 cells. Results are representative of three independent experiments with mean ± SEM, each one in triplicate.

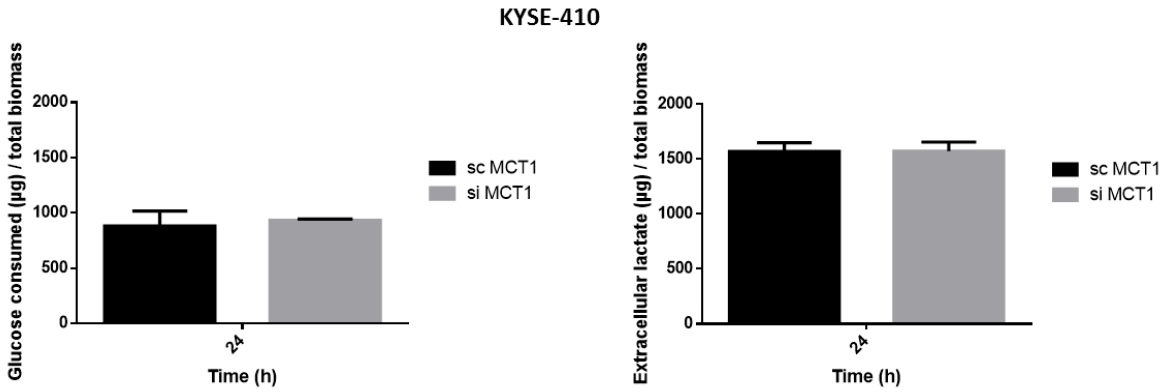

Figure 30 – Cellular metabolism of KYSE-410 siMCT1 cells. Results are representative of three independent experiments with mean ± SEM, each one in triplicate.

3.4 Effect of MCT4 down-regulation in cellular metabolism behaviour of glycolytic squamous cell carcinoma cells

In order to demonstrate the importance of the role of MCT4 expression in squamous cell carcinomas, as a mediator of lactate efflux in glycolytic tumour cells, down-regulation of MCT4 was performed used a small interference RNA (siRNA) for MCT4. Down-regulation of MCT4 in both cells was confirmed by Western Blot (Figure 31).

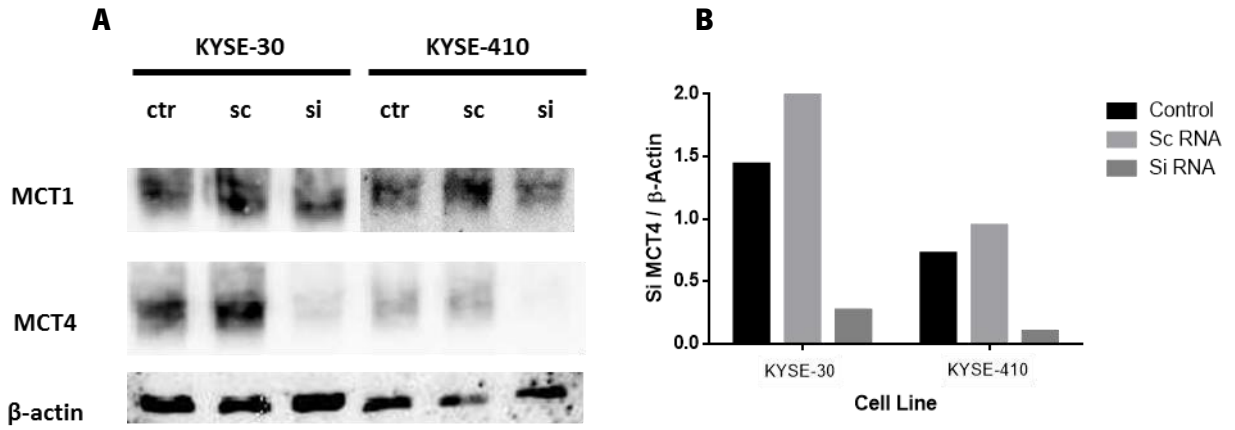

Figure 31 – Down-regulation of MCT4 expression in KYSE-30 and KYSE-410 cells with siRNA. A) Representative blot for MCT1, siMCT4 and the loading control β – Actin. B) Levels of MCT1 protein expression in KYSE-30 and KYSE-410 cells transfected with siMCT4. Ctr: control, Scr: scramble RNA; si: short interference RNA for MCT4.

3.4.1 Effect of MCT4 down-regulation on cell viability and cell metabolism of SCC cells

Cellular viability and cellular metabolism were assessed in order to demonstrate the role of MCT4 in performing the lactate efflux in cells and to see if inhibition of this protein had any effect on cell viability. For that, scramble (scr) and siMCT4 were stimulated for 48 hours and the cell biomass, the extracellular amounts of glucose and lactate were quantified. As we can see in figure 32, down-regulation of MCT4 did not lead to a significant decrease on cell viability. The lactate efflux was not affected, since there was not a decrease when in the siMCT1 is compared with the scramble (Figure 33 and 34). The most probable explanation is there is a mechanism of compensation, since as it was demonstrated in the Western blot, even though MCT4 is down-regulated, MCT1 is expressed in the silenced cells.

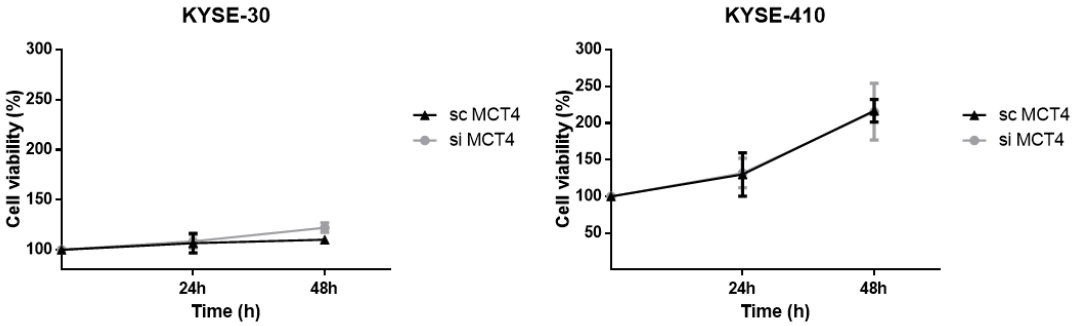

Figure 32 – Effect of MCT4 down-regulation on total cell biomass of KYSE-30 and KYSE-410 cells after 48 hours. Results are the mean ± SEM of at least three independent experiments, each one in triplicate.

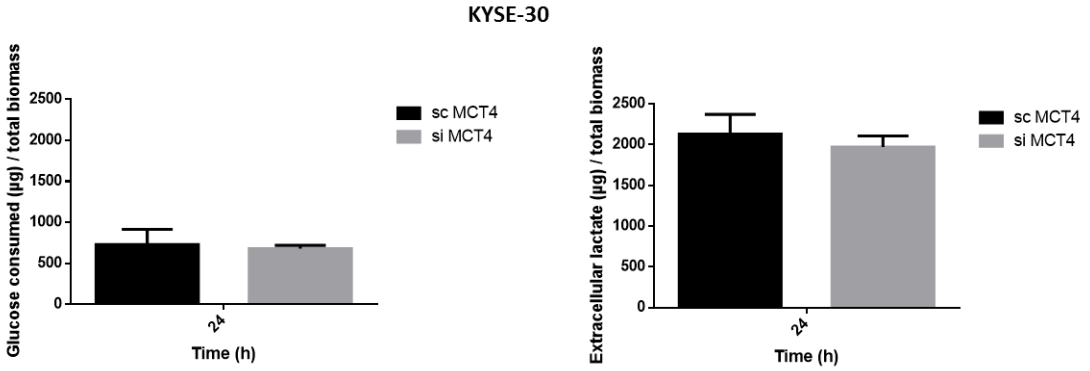

Figure 33 – Cellular metabolism of KYSE-30 siMCT4 cells. Results are representative of three independent experiments with mean ± SEM, each one in triplicate.

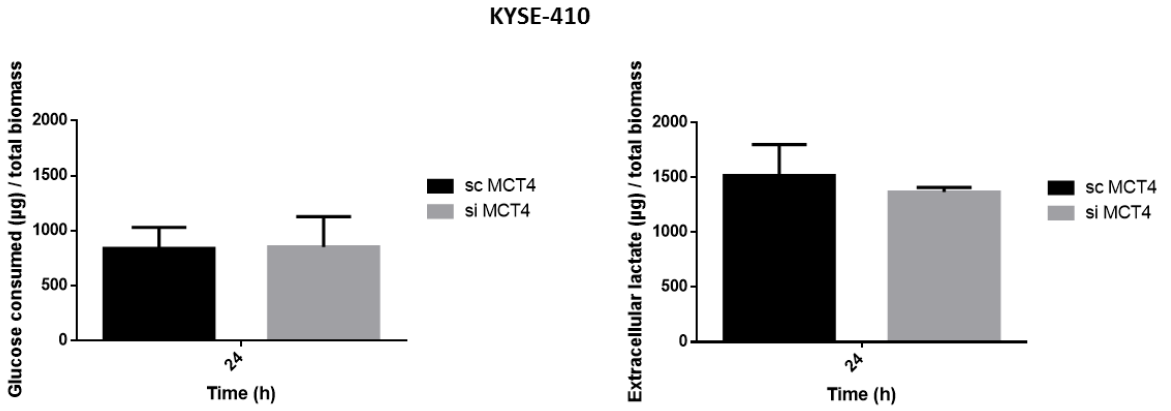

Figure 34 – Cellular metabolism of KYSE-410 siMCT4 cells. Results are representative of three independent experiments with mean ± SEM, each one in triplicate.

3.5 Effect of double MCT down-regulation in cellular metabolism behaviour of glycolytic squamous cell carcinoma cells

Since there was no decrease in cell viability or any decrease in extracellular lactate when the cells were silenced for MCT1 or MCT4, a double MCT down-regulation was performed using a small interference RNA (siRNA) for MCT1 and MCT4. Down-regulation of MCT1 and MCT4 in both cells was confirmed by Western Blot (Figure 35).

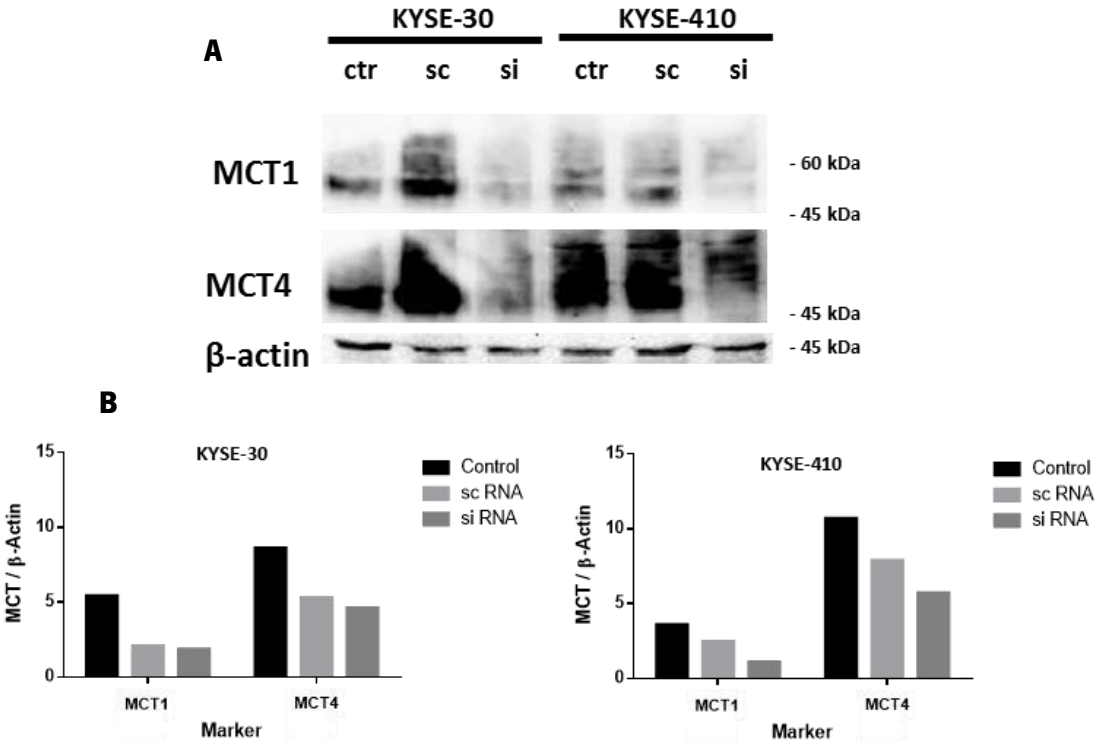

Figure 35 – Down-regulation of MCT1 and MCT4 expression in KYSE-30 and KYSE-410 cells with siRNA. A) Representative blot for siMCT1, siMCT4 and the loading control β – Actin. B) Levels of MCT1 protein expression in KYSE-30 and KYSE-410 cells transfected with siMCT1 and siMCT4. Ctr: control, Scr: scramble RNA; si: short interference RNA for MCT4.

3.5.1 Effect of double down-regulation on cell viability and cell metabolism of SCC cells

Scramble (scr) and siMCT1 + siMCT4 were stimulated for 48 hours and the cell biomass, the extracellular amounts of glucose and lactate were quantified. In Figure 36 it is possible to see that when both MCTs are down-regulated the cell viability decreases. Glucose consumption and lactate efflux were slightly affected on KYSE-30 (Figure 37). It seems that the glucose consumption and lactate efflux of KYSE-410 were not affected by the down-regulation of both MCTs (Figure 38). Thus, MCT1 and MCT4 down-regulation was not enough to impair the glycolytic rates.

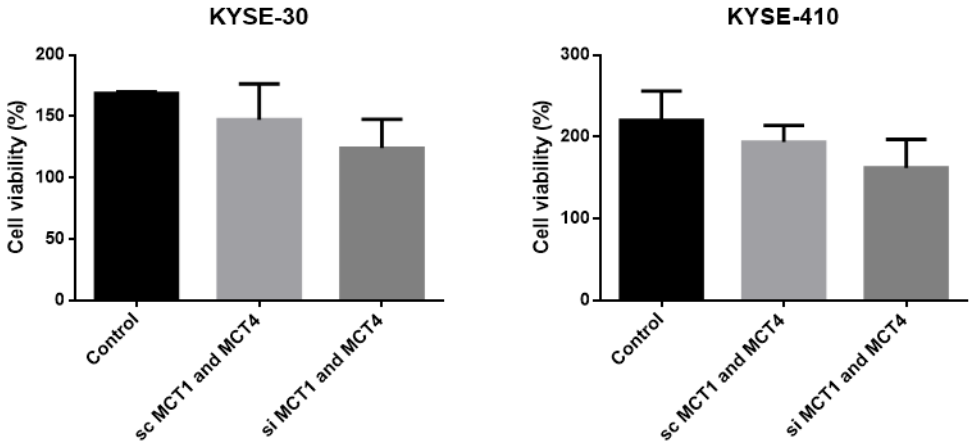

Figure 36 – Effect of MCT1 and MCT4 down-regulation on total cell biomass of KYSE-30 and KYSE-410 cells after 24 hours. Results are the mean ± SEM of at least three independent experiments, each one in triplicate.

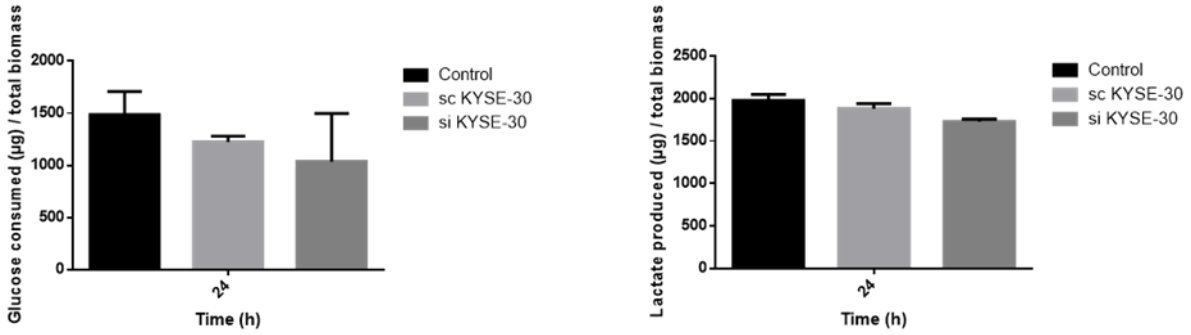

Figure 37 – Cellular metabolism of KYSE-30 siMCT1 + siMCT4 cells. Results are representative of three independent experiments with mean ± SEM, each one in triplicate.

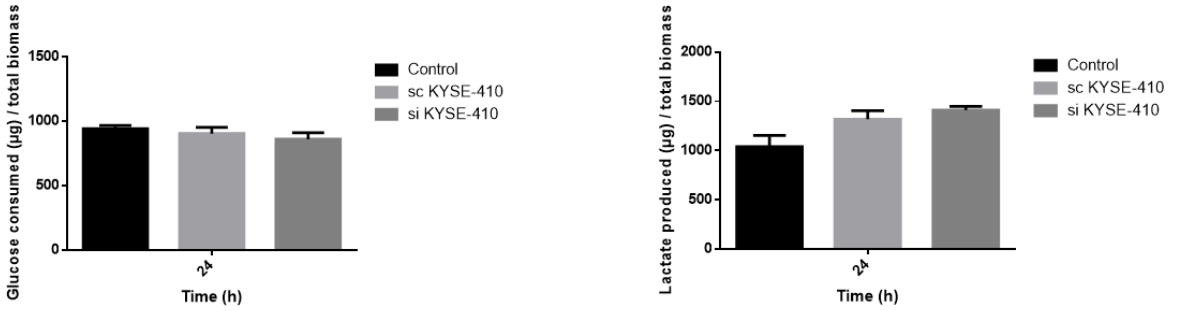

Figure 38 – Cellular metabolism of KYSE-410 siMCT1 + siMCT4 cells. Results are representative of three independent experiments with mean ± SEM, each one in triplicate.

4. DISCUSSION

Oesophageal cancer is the most frequent malignancy worldwide and the sixth leading cause of cancer-related death (117). Among the different tumour histologies of this cancer, oesophageal squamous cell carcinoma and oesophageal adenocarcinoma are the most frequent (96). This neoplasia can be detected by endoscopy followed by biopsy of suspicious areas for histopathologic diagnosis. Early-stage tumours may be suitable for endoscopy resection, while locally advanced cancers are treated with chemotherapy, chemoradiotherapy, surgical resection or combination of these (118,119). Under this regime, the prognosis for patients with this cancer remains poor with 5-year survival rates estimated at 15-25% (94). Thus, exploitation of new molecular targets becomes crucial for this neoplasia.

Reprograming of energy metabolism is an hallmark of cancer cells (4). As stated by Otto Warburg, cancer cells depend on glycolysis for the production of ATP even in aerobic conditions (10). The metabolic phenotype will lead to an accumulation of lactic acid and protons, that will provoke a change in intracellular pH. In order to avoid this problem, monocarboxylate transporters (MCTs) export the accumulated lactate across the plasma membrane, thereby protecting themselves from cellular acidosis (24). By inhibiting the lactate transport, the intracellular pH will increase and this will lead to a reduction in tumour angiogenesis, invasion and metastasis (120,121). Thus, inhibition of lactate efflux in tumour cells will cause internal acidification presenting an attractive therapeutic approach.

The present study demonstrated that tumour localization can be a low survival factor in the adenocarcinoma subtype. When the adenocarcinoma affects the middle part of the oesophagus the patient has a low probability of survival, nevertheless, it is very rare this subtype affecting only the middle part of the oesophagus, since it is more predominant in the distal part of the oesophagus. The oesophagus is an organ that is not supported by other organs in the human body and due to that features, the localization of the neoplasia is important. As it was seen above the main treatments for this cancer include chemoradiotherapy, neoadjuvant chemoradiotherapy and surgery and depending on the characteristics of the patient and of the neoplasia these treatment options could reduce the health-related quality of life (HRQoL). So, if the neoplasia is situated in the middle of the oesophagus and it needs a surgical approach this will lead to another problem, since the tissue of the oesophagus has its own characteristics like, the peristaltic movements that aid in the transport of food into our stomach. If part of this tissue is removed there will be a problem when it concerns connection of the part that was removed, and this could lead to a decline in the HRQoL. Another feature that has been demonstrated to be a poor prognostic factor is the age. Patients that are older than 61, have little chance of survival, because elderly patients have more

difficulty in leading with the disease and the aggressive treatments, since they normally are physically and mentally worn out. For squamous cell carcinoma, only gender was demonstrated to be a low survival prognostic factor, however this results do not correlate with the existing data, since there's a higher prevalence of the disease in males than in females and that may be because of the sex hormones that have an protective effect on women (100). So, this result is being since this series of tissue have very small number of women and this produces this sort of variation on the results. Even though it is not statistically significant, the lesion size seems to be related with a poor outcome in squamous cell carcinoma, this has some truth, since if the lesion has a bigger size, the damage that will provoke will be bigger.

The presence of metabolic remodelling has been described in oesophageal carcinomas (106,107). Several studies have been drawing attention to the role of key metabolic enzymes and their association with aerobic glycolysis in OC, trying to provide a new molecular therapeutic targeting in adenocarcinoma and squamous cell carcinoma of the oesophagus (108–110). The studies focus on the importance of GLUT1 (108,111), LDH-A (112), CA IX (109,114) and HIF1-α (110,115) in the metabolic remodelling of oesophageal cancer. Regarding to the expression of MCTs and the analysed metabolic markers, our results showed that MCT1, MCT4, CD147, HKII, LDH-A, GLUT1 and CA IX were associated with both histological subtypes since their expression was positive. PDK expression was only positive for adenocarcinoma and HIF1-α was positive for squamous cell carcinoma. However, MCT2 was not associated with either subtype of oesophageal cancer. Our results show that only MCT4 and CA IX can be considered has a poor prognostic factors for squamous cell carcinoma. Even though in our results, the other markers did not have any statistical significance the metabolic markers that were analysed were very importantly expressed in the two tumor types, but because the expression was almost ubiquitous, it was not possible to draw a differential meaning between them and the biological behaviour of the tumors. Nevertheless, there is some literature that demonstrates that these markers can be used has indicators of poor prognosis. A study of Huhta *et al*, showed that there is an increase in markers of tumour metabolism that occurs during carcinogenesis and leads to the progression of adenocarcinoma and that MCT1 and MCT4 are prognostic factors for OAC (122). Regarding CD147 expression, a meta-analysis performed by Wang *et al* indicate that high CD147 expression in patients with oesophageal cancer was associated with worse survival outcomes and common clinicopathological indicators of poor prognosis (123). Li and colleagues have demonstrated that the expression of LDH-A may be a prognostic indicator for poor survival in OSCC patients (124). In other solid tumours, the study of GLUT-1, CAIX and HIF-1α in tissues has received much attention to understand how they correlate with malignancy progression.

Concerning GLUT1 expression, its expression was considered a poor survival marker for oesophageal squamous cell carcinoma (125) and oesophageal adenocarcinoma (126), however our study demonstrated that GLUT1 was not significantly express in both carcinomas types. Carbonic anhydrase IX is strongly expressed in oesophageal tissues and it appears to facilitate metastasis leading to a worse prognostic (109,114), and this is in concordance with this study. HIF1-α has an increased expression in oesophageal squamous cell carcinoma and it has an important role in the malignant features of OSCC resulting in a significant poorer radiochemotherapy outcomes and 2-year overall survival (108,110,115); however, in our casuistic, HIF1- α was not significantly express in both carcinomas types.

Characterization of cellular localization for MCTs expression is not always assessed in the literature. As it is known, MCTs are important mediators of intracellular homeostasis, through the intracellular pH regulation and maintenance of glycolytic activity. Thus, their plasma membrane expression in tumour cells is essential for their functions (44). When analysing the immunohistochemistry data, it is possible to verify that the squamous cell carcinoma has a more glycolytic profile than the adenocarcinoma, and this is in accordance with the immunofluorescence and Western blot of the studies *in vitro*. These findings suggest that MCT1 and MCT4 are responsible for the maintenance of glycolytic acidic-resistant phenotype in squamous cell carcinoma, by the efflux of lactate. The adenocarcinoma, even though it is not so glycolytic it still expresses all the proteins that were tested. This study also demonstrated that MCT1 and MCT4 are present in higher amounts in the plasma membrane. So, we verified that MCT1 and MCT4 plasma membrane was associated with CD147 expression and it was observed that several cases of squamous cell carcinoma samples presented plasma membrane expression comparable to MCT1 expression.

Overall, these results show that MCT1 and MCT4 have an important role in the efflux of lactate that is associated to the glycolytic acid-resistance phenotype of the squamous cell carcinoma of the oesophagus.

As stated before, there are various types of inhibitor of MCTs, however there is no specific inhibitor for each MCT isoforms. Thus, specific MCT1 and MCT4 inhibition is important to demonstrate the role of MCT1 and MCT4 in the lactate efflux and their pH regulation and understand its potential effects on cell viability. The results herein obtained have shown that MCT 1 inhibition did not lead neither to a decrease on cell viability nor a decrease in lactate efflux. This could be due to the fact that the expression of MCT4 was untouched even though MCT1 was inhibit. Consequentially, if we assume that MCT4 was expressed, this means that the transport of lactate to out of the cell was still happening, since MCT4 was active. Since the lactate efflux was still occurring, the regulation of the intracellular pH was being maintained and

therefor the cells were still viable. The same phenomenon did occur when MCT4 was inhibited, since the expression of MCT1 was unaffected by the inhibition of the other isoform, and therefore the mechanism of compensation was still occurring leading to the continuous lactate efflux and pH regulation. As the inhibition of a single MCT isoform did not lead to a decrease in lactate efflux and cell viability, the next step was to attempt a double inhibition, i.e., both MCT1 and MCT4 were inhibited. When this inhibition occurred, it was possible to see a dismal decrease in the cell viability, although it was not statistically significant for both cell lines of squamous cell carcinoma tested. Regarding the lactate efflux, there was a small decrease for KYSE-30, but was not significant. Even though both MCT forms were inhibited there was still a continuous lactate efflux. This phenomenon may be due to the fact that lactic acid can also diffuse across the membrane as a weak acid, and it does not matter if the lactate transporters are inhibited (30). A model for lactate transport proposed by Michels and co-workers (127), suggests that a lactate transport system with variable stoichiometry can efficiently transform free energy existing in the form of a lactate chemical-potential gradient into a proton-motive force. This model is based upon the general model of carrier mediated proton-substrate cotransport proposed by Rottenberg (128). Lactate is envisioned as having an acidic proton that dissociates with a pH, in the physiological range. At low external pH, the lactate is uncharged, and the mode of transport is represented by the stoichiometry H^+/ L^-. In other words, the transport of a single lactate molecule is tightly coupled to the transport of a single proton. At higher external pH, the lactate proton dissociates, and the transport stoichiometry becomes $(H^+)_2L^-$. Basically, when the MCTs are silenced, the intracellular pH will decrease leading to a the cellular acidosis, however when this happens lactic acid transforms into a permeant acid, this condition could promote a slow but continuous extrusion of lactic acid from the cellular space to allow the maintenance of glycolysis (30,129).

In conclusion, our study demonstrated that decreased expression of MCT1 and MCT4 is responsible for decreased cell viability. However, further studies will be needed to observe what this dual inhibition may cause in proliferation and migration, only in this way will it be possible to know if MCTs could be a possible therapeutic target for use in this type of cancer.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Carcinogenesis is a multistep process marked by the reprograming of cellular energy metabolism of cancer cells (4). MCTs are key players in high glycolytic metabolism and acid resistant phenotype allowing the transport of formed lactate couple to a proton (42). This work reveals some poor survival factors for each histological subtype of oesophageal cancer and that MCT4 is a prognostic factor for squamous cell carcinoma. After analysing the metabolic characterization, it is possible to affirm that the squamous cell carcinoma presents a more glycolytic phenotype than the adenocarcinoma, which is supported by the *in vitro* studies. Regarding MCT inhibition, only in the inhibition of both MCT isoform was a slight decrease in cell viability.

Despite the findings of the present work, many questions remain to be answered, thus, additional experiments need to be carried out in the near future to complement this work.

To complement the results obtained in oesophageal carcinoma tissues, it would be important to investigate the expression of MCTs in hypoxic regions and characterize *in vitro* its metabolic behaviour in hypoxia.

Additional *in vitro* studies for oesophageal adenocarcinoma cell lines, in order to see if this histological type has the same metabolic behaviour as the oesophageal squamous cell carcinoma cell lines. MCT1 activity inhibition, with specific MCT1 inhibitors (developed by AstraZeneca), will be important to confirm the results obtained in this work. It would be interesting performing functional assays like cell migration, cell proliferation and apoptosis, to see how inhibition of MCTs would affect these aspects of tumoral cells.

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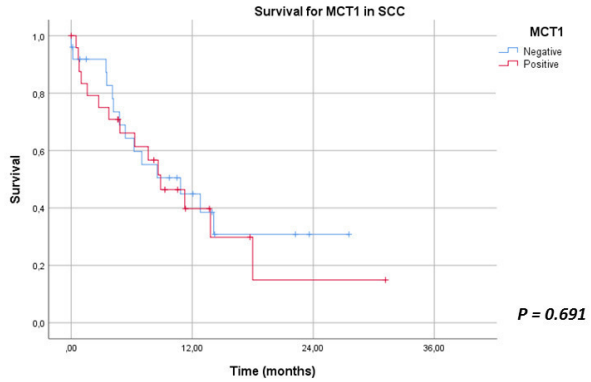
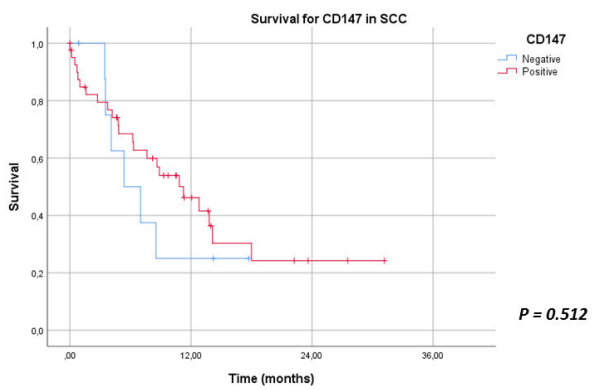
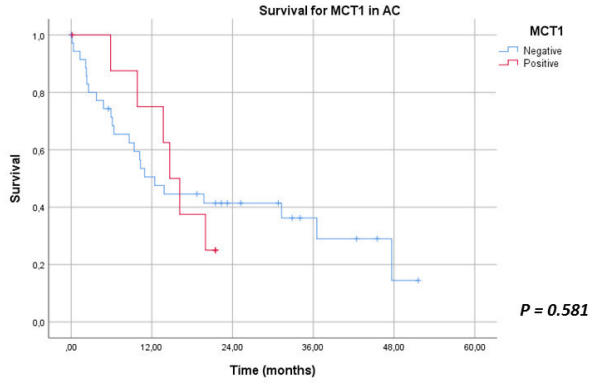
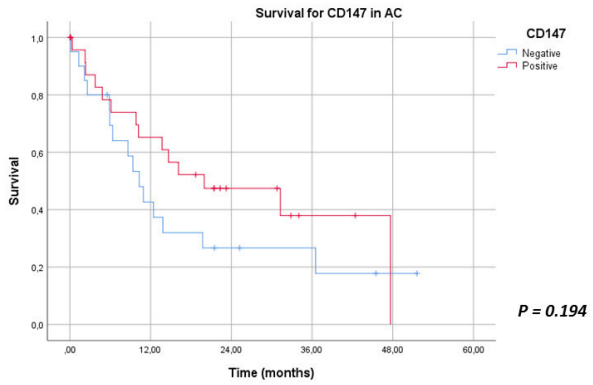
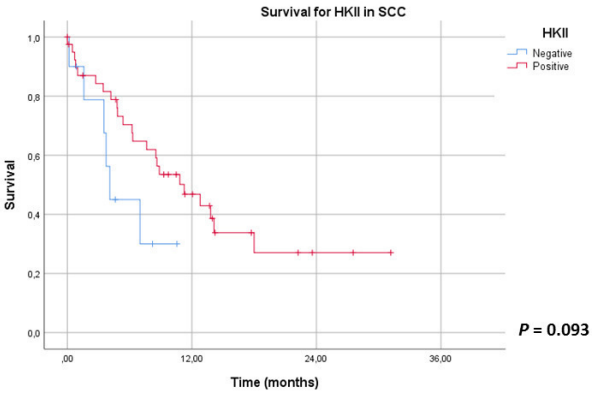
APPENDIX I

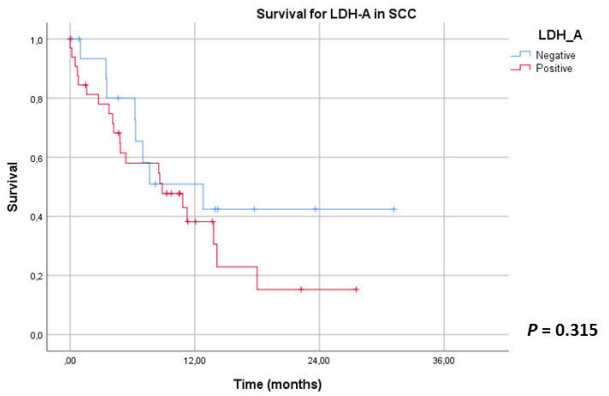


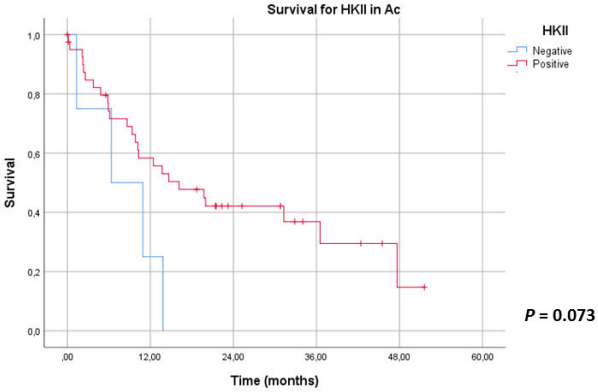


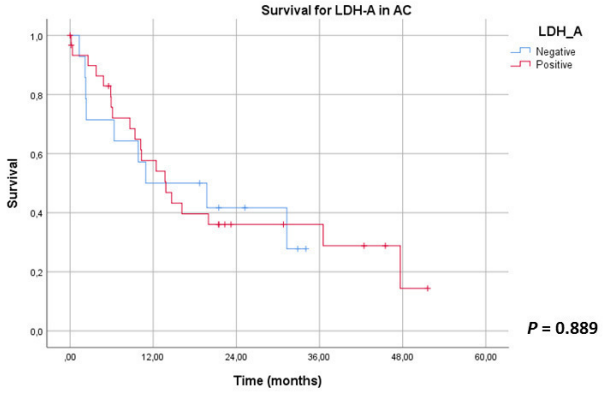




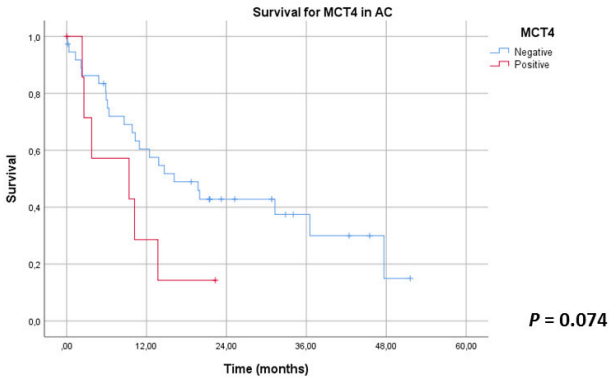








Kaplan-Meier Curves of survival for MCT1, CD147, HK II and LDH-A in OSCC and OAC patients.



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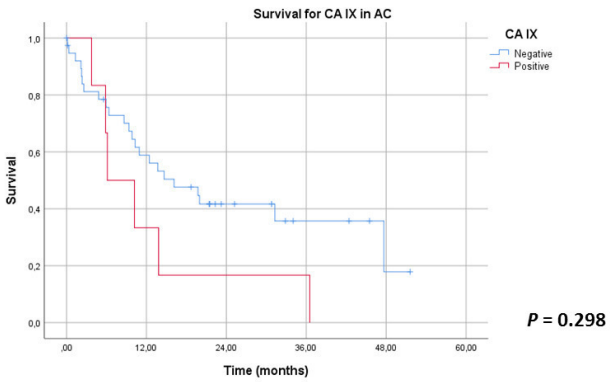

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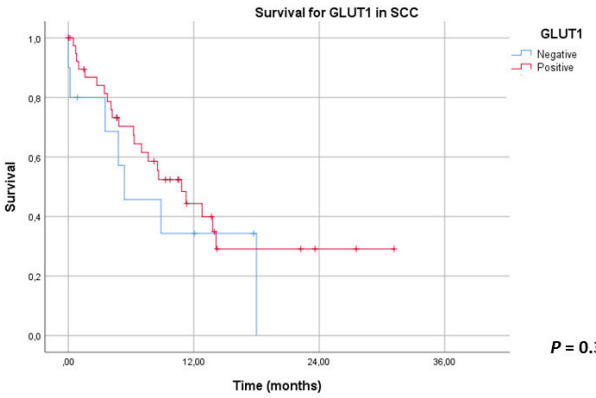

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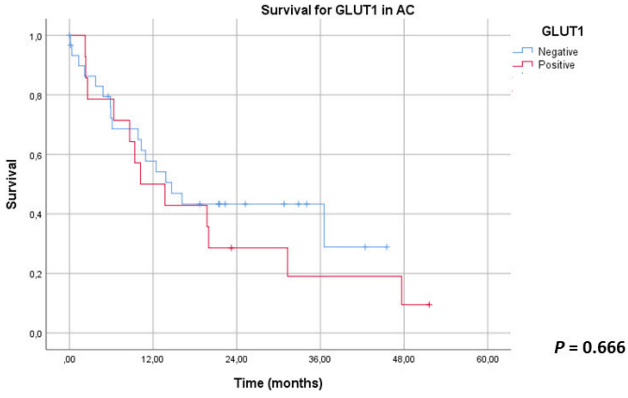

***P* = 0.666**

Kaplan-Meier Curves of survival for MCT4, CA IX, GLUT 1 in OSCC and OAC patients.