Universidade do Minho
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O papel dos transportadores de monocarboxilatos em tumores sólidos

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Tese de Doutoramento
Ciências da Saúde – Ciências da Saúde

Trabalho efectuado sob a orientação da
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ABSTRACT / RESUMO

ABSTRACT

Most solid tumours rely on glycolysis for energy production, even in conditions of high oxygen tension (‘‘aerobic glycolysis’’, also known as the Warburg effect), giving rise to enhanced lactate production. This lactate production is responsible for extracellular acidification, which, conditioning the tumour environment, favours tumour invasion and suppresses anticancer immune response. Transport of lactate across the plasma membrane is mediated by a family of proton-coupled monocarboxylate transporters (MCTs), which comprises 14 members. The isoforms MCT1-MCT4 are proton symporters that exhibit different affinities for lactate, leading to different levels of tissue expression. Since some MCT isoforms (especially MCT1 and MCT4) play a role in the intracellular pH homeostasis, by exporting the accumulating lactic acid, they are up-regulated in glycolytic tumours, where high levels of lactate are produced, such as high grade gliomas, colorectal carcinomas and lung cancer. However, the role of MCTs in tumours is far from being well understood and their potential as therapeutic targets is poorly explored, being the main aim of this work to further elucidate the significance of MCT expression in solid tumours.

Thus, MCTs expression and their clinic-pathological value were evaluated in human series of colorectal, cervical, gastric and breast carcinomas. Also, MCT regulation by chaperones was further investigated by analysing CD147 in cervical, gastric and breast carcinomas series, as well as performing a screening in colorectal, breast, lung and ovary tumour samples, where CD44 expression was evaluated, as a putative MCT chaperone, in addition to CD147. Finally, some *in vitro* studies were performed, to determine the contribution of MCTs to cancer cell metabolic profile and viability.

Importantly, up-regulation of MCTs, in particular MCT1, was found in colorectal, cervical and breast carcinomas, but not in gastric carcinomas, which, in fact, showed a decrease of MCT4 along towards malignancy. Also, MCT1 was associated with poor prognosis, especially in breast carcinomas, as well as in gastric carcinoma where MCT1/CD147 co-expression was associated with poorer patient prognostic. Also, as anticipated, CD147 was found to be co-expressed with both MCT1 and MCT4 in the large series studied (cervical, gastric and breast carcinomas). In the tumour screening, CD44 was only associated with MCT1 in lung cancer, however, series were very small and some results warrant further attention. Finally, the *in vitro* studies showed a CHC-induced inhibition of cell proliferation in human breast cancer cell lines that was, in some cases, accompanied by metabolic alterations.

In conclusion, the results presented in this thesis have an important impact on the comprehension of MCT contribution to malignant phenotype and pave the way for further studies aiming to the development of cancer therapies directed to MCTs.

RESUMO

A maioria dos tumores sólidos depende da glicólise para produção de energia, mesmo em condições de alta pressão parcial de oxigénio ("glicólise aeróbia", também conhecida como o efeito de Warburg), dando origem a uma maior produção de lactato. Esta produção de lactato é responsável pela acidificação extracelular, o que condiciona o ambiente do tumor, favorecendo a invasão tumoral e a supressão da resposta imune contra o tumor. O transporte de lactato através da membrana plasmática é mediado pelos transportadores de monocarboxilatos (MCTs), que pertencem a uma família composta actualmente por 14 membros. O transporte mediado pelas isoformas MCT1-MCT4 é um simporte com protões e cada isoforma possui afinidade distinta para o lactato, apresentando uma distribuição diferente nos vários tecidos. Uma vez que algumas isoformas (especialmente o MCT1 e o MCT4) desempenham um papel na homeostasia intracelular, ao exportar o ácido láctico acumulado, está descrito um aumento destas isoformas em alguns tumores glicolíticos, onde são produzidos níveis elevados de lactato, como seja em gliomas de alto grau, carcinomas colorectais e cancro do pulmão. No entanto, o papel dos MCTs em tumores está longe de ser completamento elucidado e o seu potencial como alvo terapêutico ainda se encontra pouco explorado, sendo o objectivo principal deste trabalho caracterizar o papel dos MCTs em tumores sólidos.

Assim, a expressão e valor clínico-patológico dos MCTs foram avaliados em séries humanas de carcinoma colorectal, do colo do útero, do estômago e da mama. Para além disso, a regulação dos MCTs por proteínas chaperonas foi investigada, analisando a expressão da CD147 nas séries de carcinoma do colo do útero, do estômago e da mama, assim como a realização de um estudo-piloto onde a expressão da CD44, outra possível chaperone dos MCTs, foi avaliada em tumores colorectais, da mama, do pulmão e do ovário, para além da CD147. Finalmente, alguns estudos *in vitro* foram realizados de modo a elucidar a contribuição dos MCTs para o perfil metabólico e viabilidade das células tumorais.

De notar que um aumento na expressão dos MCTs, em particular do MCT1, foi encontrado nos carcinomas colorectal, do colo do útero e de mama, mas não nos carcinomas gástricos, que, na realidade, mostraram uma diminuição do MCT4 com o aumento da malignidade. Além disso, a expressão do MCT1 foi associada a um pior prognóstico, em especial nos carcinomas da mama, assim como no carcinoma gástrico, onde a co-expressão do MCT1 com a CD147 foi associada a um pior prognóstico do paciente. Como antecipado, a CD147 estava co-expressa quer com o MCT1 quer com o MCT4, quando avaliado nas séries de maior dimensão (carcinomas do colo uterino, gástrico e da mama). No estudo-piloto, a CD44 estava associada apenas com o MCT1 no

cancro do pulmão, no entanto, o tamanho das séries era muito reduzido e alguns dos resultados obtidos merecem ser explorados no futuro. Finalmente, os estudos *in vitro* mostraram que o tratamento com CHC induz uma inibição da proliferação celular em linhas celulares de tumor da mama, a qual foi, em alguns casos, acompanhada por alterações metabólicas.

Em conclusão, os resultados apresentados nesta tese têm um grande impacto na compreensão da contribuição dos MCTs para o fenótipo maligno e abrem caminho para estudos posteriores que visem ao desenvolvimento de terapias anti-tumorais direccionadas aos MCTs.

CONTENTS

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ABBREVIATIONS LIST

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AE1	*anion exchanger 1*
AML-1a	*acute myeloid leukemia-1a*
ANF	*albumin negative factor*
AP	*activated protein*
CA	*carbonic anhydrase*
CHC	*α-cyano-4-hydroxycinnamate*
COX	*cyclooxygenase*
CREB	*cAMP-response element-binding protein*
DIDS	*4,4'-diisothiocyanostilbene-2,2'-disulphonate*
FdG-PET	*^{18}F-fluorodeoxyglucose positron emission tomography*
GBM	*glioblastoma multiforme*
GLUT1	*glucose transporter 1*
HIF-1α	*hypoxia-inducible transcription factor-1alpha*
HMGCoA	*3-hydroxyl-3-methylglutaryl coenzyme A*
HPV	*human papillomavirus*
IGF-IR	*insulin-like growth factor receptor type I*
INF-γ	*interferon-gamma*
LDH-A	*lactate dehydrogenase A*
MCT	*monocarboxylate transporter*
MZF1	*myeloid zinc finger 1*
NF-E2	*nuclear factor erythroid 2*
NF-κB	*nuclear factor-kappaB*
NHE1	*Na^+/H^+ exchanger 1*
NMP1	*nuclear matrix protein-1*
Nrf2	*NF-E2 related factor 2*
NSAIDs	*non-steroidal anti-inflammatory drugs*
pCMBS	p-*chloromercuribenzene sulphonate*
PDH	*pruvate dehydrogenase*
PDK1	*pyruvate dehydrogenase kinase 1*
PGC-1α	*peroxisome proliferator-activated receptor gamma, co-activator 1 alpha*
PKC	*protein kinase C*
PPARα	*peroxisome proliferator-activated receptor alpha*

PPRE	*peroxisome proliferator-activated receptor response elements*
PrP	*prion-related protein*
REMP	*retinal epithelial membrane protein*
siRNA	*small-interfering RNA*
SP1	*simian-virus-40-protein-1*
Sp1	*stimulating protein-1*
T_3	*triiodothryonine*
TCA	*tricarboxylic acid*
TMD	*transmembrane domain*
TNF-α	*tumour necrosis factor-alpha*
TSH	*thyroid-stimulating hormone*
USF	*upstream stimulatory factor*
VEGF	*vascular endothelial growth factor*

AIMS AND THESIS LAYOUT

AIMS AND THESIS LAYOUT

Accumulation of lactate in the tumour microenvironment is a widely described event and this lactate has been associated with poor prognosis in cancer patients. Also, lactate has been recently described as the key metabolic intermediate in the metabolic symbiosis between glycolytic cancer cells and oxidative cancer cells. Monocarboxylate transporters (MCTs), as facilitators of lactate efflux from cells, mainly the isoforms 1 and 4, are key proteins in cancer cell metabolism and survival. Actually, these membrane proteins have been pointed out several times as promising cancer therapeutic targets. However, studies on MCT contribution to the malignant phenotype are still scarce and more basic scientific efforts on this matter are needed, which will probably result in important findings, with clinical implications.

Therefore, the main aim of this thesis was to further characterise MCT expression in different human solid tumours, as well as to give a contribution to understand the role of MCT in cancer cell hyper-glycolytic phenotype, providing evidence for the exploitation of MCTs as potential targets for cancer therapy.

In **Chapter 1**, a general introduction to the thesis subject is provided, with special emphasis on MCT family members, their role in homeostasis and disease, as well as their regulation and inhibition. The current knowledge on MCT contribution to tumour microenvironment, lactate production and its contribution to the malignant phenotype and the potential of MCTs as targets in cancer therapy are also reviewed.

In **Chapters 2-5**, MCT characterisation in human solid tumours, including results already published, is presented, giving a contribution to the knowledge on MCT behaviour in cancer metabolic adaptations. Characterisation of MCT expression in colorectal carcinoma is presented in **Chapter 2**. In **Chapter 3**, MCT expression, as well as expression of MCT1 and 4 chaperone, CD147, in cervical carcinoma are shown. Characterisation of MCT and CD147 expression in gastric carcinomas is included in **Chapter 4**. Finally, **Chapter 5** provides the results obtained in breast cancer, both in human samples and in *in vitro* models. Besides the published results on MCT and CD147 characterisation in breast carcinoma, **Chapter 5** also includes results, already submitted for publication, where the expression of MCTs was associated with proteins implicated in the metabolic adaptation to hypoxia, i.e. CAIX and GLUT1. Also, unpublished results of *in vitro* MCT inhibition in human breast cancer cell lines, to evaluate the contribution of MCTs for cancer cell metabolic profile and survival, are presented in **Chapter 5**.

Moreover, as MCT regulatory mechanisms are still poorly elucidated, **Chapter 6** presents the published results of a screening study (in a commercial tissue microarray containing breast carcinomas, colon adenocarcinomas, non-small cell lung cancer and ovarian adenocarcinomas), where the recently described MCT regulation by CD44 is explored, in addition to CD147.

Finally, in **Chapter 7**, the main conclusions of **Chapters 2-6** are summarised and further discussed, and some important future directions are suggested.

Chapter 1. GENERAL INTRODUCTION

1.1. MONOCARBOXYLATE TRANSPORTERS

Monocarboxylic acids play a major role in cellular metabolism, with lactate, the end product of glycolysis, being especially important. For some highly glycolytic tissues such as red blood cells, white muscle and tumour cells, which rely on glycolysis for energy supply, lactate is produced in high rates and so must be rapidly expelled out of the cell, to maintain high rates of glycolysis. On the other hand, in brain or cardiac and red muscles, where lactate is oxidised as a respiratory fuel, or kidney and liver were lactate is the major gluconeogenic substrate, lactate must be rapidly transported into the cell [1]. Transportation of monocarboxylates through the plasma membrane was originally thought to be via non-ionic diffusion of the free acid, however, following demonstration that lactate and pyruvate transport into human erythrocytes could be strongly inhibited after treatment with chemicals [2], a specific monocarboxylate transport mechanism was recognised. After extensive characterisation of monocarboxylate transport in different cell types like erythrocytes, cardiac myocytes and hepatocytes, among others, the different characteristic observed led to the hypothesis of the existence of a family of monocarboxylate transporters (for review see [3]).

1.1.1. THE MCT FAMILY

The monocarboxylate transporter (MCT) family is presently composed by 14 members (Table 1), and belongs to the major facilitator superfamily [4]. MCTs are encoded by the *SLC16* gene family [5], which is conserved among species, including rat, mouse, chicken and others. Hydropathy plots predict 10-12 α-helical transmembrane domains (TMD) for MCT members, with both N- and C-termini located within the cytoplasm [5], as illustrated in Figure 1 for MCT1. Like members of other families, MCTs exhibit the highest sequence conservation in TMDs and in the shorter loops between TMDs. In contrast, the hydrophilic regions (N- and C-termini as well as the loop between TMDs 6 and 7) show little conservation, indicating that these regions are unlikely to be directly involved in transport, being probably critical in other functional aspects such as substrate specificity or regulation of activity [6]. Theoretical predictions [6] and experimental evidence [7,8] indicate that none of the MCT family members is glycosylated. To function, an MCT translocates a proton and a monocarboxylate through the plasma membrane by an ordered mechanism in which H^+ binding is followed by monocarboxylate binding to the protonated

transporter [9]. Therefore, MCT activity is dependent on both substrate concentration and proton gradient.

Table 1. The human SLC16 family of transporters (from [10]).

MCT	UniGene name	Alternate (*former) Name	Sequence accession ID	Human gene locus	Tissue distribution	Transport mechanism
MCT1	SLC16A1		NM_003051	1p13.2	Ubiquitous	H^+ cotransporter exchanger
MCT2	SLC16A7		NM_004731	12q14.1	Testis, liver, kidney, skeletal muscle, heart, brain, spleen, pancreas	H^+ cotransporter
MCT3	SLC16A8	REMP	NM_013356	22q13.1	Retinal pigment epithelium (RPE), choroids plexus, aorta, placenta, kidney	H^+ cotransporter
MCT4	SLC16A3	(*MCT3)	NM_004207	17q25.3	White muscle, white blood cells, tumors, RPE, brain kidney, placenta, small intestine, lung, heart	H^+ cotransporter
MCT5	SLC16A4	(*MCT4)	NM_004696	1p13.3	Placenta, intestine, colon	Orphan
MCT6	SLC16A5	(*MCT5)	NM_004695	17q25.1	Kidney, muscle, placenta, intestine, brain, heart, pancreas, prostate, lung	Facilitated diffusion
MCT7	SLC16A6	(*MCT6)	NM_004694	17q24.2	Pancreas, brain, muscle	Orphan
MCT8	SLC16A2	XPCT (*MCT7)	NM_006517	Xq13.2	Liver, brain, kidney, heart, placenta	Orphan
MCT9	SLC16A9		BN000144	10q21.2	Endometrium, testis, ovary, breast, brain, kidney, adrenal, retina	Orphan
MCT10	SLC16A10	TAT1	NM_018593	6q21-q22	Intestine, kidney, skeletal muscle, heart, liver, placenta	Facilitated diffusion/ exchanger
MCT11	SLC16A11		NM_153357	17p13.2	Skin, lung, ovary, breast, pancreas, RPE, choroid plexus	Orphan
MCT12	SLC16A12		ENSG000 00152779	10q23.3	Kidney	Orphan
MCT13	SLC16A13		BN000145	17p13.1	Breast, bone marrow	Orphan
MCT14	SLC16A14		BN000146	2q36.3	Brain, heart, ovary, breast, lung, pancreas, RPE, choroid plexus	Orphan

Although lactate is the monocarboxylate whose transport across the plasma membrane is quantitatively more important, MCTs are also important for the transport of many other metabolically important monocarboxylates such as pyruvate, the branched-chain oxoacids derived from leucine, valine and isoleucine, and the ketone bodies acetoacetate, *β*-hydroxybutyrate and acetate [1]. Consequently, MCTs have a central role in mammalian cell metabolism and are critical for the communication between cells [3], as illustrated in Figure 2.

Besides being a family of 14 members, only the first four (MCT1-MCT4) have been demonstrated experimentally to facilitate the proton-linked transport of metabolically important monocarboxylates [8,11-13]. **MCT3**, formerly known as REMP (from retinal epithelial membrane

protein), was firstly described the chicken retinal pigment epithelium [14] and then functionally characterised and renamed MCT3 [8]. Subsequent studies showed the very particular distribution of MCT3, limited to the retinal pigment and choroid plexus epithelia [15-18], indicating that these isoform seems to be a very specialised MCT. Therefore, this introduction will only focus on **MCT1**, **MCT2** and **MCT4** isoforms, whose function is responsible for the name of the family of transporters.

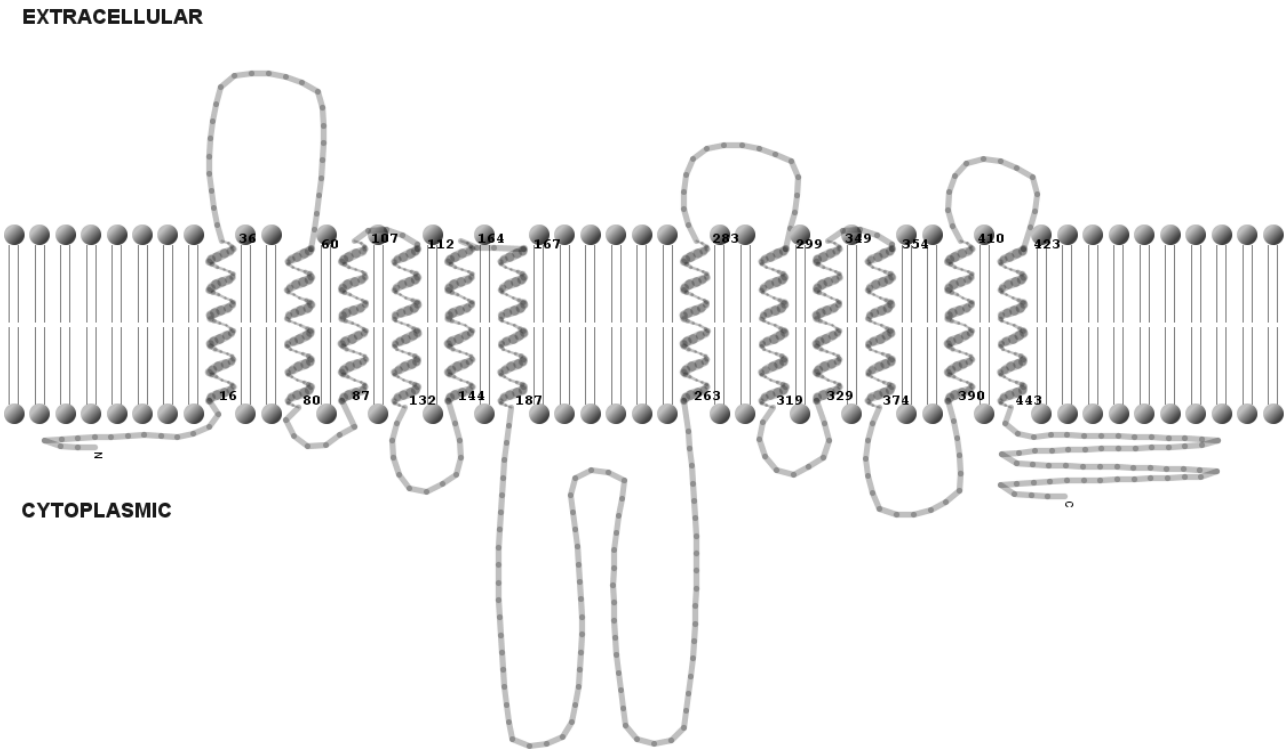

Figure 1. MCT1 protein diagram (from [19], see Appendix I).

1.1.1.1. MCT1

Most of the early functional studies on plasma membrane lactate transport were performed using red blood cells, since a homogeneous population of these cells could be readily obtained. However, MCTs were biochemically studied for many years before their molecular characterisation was performed [2,20-23]. The discovery and cloning of a mutant allele encoding a protein with preferential transport of the cholesterol precursor mevalonate (Mev) in Chinese hamster ovary cells resulted in new information about the molecular features of MCTs. When characterising the Mev mutation [24], it was found that the wild-type protein preferentially transported pyruvate [25]. This novel protein was designated monocarboxylate transporter 1

(MCT1) and kinetically resembles the biochemically characterised erythrocyte MCT.

Human MCT1 gene, *SLC16A1* (for review see [19], Appendix I), firstly cloned in 1994, by Garcia and colleagues [26], is located chromosome 1 (1p13.2-p12). Structural gene organisation as well as isolation and characterisation of *SLC16A1* promoter was achieved in 2002, by Cuff and Shirazi-Beechey [27]. *SLC16A1* comprises 5 coding exons [27] and, although there are no evidences for alternative splicing in the 5' and 3' untranslated regions (UTR) [1], 6 transcripts have been identified, 4 resulting in proteins of different sizes and 2 with no translation product (ENSG00000155380 [28]). MCT1 functional protein is composed by 500 amino acids and has a molecular weight of 53,958 Daltons (P53985, [29]).

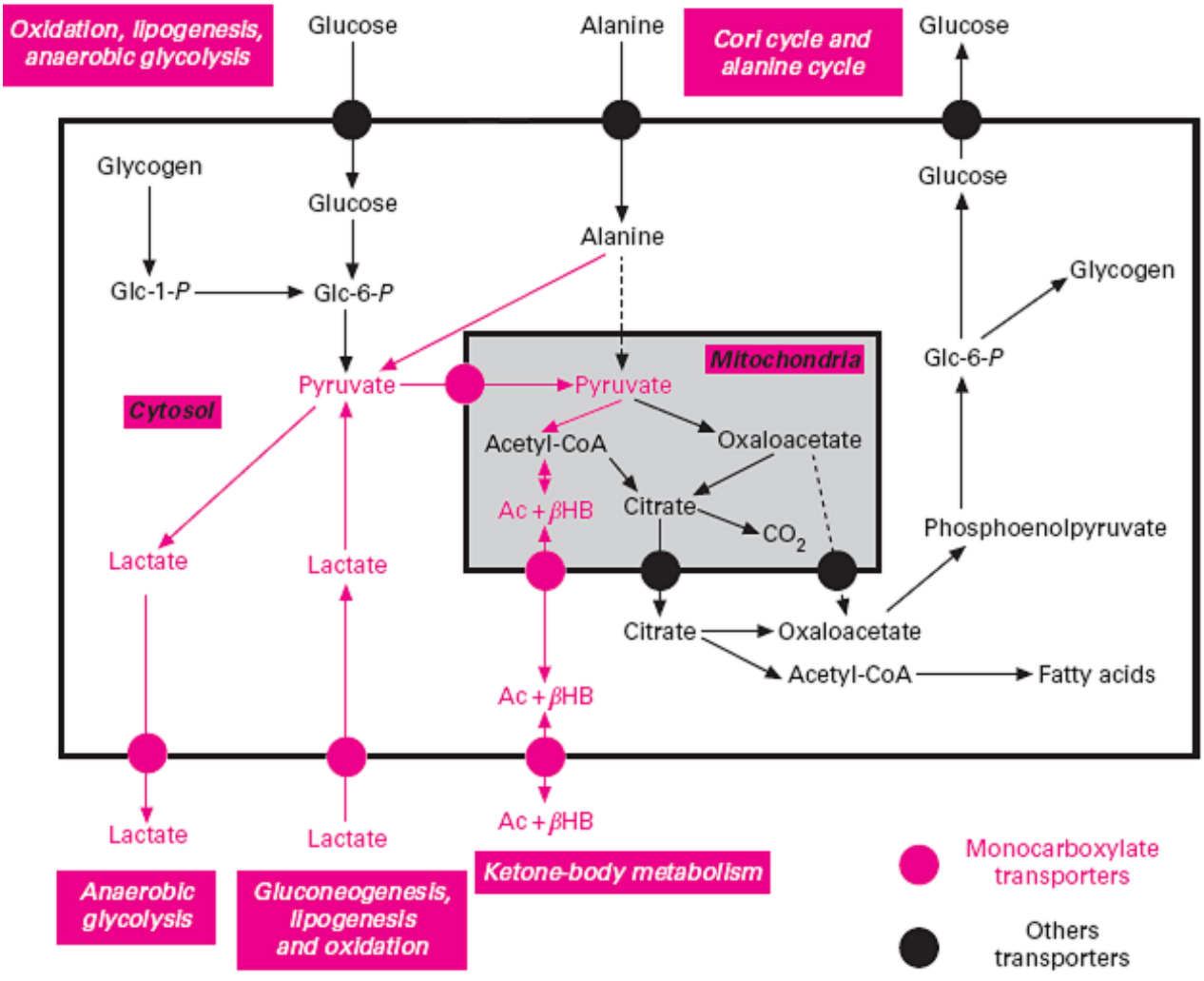

Figure 2. Metabolic pathways involving monocarboxylate transport across the mitochondrial and plasma membranes (adapted from [1]). Abbreviations: Ac, acetoacetate; βHB, β-hydroxybutyrate; Glc-1-P, glucose 1-phosphate; Glc-6-P, glucose 6-phosphate.

MCT1 has an ubiquous distribution in human tissues, with higher expressions in heart and

muscle [6,12,30]. Although conserved among species, MCT1 expression levels in tissues is species-specific [31]. The wide pattern of expression observed for MCT1 may be explained by its substrate affinities that indicate that MCT1, with intermediate substrate affinities, may be involved in both uptake and efflux of monocarboxylates from cells (Table 2). MCT1 transports a variety of substrates including short chain (C2-C5) unbranched aliphatic monocarboxylates such as acetate and propionate. Monocarboxylates with C2 or C3 substitutions (excluding amino- and amido-substitutions) are also transported or even preferred (e.g. pyruvate, L-lactate, acetoacetate and *β*-hydroxybutyrate) [32-34]. The simplest monocarboxylate, formate, is a poor substrate (K_m>100 mM) whereas bicarbonate, dicarboxylates, tricarboxylates and sulphonates are not transported [5]. Also, as can be seen in Table 2, MCT1 is stereoselective for lactate but not for *β*-hydroxybutyrate.

Although plasma membrane expression is required to the export or uptake of monocarboxylates, MCT1 has also been described to be expressed in mitochondria [35-41] and peroxisome [42]. In these organelles, MCT1 is believed to participate in a lactate oxidation complex to maintain organelle redox and proper functioning.

Table 2. K_m values (mM) of different mammalian MCT isoforms for a range of monocarboxylates.

	MCT1[33,34,43]	**MCT2**[44]	**MCT4**[13,45]
L-Lactate	2.2-4.5	0.7	28.0-34.0
D-Lactate	51.0		519.0
Pyruvate	0.6-1.0	0.08	153.0
L-*β*-hydroxybutyrate	8.1-11.4		824.0
D-*β*-hydroxybutyrate	8.1-10.1	1.2	130.0
Acetoacetate	5.5	0.8	
Propionate	1.5		
Acetate	3.5		

1.1.1.2. MCT2

In 1995, the second MCT isoform was cloned and sequenced from a hamster liver cDNA library [46]. The *SLC16A7* gene, which encodes for MCT2, is located in chromosome 12 (12q13) [12], comprises 5 coding exons and, although only one transcript is identified in a public database for human *SLC16A7* (ENSG00000118596, [28]), there is evidence for alternatively spliced mRNA species in human and rat [12,31], but no evidence of splice variants of the protein. This isoform

shares approximately 50% sequence identity with MCT1, contains 478 amino acids and has a molecular weight of 52,186 Da (O60669, [29]).

Northern blot analysis of human tissues showed a much more restricted tissue distribution of MCT2, as compared to MCT1, with MCT2 mRNA found in spleen, heart, kidney, pancreas, skeletal muscle, brain, and leukocyte [12]. These results were subsequently validated by Western blot analysis [30]. The fact that, when both MCT1 and MCT2 are expressed in the same tissue, the expression pattern is cell-specific, suggests a distinct functional role between these isoforms [46-49]. Subsequent expression of human MCT2 in *Xenopus* oocytes revealed its unique biochemical feature of facilitating the proton-linked transport of a range of monocarboxylates, especially pyruvate, with a considerable high affinity, supporting the previous evidence of an alternative biological role [12,44]. Therefore, MCT2 emerges as a high affinity transporter (Table 2), being adapted to perform the uptake of monocarboxylates into cells. As a result, MCT2 is found in tissues that use lactate as a respiratory fuel, like brain [30,48-51] or cardiac and skeletal muscle [30,46], and kidney and liver were lactate is the major gluconeogenic substrate [30,31,46]. As MCT1, MCT2 is also found in mitochondria [38,41].

1.1.1.3. MCT4

In 1998, Price and colleagues subsequently cloned four additional human MCT isoforms [6]. These are now referred to as MCT4, MCT5, MCT6 and MCT7, formerly known as MCTs 3-6 [1]. When these four new sequences were identified, one of them, was more closely related to chicken MCT3 than to MCT1 and MCT2, and, despite the much broader tissue distribution [6], the protein was named mammalian MCT3 (*SLC16A3*) [6]. However, Philp and colleagues subsequently cloned rat and mouse MCT3 (*SLC16A8*). This new MCT3, having an expression confined to the retinal pigment epithelium [16,17], as the chicken MCT3 [8,14], suggests that this was the true mammalian equivalent to the chicken MCT3. Therefore, the MCT3 identified by Philp and colleagues maintained the name, while the four isoforms identified as MCT 3-6 by Price and colleagues were renamed MCT 4-7 [1].

The human *SLC16A3* gene (for review see [52], Appendix II), which encodes for MCT4, is located in chromosome 17 (17q25.3), comprises 5 exons and 3 transcripts, with different initiation sites but no difference in protein product has been identified (ENSG00000155380, [28]). The protein is constituted by 465 residues, corresponding to a molecular weight of 49,469 Da (O15427, [29]).

Like MCT1, and in opposition to MCT2 and MCT3, MCT4 has a broader distribution, being particularly observed in highly glycolytic tissues such as white skeletal muscle fibers, astrocytes, and white blood cells [6,13,53]. This led to the hypothesis that MCT4 may be of particular importance in tissues that rely on glycolysis to meet their energy demands, producing high amounts of lactate that need to be rapidly exported [1,53]. In fact, the kinetic properties of MCT4 show that this isoform is adapted to the export of lactate [13,45]. MCT4 shows a much lower affinity for substrates than MCT1 and MCT2 (Table 2), with K_m values of around 30 mM for L-lactate [13,45] and 150 mM for pyruvate [45].

1.1.1.4. OTHER MCT ISOFORMS

Although performing functions different from the family related isoforms MCT1-MCT4, other MCT isoforms have been characterised in the last years. *SLC18A10* gene encodes for an aromatic amino-acid transporter (T-type amino-acid transporter 1, named **TAT1** rather than MCT10) [54] and has been also recently described, in parallel with **MCT8** (*SLC16A2*) [55], as a thyroid hormone transporter [56]. Importantly, mutations in *SLC16A2* have been associated with X linked severe mental retardation and neurological dysfunction [57-61]. **MCT6** (*SLC16A5*) transports bumetanide, but neither L-lactic acid nor L-tryptophan. Butemanide transports is sensitive to pH and membrane potential but does not depend on proton gradient [62]. Although the substrate for **MCT12** (*SLC16A12*) is still unknown, recent studies suggest a function in the establishment and/or maintenance of homeostasis in the eye lens and probably also in the kidney, and a mutation *SLC16A12* has been associated with development of cataracts [63,64]. Furthermore, *SLC16A12* has been identified as a possible biomarker for colon, prostate and breast carcinoma, due to gene hypermethylation [65]. Finally, a polymorphism in *SLC16A9* (encoding **MCT9**) was found to be associated with altered serum uric acid [66], however MCT9 substrate is still unknown, as for the remaining members of the family (MCT5, MCT7, MCT11, MCT13 and MCT14).

1.1.2. ROLE OF MCTS IN METABOLIC HOMEOSTASIS

As already mentioned, monocarboxylate transporter across the plasma membrane, both efflux and uptake, is of extreme importance to maintain the cellular metabolic homeostasis. Rapid

efflux of lactic acid across the plasma membrane is essential to all mammalian cells that are glycolytic or become glycolytic due to environmental conditions like hypoxia [3]. Lactic acid may be exported by MCT1; however, in cells that rely on glycolysis for energy production, MCT4 appears to be the major isoform. MCT4 has a high K_m for pyruvate, so, unlike MCT1, ensures that this metabolite is not lost from the cell but reduced to lactic acid, regenerating NAD^+, therefore allowing glycolysis to continue [5]. Uptake of lactate is also of great importance in tissues such as liver and kidney, where lactic acid is transported into the cell to support gluconeogenesis and lipogenesis, and in tissues like heart, skeletal muscle and brain, where lactate, together with ketone bodies, is used as a major respiratory fuel [1]. Both skeletal muscle and heart express MCT1 to meet this role, while in liver, kidney and brain both MCT1 and MCT2 can be used, with the latter providing a higher affinity lactate uptake mechanism [1]. Despite the role of MCTs in a wide tissue range, no detectable MCT activity is found in the β-cells of the Islets of Langerhans in the endocrine pancreas. This phenomenon is also of major physiological relevance, since, by not allowing the for pyruvate and lactate, that could enter the citric acid cycle and produce ATP, ensures that adequate amounts of insulin are secreted [67].

Importantly, lactate is a quantitatively important oxidisable substrate and gluconeogenic precursor, being responsible for coordination of intermediary metabolism in diverse tissues. This role of lactate as oxidative and gluconeogenic substrate, as well as in cell signalling, is explained by the "**cell-cell**" and "**intracellular lactate shuttle**" concepts [68-70]. According to the "cell-cell lactate shuttle" hypothesis, lactate produced in glycolytic cells will be utilised continuously under fully aerobic conditions. This is the case of lactate exchanges between glycolytic astrocytes and oxidative neurons [71], between white-glycolytic and red-oxidative fibres within a working muscle, and between tissues of net release of lactate and gluconeogenic tissues [72]. In the "intracellular lactate shuttle", mitochondria play a fundamental role in the oxidative catabolism of lactate, thanks to a lactate oxidation complex composed of lactate dehydrogenase (LDH) and MCTs, which is associated with the electron transport chain [39,40,73]. An "intracellular lactate shuttle" is also present in the peroxisome [42]. As lactate is more reduced than pyruvate, lactate oxidation to pyruvate (or exchange with pyruvate, and subsequent oxidation) induces a change in the cell redox balance. Hence, lactate production in one compartment and its removal from another, whether the compartments are at the cellular or tissue level, represents a major signalling mechanism [5]. In this regard, MCTs, as responsible for lactate shuttling, play an important role in metabolic coordination, linking glycolysis to oxidative metabolism and gluconeogenesis [69], as well as providing some harmonisation to the cytosolic redox potential [5].

Two cell-cell lactate shuttles have been described with more detail, one in the **brain** and the other in **skeletal muscle**, as presented bellow.

1.1.2.1. BRAIN LACTATE SHUTTLE

The classical view of neuroenergetics describes that normal brain is restricted almost exclusively to glucose as the substrate for energy production, which is directly delivered to neurons via the blood supply [74]. However, recent evidence revealed a more complex metabolic pathway, where astrocytes play a role in providing lactate as an additional energy source for neurons (for review see [75-77]).

It was previously shown that lactate produced by muscle during exercise could be used by the brain [78-80], as well as that astrocytes have the capacity of producing lactate in the presence of normal oxygen levels [81]. With the description of MCT cell-specific expression by both *in vitro* and *in vivo* studies, new evidence for a complementary metabolism between neurons and astrocytes was provided [48-51,82-84]. MCT1 expression is found in astrocytes [48-51,82-84], as well as in brain endothelial cells [51,82]. Although firstly reported in astrocytes [82,84], MCT2 is exclusively found in neurons [48-51,83], while MCT4 presents a strong expression in the plasma membrane of astrocytes [51]. Thus, a mechanism of cell-cell lactate shuttle between astrocytes and neurons (Figure 3) is consistent with, on one hand, the fact that neurons contain more mitochondria than astrocytes [85], hence neurons are mainly oxidative while astrocytes are mainly glycolytic, and, on the other hand, the K_m values of each MCT isoform (Table 2). Thus, glycolytic astrocytes consume glucose supplied by the blood flow and the resultant lactate is exported through MCT1 and MCT4 to the extracellular space; this lactate will be transported by the high affinity MCT2 into the oxidative neurons, to be used as a energy substrate for mitochondrial oxidation [51,71,75-77]. As mentioned above, MCT activity is not limited to plasma membrane as neurons contain a mitochondrial lactate oxidation complex that has the potential to facilitate both intracellular and cell-cell lactate shuttles in brain [73].

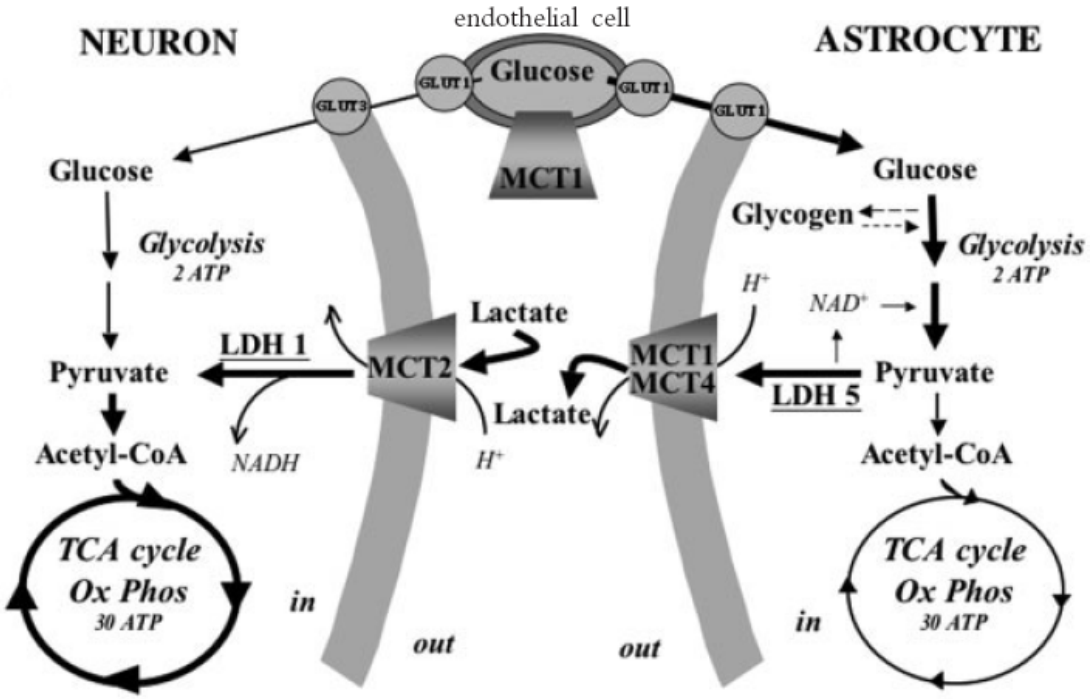

Figure 3. Schematic illustration of lactate shuttle between astrocytes and neurons (adapted from [51]). Abbreviations: GLUT, glucose transporter; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; Ox Phos, oxidative phosphorylation; TCA, tricarboxylic acid.

1.1.2.2. SKELETAL MUSCLE LACTATE SHUTTLE

It is known that lactate is released from diverse tissues like skeletal muscle, skin and red blood cells, but the high glycolytic rates of skeletal muscle makes this tissue the main producer of lactate in the body. This lactate derived from glycolytic skeletal fibers may be consumed by adjacent oxidative skeletal fibers or enter the blood flow and be taken up by other tissues such as heart and brain to be used as a respiratory fuel, or liver as substrate of gluconeogenesis [1]. MCT1 and MCT4 are both found in skeletal muscle; however, their relative amounts are dependent on the type of muscle fiber. MCT1 expression is prominent in slow twitch (red) muscle fibers [30,53,86-89], which present oxidative metabolism, and correlates with the concentration of mitochondria [86]. In contrast, MCT4 is preferentially expressed in glycolytic fast twitch (white) muscle fibers [30,53,87-89]. MCT fiber-specific distribution and differences in MCT affinity for lactate (Table 2), support the hypothesis of a cell-cell lactate shuttle in skeletal muscle where lactate produced by glycolytic fibers is exported by the low affinity MCT4 [13,45], and subsequently imported by oxidative fibers through MCT1, which has a higher affinity for lactate than MCT4. The major role

of MCTs in muscle function is supported by the increase in the density of both MCT isoform, especially MCT1, after training periods [90-98].

1.1.3. MCTs IN DISEASE

Considering the fundamental role of lactate transporters in key metabolic processes like glycolysis and gluconeogenesis, it is not surprising that alterations in MCT expression or function are associated with physiological dysfunctions. In fact, MCT4 overexpression was reported in a patient with a mitochondrial myopathy [99] and MCT4 down-regulation (35% lower expression) was described in the *vastus lateralis* muscle of patients with chronic obstructive pulmonary disease [100]. However, these alterations in MCT4 expression appear to be more a consequence rather than a cause of the disease.

In contrast, *SLC16A1* mutations associated with **exercise-induced hyperinsulinism** [101] and **erythrocyte lactate transporter defect** [102], as well as a decrease in *SLC16A1* gene transcription in **inflammatory bowel diseases**, have been described and will be further explored below.

1.1.3.1. EXERCISE-INDUCED HYPERINSULINISM

Exercise-induced hyperinsulinism is characterised by inappropriate insulin secretion by pancreatic β-cells, especially during anaerobic exercise, in response to exogenous catabolic metabolites such as lactate and pyruvate [103-105]. As previously mentioned, pancreatic β-cells show no MCT activity [67], however, an increase of MCTs in β-cells plasma membrane, will allow the entry of pyruvate into the cell and a consequent increase in ATP production and insulin secretion. Although no mutations were detected in the coding regions of eight MCT genes in earlier studies [104], sequencing of the 5' UTR and promoter regions of the *SLC16A1* gene brought new insights on the mechanism for this disease [101]. By studying affected members of a Finnish family, segregating autosomal dominant exercise-induced hyperinsulinemic hypoglycemia, Otonkoski and colleagues identified two functional alterations in *SLC16A1*. First, a +163G-A transition in exon 1, located within a binding site for nuclear matrix protein-1 (NMP1) and predicted to disrupt the binding sites of 2 potential transcriptional repressors (albumin negative factor (ANF) and acute myeloid leukemia-1a (AML-1a)), and, second, a 25-bp insertion

between nucleotides -23 and -24, introducing additional binding sites for the ubiquitous transcription factors simian-virus-40-protein-1 (SP1), upstream stimulatory factor (USF) and myeloid zinc finger 1 (MZF1). The first variation leads to a 3.5-fold increase in transcription while the second variation leads to a 10-fold increase in transcription. These mutations were not found in 94 Finnish and German controls, excluding the probability of being polymorphisms [101].

1.1.3.2. ERYTHROCYTE LACTATE TRANSPORTER DEFECT

In 1986, a rare condition in which apparently healthy patients suffered severe chest pain and muscle cramping after vigorous exercise, was associated with an impaired capacity of lactate transport by skeletal muscle and red cells [106]. More recently, Merezhinskaya and colleagues identified three heterozygous transitions in the *SLC16A1* gene, in patients with erythrocyte lactate transporter defect. Firstly, a 610A>G transition (resulting in a lys204-to-glu (K204E) substitution in a highly conserved residue); secondly, a 1414G>A transition (resulting in a gly472-to-arg (G472R) substitution halfway along the cytoplasmic C-terminal chain, in a non-conserved residue); and thirdly, a 1470A>T transition (resulting in a glu490-to-asp (E490D), which revealed to be a common polymorphism) were identified in patient's muscle biopsies. Erythrocyte lactate clearance in patients with the 2 mutations was 40 to 50% that of normal control values, while for patients with the polymorphism erythrocyte lactate clearance was 60 to 65% of mean normal [102]. However, heterologous expression of the MCT1 K204E mutant, considered the most important alteration due to its location in a conserved residue, failed to support any difference in its properties from wild type MCT1 [5]. Thus, it remains unclear if mutations in MCT1 are responsible for cryptic exercise intolerance.

1.1.3.3. INFLAMMATORY BOWEL DISEASES

Butyrate, a monocarboxylate commonly referred to as a short-chain fatty acid, is produced in the lumen of the colon by microbial fermentation of dietary carbohydrates that escape digestion in the small intestine. This monocarboxylate is the major energy source for colonic epithelial cells and exerts a variety of effects important to intestinal health and function [107]. Impaired oxidation of butyrate has been associated with inflammatory bowel disease, but the mechanism was not known [108,109]. Recently, it was observed that inflammation caused down-regulation of MCT1

expression in the colonic tissue, the MCT isoform responsible for butyrate uptake in colonic epithelial cells [110]. Also, treatment of intestinal epithelial cell lines with pro-inflammatory cytokines (interferon-gamma (INF-γ) and tumour necrosis factor-alpha (TNF-α) induced down-regulation of MCT1 gene transcription, which was associated with a reduction in butyrate uptake and subsequent oxidation. Thus, reduction in MCT1-mediated butyrate uptake is the cause of butyrate oxidation deficiency in intestinal inflammation [111].

1.1.3.4. CANCER

It is known that tumour cells are highly glycolytic, producing lactic acid in excess, and both biochemical and molecular evidence suggest the up-regulation of MCTs in some tumour cells [112]. However, since the role of MCTs in solid tumours is the main subject of this thesis, a more detailed review of the state of the art is included latter on.

1.1.4. MCT REGULATION

Reports on MCT expression variations, especially MCT1, have been described in different physiological as well as pathological conditions including, among others, MCT up-regulation in skeletal muscle in response to training [90-98], down-regulation after muscle denervation [53] and in inflammatory bowel diseases [111], changes in MCT expression during development [113-117] or substrate-induced MCT1 up-regulation [118-120]. Importantly, regulatory mechanisms vary among MCT isoforms, which allow induction of specific isoforms upon different stimuli, adapting cells to different energy demands.

Although the regulatory mechanisms of MCT expression are far from being completely unveiled, plenty of evidence indicates that MCTs are regulated at various points up to the functional protein, including both **transcriptional**, and **post-transcriptional** [31,88,95,119,120] regulation that affects protein amounts as well as regulators of **transporter activity**, like chaperone proteins. Further, **hormone** regulation has also been described for MCTs, as well as regulation by signalling pathways like insulin-like growth factor receptor type I (IGF-IR) activation, which up-regulates MCT1 [121].

1.1.4.1. TRANSCRIPTIONAL LEVEL

Transcriptional regulation of genes is a complex mechanism involving intervention of many regulatory proteins, including transcription factors, functioning as enhancers or repressors of gene transcription [122]. Analysis of *SLC16A1* 5'-flanking region allowed the identification of putative binding site sequences for the transcription factors USF, nuclear factor-kappaB (NF-κB), activated protein 1 and 2 (AP1 and AP2) and stimulating protein-1 (Sp1) [27]. In fact, USF1 and USF2 have been described as potential repressor proteins for MCT1 [123], NF-κB pathway has been involved in the butyrate-induced MCT1 up-regulation [124], while AP2 has been associated to protein kinase C (PKC)-dependent stimulation of the *SLC16A1* promoter [125].

Additionally, lactate-induced increase in MCT1 has been linked to activation of NF-κB and nuclear factor erythroid 2 (NF-E2) pathways, as well as cAMP-response element-binding protein (CREB) and NF-E2 related factor 2 (Nrf2) transcription factors, the last three elements possessing also putative transcription binding sites in the *SLC16A1* 5'-flanking region [120].

More recently, the co-activators peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PGC-1α) [126] and peroxisome proliferator-activated receptor alpha (PPARα) [127,128] have been associated with MCT1, but not with MCT2 and MCT4 up-regulations. In fact, promoter analyses of MCT1, MCT2 and MCT4 have shown that the *SLC16A1* promoter contains two peroxisome proliferator-activated receptor response elements (PPRE) while *SLC16A7* and *SLC16A3* each contain one PPRE [126].

In expression studies, *SLC16A1* was also shown to be activated by ***c-myc*** and *n-myc* proto-oncogenes [129-131], while the pro-inflammatory cytokines IFN-γ and TNF-α, have also been implicated in the transcriptional control of MCT1, by down-regulating *SLC16A1* transcription [111].

Hypoxia conditions, which are known to induce the glycolytic phenotype, are also associated with altered expression of MCTs [132-138]. The first report described a tissue-specific change in MCT expression after chronic hypoxia, where MCT1 did not change in heart, soleus, or gastrocnemius muscles, while MCT4 increased significantly in heart muscle. However, in the plantaris muscle, both MCT1 and MCT4 showed a significant decrease after chronic hypoxia [132]. A subsequent study suggested that the increase in neuronal, astrocytic and endothelial MCT1 expression, observed after permanent occlusion of the left middle cerebral artery, is mediated by the hypoxia-inducible factor-1alpha (**HIF-1α**) [133], the major transcriptional regulator of adaptation to hypoxic stress; however, this view was promptly contested by Ullah and collaborators who, after performing functional studies with MCT1, MCT2 and MCT4 promoters,

showed that only MCT4 promoter was activated by hypoxia and that this response was mediated by HIF-1α [135]. Additionally, MCT4, but not MCT1, was shown to be up-regulated by hypoxia in human bladder cancer cells [134] and in trophoblast cells [136], and MCT1 and hypoxia were described as being mutually exclusive [137]. However, recent evidence describe a hypoxia-mediated increase in both MCT1 and MCT4 and decrease in MCT2, with MCT1 and MCT4 change being HIF-1α-dependent [138]. From this, one can conclude that evidence so far, especially on MCT1 regulation by hypoxia, is very controversial and more efforts have to be made to enlighten the knowledge on MCTs' regulation by hypoxia.

1.1.4.2. POST-TRANSCRIPTIONAL LEVEL

The relatively long 3'UTR of *SLC16A1* (1.6kb), on which initiation factors and regulatory proteins interact to enhance or repress translation [139], suggests that MCT1 expression, but not MCT2 nor MCT4 which have much shorter 3'UTRs, might also depend on translational regulation [1,5].

MicroRNAs (miRNAs) are a group of small non-coding RNAs (approximately 22 nucleotides) that play a critical role in a variety of biological processes, like development, differentiation and apoptosis. Mature miRNAs negatively regulate their targets through complementary sequence pairing with the 3′ UTR of mRNA targets, inducing transcript degradation or translational repression (for review see [140]). One of the most well characterised miRNAs in mammalian nervous system is miR-124, which has been described to regulate MCT1; miR-124 regulates *SLC16A1* through binding to its 3′ UTR and MCT1 protein level is reduced after miR-24 transfection [141]. Regulation of *SLC16A1* mRNA stability has already been described in the context of MCT1 regulation by butyrate [119].

1.1.4.3. TRANSPORTER ACTIVITY LEVEL

Other factors that may regulate functional expression of MCTs are accessory proteins that are involved in trafficking and anchoring of membrane proteins to specific cellular locations. In this context, CD147 emerges as the major and better studied regulator of MCT expression.

CD147, also known as basigin, EMMPRIN, OX-47 and HT7, is a broadly distributed plasma membrane glycoprotein discovered in 1982 [142], which belongs to the immunoglobulin

superfamily [143]. Originally identified as a tumour surface protein with the ability of inducing matrix metalloproteinases, with a critical role in tumour progression, CD147 expression is found in cells other than tumour cells where it plays an important role in fetal, neuronal, lymphocyte and extracellular matrix development as well as tissue repair (for review see [144,145]). Co-immunoprecipitation and chemical cross-linking studies showed that CD147 specifically interacts with MCT1 (Figure 4) and MCT4, but not MCT2. Also, co-transfection of CD147 allowed active expression of MCT1 and MCT4, but not MCT2, in the plasma membrane of MCT-transfected cells. When CD147 was not co-transfected, MCT accumulated in a peri-nuclear compartment [146]. The close association between CD147 and both MCT1 and MCT4 within the plasma membrane was confirmed by co-localisation studies [67,147,148].

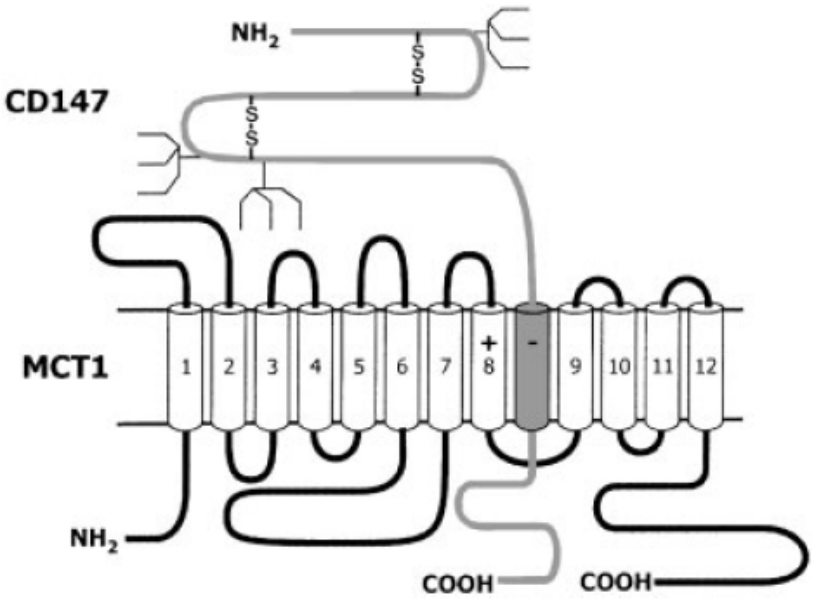

Figure 4. Monocarboxylate transporter 1 (MCT1) and CD147 predicted interaction (from [146]). This interaction is proposed to involve an arginine residue within the transmembrane segment 8 of MCT1 and a glutamic acid residue in the transmembrane segment of CD147.

Importantly, subsequent studies showed that, besides being important for MCT membrane location [149-153], CD147 has also a role in MCT1, 3 and 4 expression [18,151,153,154] and improvement of MCT activity [149,155]. On the other hand, MCT1 and MCT4 have also shown to be regulators of CD147 maturation and trafficking to the plasma membrane [150,151]. As mentioned above, although also requiring an ancillary protein for proper expression, MCT2 does not interact with CD147 nor is influenced by CD147 expression [18,146]. This function is fulfilled by another member of the immunoglobulin superfamily, the gp70 protein [156], which, in co-

immunoprecipitation and co-localisation studies, was found to interact with MCT2 [155]. Gp70 has also been described as interacting with MCT1 in rat erythrocytes [157].

More recently, other players were included in the MCT/CD147 complex. The widely distributed transmembrane glycoprotein **CD44** and its main ligand, hyaluronan, were implicated in the regulation of lactate efflux and membrane localisation of MCTs, in human breast carcinoma cells [158]. In fact, CD44 co-immunoprecipitates with MCT1, MCT4 and CD147 and co-localises with these proteins in the plasma membrane. Perturbation of endogenous hyaluronan, using hyaluronan oligosaccharides, induced intracellular accumulation of CD44, MCT1 and MCT4. These observations suggest that constitutive interactions between hyaluronan, CD44 and CD147 regulate MCT localisation and function of MCT1 and MCT4 [158]. Importantly, a role of CD44 activation has been described in cell growth control, adhesion, migration, invasion, and chemoresistance [159-161], which may count on the contribution of MCTs.

Additionally, other modulators of transporter activity may directly affect MCT function, independently of affecting protein amounts and location, including carbonic anhydrase (CA) II, which enhance both MCT1 and MCT4 activity by direct binding [162-164], prion-related protein (PrP) [165] and intracellular calcium [166].

1.1.4.4. HORMONAL REGULATION

Hormonal regulation of MCTs may involve transcriptional and post-transcriptional mechanisms. MCTs' regulation by hormones has been firstly described in 2002, as luminal leptin was shown to significantly up-regulate MCT1-mediated butyrate uptake, in Caco2-BBE cell monolayers. This increased uptake was achieved through two distinct mechanisms: an increase in the intracellular pool of MCT1 protein, with no changes in CD147 amounts, and translocation of MCT1/CD147 to the apical membrane of Caco2-BBE cell monolayers [167]. Shortly after, the hormones thyroid-stimulating hormone (TSH), noradrenaline, triiodothryonine (T_3) and somatostatin were also described as modulators of MCT expression [168-171]. TSH regulates MCT1 protein expression in rat thyroid cells, increasing *SLC16A1* transcription, and also increases CD147 protein levels [170]; noradrenaline induces MCT2, but not MCT1 expression, in mouse neurons, at the translational level, with the requirement of an yet unknown transcriptional step [168]; MCT4, but not MCT1, is induced by T_3 in rat skeletal muscle [169]; and somatostatin increases MCT1 association with CD147 at the plasma membrane, with an increase in the apical membrane levels of MCT1 protein in parallel to a decrease in the intracellular MCT1 pool [171].

1.1.5. INHIBITION OF MCT ACTIVITY

Several agents are known to inhibit MCT activity, in an isoform-dependent manner. These include classical inhibitors like α-cyano-4-hydroxycinnamate (CHC) or quercetin, inhibitors that influence MCT activity in a specific manner like AstraZeneca inhibitors, and molecules that, being used in particular contexts, have also been described as inhibitors of MCT activity like cholesterol synthesis inhibitors (statins) and non-steroidal anti-inflammatory drugs (NSAIDs) (for review see [10]).

1.1.5.1. CLASSICAL INHIBITORS

Identification of MCT inhibitors was achieved during characterisation of MCT1, as the most studied MCT isoform. Bulky or aromatic monocarboxylates were among the first inhibitors of lactate transport to be identified, which, as substrate analogues, are competitive inhibitors of MCT1 [3]. Later on, other types of molecules were identified as inhibiting MCT1 activity, as well as the activity of other isoforms.

Classical reversible inhibitors of MCTs fall into three broad categories (for review, see [3,5,11]):

(1) Bulky or aromatic monocarboxylates like phenyl-pyruvate and **CHC**. In this category, the derivatives of α-cyanocinnamate are the most potent, with K_i values of 50-500 μM. However, although CHC is often used as a MCT1 specific inhibitor [137,172,173], it also inhibits the mitochondrial pyruvate transporter with a K_i<5 μM, as well as the anion exchanger 1 (AE1), which is responsible for Cl^-/HCO_3^- membrane exchange [174].

(2) Amphiphilic compounds with widely divergent structures like bioflavonoids (e.g. phloretin and quercetin) [175] and inhibitors of anion transport (e.g. niflumate). Phloretin and quercetin are particularly potent inhibitors of MCT1 but, like the majority of MCT classical inhibitors, they also inhibit other membrane transport systems.

(3) Stilbenedisulphonates (e.g. 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS)). These inhibitors are some of the most effective MCT inhibitors with K_i<40 μM, however, with much lower affinity than for AE1.

Additionally, miscellaneous inhibitors including thiol reagents, such as the organomercurial thiol reagent *p*-chloromercuribenzene sulphonate (pCMBS), and amino reagents (e.g. pyridoxal phosphate and phenylglyoxal) irreversibly inhibit MCTs (for review, see [3,5,11]).

Importantly, these inhibitors have different affinities among MCT isoforms. MCT2 is more sensitive to CHC, DIDS and phloretin than MCT1 [44], but is not sensitive to pCMBS, due to the different ancillary protein [155]; MCT3 is insensitive to CHC, pCMBS and phloretin [176]; and MCT4, although also sensitive to CHC, DIDS and pCMBS, inhibition is achieved with a much lower affinity, which is in accordance with the low affinity characteristics of this transporter [13,45].

1.1.5.2. SPECIFIC INHIBITORS

As mentioned above, 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.

T cell activation is dependent on glycolysis and this leads to a high rate of lactate production, due to a higher energetic demand during proliferation and cytokine production [177]. In the immunological context this may be of great importance, as inhibition of T cell activation has therapeutic implications in immunosuppressive therapy. It was in this perspective that specific MCT1 inhibitors arose, when MCT1 was identified as the target for newly developed **AstraZeneca immunomodulatory compounds**, that potently inhibit human and rat T lymphocyte activation [178,179]. These compounds have shown promising results in allograft rejection both in mouse and rat [180,181], having also been used in the context of the astrocytes-neuron lactate shuttle, where inhibition of both MCT1 and MCT2 was achieved [182]. In fact, some of these compounds appear to be MCT isoform specific [178], while others, although binding with higher affinity to MCT1, also bind to other MCT isoforms [182].

1.1.5.3. OTHER INHIBITORS

Lonidamine is a derivative of indazole-3-carboxylic acid, which for a long time, has been known to inhibit glycolysis in cancer cells. Although this action was originally attributed to hexokinase inhibition [183], further studies revealed that lonidamine inhibits lactate efflux from cancer cells [184,185], through inhibition of MCT1 and MCT4 [130]. Actually, despite a lack of knowledge of its precise mechanism of action, lonidamine has been effective in clinical trials against various tumours, especially as a sensitizer to other chemotherapies [186].

Statins are 3-hydroxyl-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors, which are used as safe and effective drugs for managing hypercholesterolemia [187]. Lipophilic compounds, such as fluvastatin, atorvastatin, lovastatin acid, simvastatin acid and cerivastatin, have monocarboxylate structures within the compounds, making these molecules putative MCT substrates. Actually, these statins have been shown to be MCT4 substrates and, therefore, inhibitors of lactate and other substrates' transport [188-190]. The interaction of simvastatin with MCT4 accounts for the simvastatin-induced muscular toxicity, observed in MCT4 expressing skeletal muscle but not in MCT1 expressing heart muscle [191].

Although apparently being transported through a transport system other than MCT in trophoblast BeWo cells [192], **NSAIDs** have been described as partially transported by MCT1 in Caco-2 cells [193], and also described as potent inhibitors of lactate transport [188,189,192].

1.2. ROLE OF MCT IN CANCER CELL METABOLIC PHENOTYPE

The role of MCTs in physiological homeostasis is widely accepted and described in detail in some tissues. Also, as mentioned previously, MCT has also a role in disease. However, in what concerns tumour biology, a lot of work is needed to shed some light in that area. Even though, if one looks at the microenvironmental scenario and molecular events that occurs in carcinogenesis, it is possible to anticipate an important contribution of MCTs in the progression to malignancy.

1.2.1. CANCER HALLMARKS: CONTRIBUTION OF MICROENVIRONMENT

Over the last years, much attention has been given to the genetic and epigenetic alterations occurring in cancer development, which ultimately leads to emergence of invasive cancer. In fact, conceptual models of epithelial carcinogenesis are typically based on Darwinian dynamics and depict a sequence of heritable changes, as described in the genetic model of colorectal tumourigenesis described by Fearon and Volgestein [194], that give rise to a population of cells possessing the hallmarks of invasive cancer (Figure 5) [195]. Therefore, in general, cancer evolution consists in accumulation of alterations in oncogenes, proto-oncogenes and tumour suppressor genes that will increase growth-promotion signals and decrease growth inhibitors [196]. However, this classical view does not account for the other component of Darwinian dynamics, where the environment exerts a selection force that will only allow the emergence of

successful adaptations. In this context, the specific changes that occur in tumour microenvironment are emerging as key components in carcinogenesis.

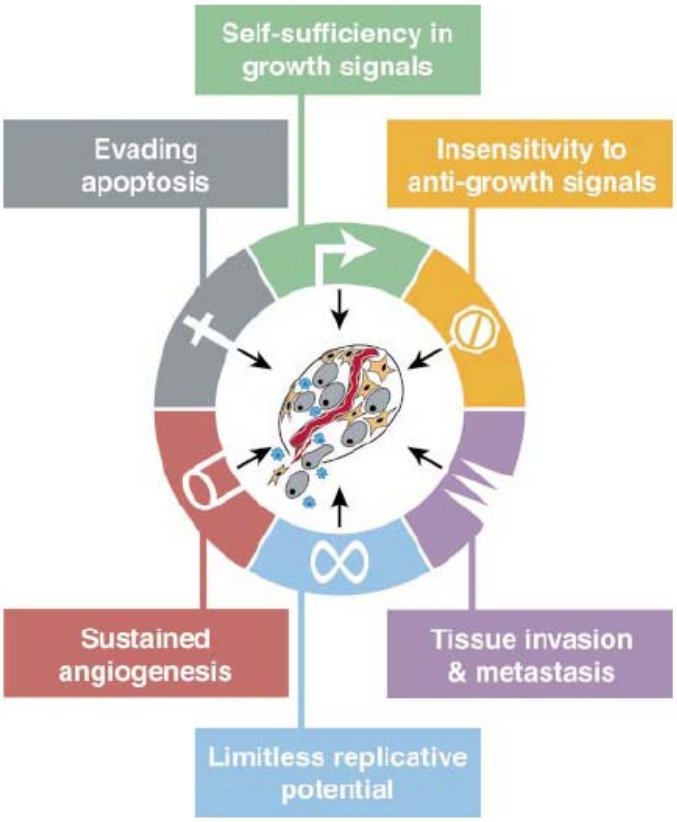

Figure 5. The hallmarks of cancer. Although through various mechanistic strategies, all cancers have acquired the same set of functional capabilities during their development (from [195]).

1.2.1.1. THE WARBURG EFFECT

More than half a century ago, Otto Warburg demonstrated that cancer cells rapidly convert the majority of glucose into lactate, even in the presence of sufficient oxygen to support mitochondrial oxidative phosphorylation [197]. This phenomenon is presently known as "aerobic glycolysis" or "**Warburg effect**" (Figure 6). Although Warburg's hypothesis that impaired mitochondrial metabolism underlies the high rates of glycolysis has proven incorrect [198-200], the original observation of increased glycolysis in tumours has been confirmed repeatedly. In fact, this increased glucose uptake by cancer cells is the rationale behind the whole-body non-invasive ^{18}F-fluorodeoxyglucose positron emission tomography (FdG-PET) technique. This widespread clinical application is used for diagnosis, initial staging, restaging, prediction, monitoring of treatment response and surveillance in a variety of cancers [201].

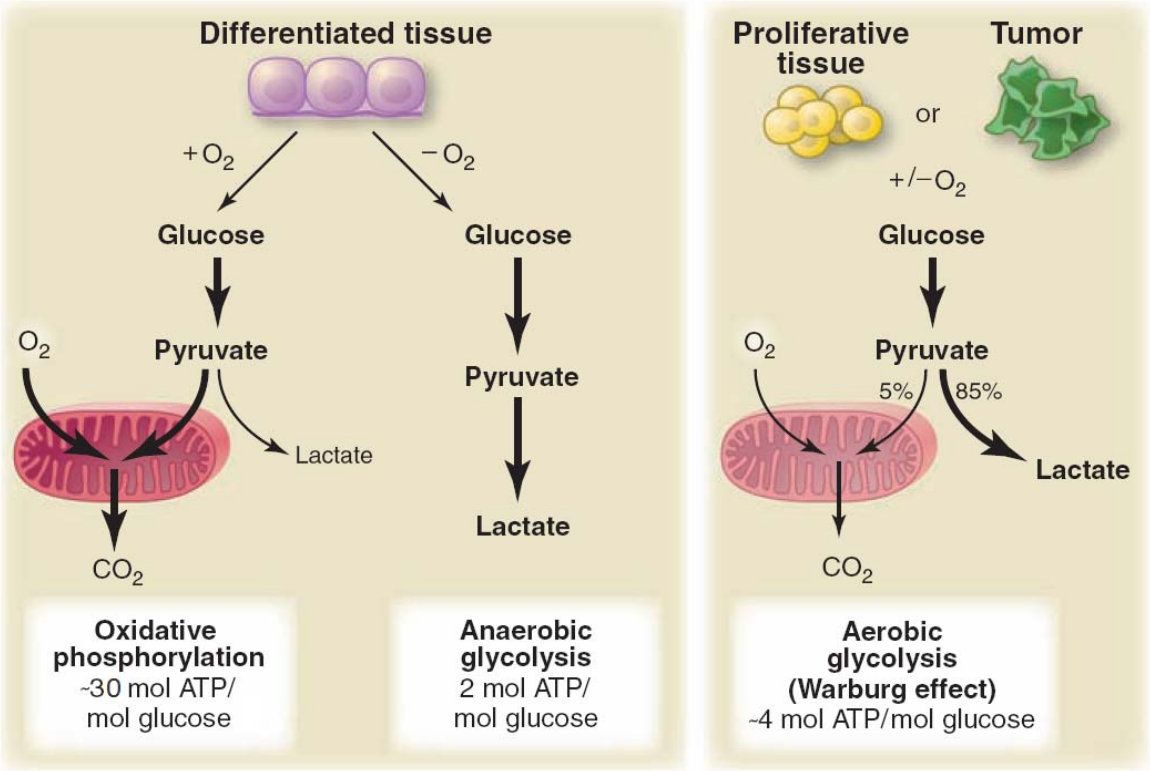

Figure 6. Schematic representation of the main differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis (adapted from [202]).

Aerobic glycolysis, where metabolism of glucose to lactate generates only around 4 ATP per molecule of glucose (Figure 6), is a much less efficient energetic pathway than mitochondrial oxidative phosphorylation, where complete oxidation of one molecule of glucose through yields around 30 ATP [203]. Additionally, the metabolic products of glycolysis, including lactic acid and H^+, cause a consistent acidification of the extracellular space [204-207], which might result in cellular toxicity. Therefore, why do cancer cells engage into the glycolytic phenotype, where only a fraction of the energy is obtained from glucose and possibly harmful products are produced? At first glance, cancer cells have no proliferative advantage in having a glycolytic metabolism, however, as discussed below, this apparent deleterious feature is, in fact, the key cellular trait that allows selection for cancer cells [208].

1.2.1.2. MICROENVIRONMENTAL HYPOXIA AND ACIDOSIS: THE EMERGENCE OF THE HYPER-GLYCOLYTIC ACID-RESISTANT PHENOTYPE

Epithelial cells and the underlying stroma are physically separated by a basement membrane. As carcinogenesis occurs, the epithelial cell layer becomes increasingly thicker, driving cells towards a lumen that is further away from blood supply, as blood vessels are confined to the stroma (Figure 7). Therefore, early carcinogenesis and development of the malignant phenotype occur in an avascular environment, and cancer cells become dependent on glucose and oxygen diffusion through blood vessels and basement membrane to fulfil the major metabolic demands [208,209]. The first limiting substrate for cell growth is oxygen, with hypoxia expected at distances between 100 to 150 µm from the vessel wall [210-212], while glucose diffusion distances are substantially larger [196,211,213].

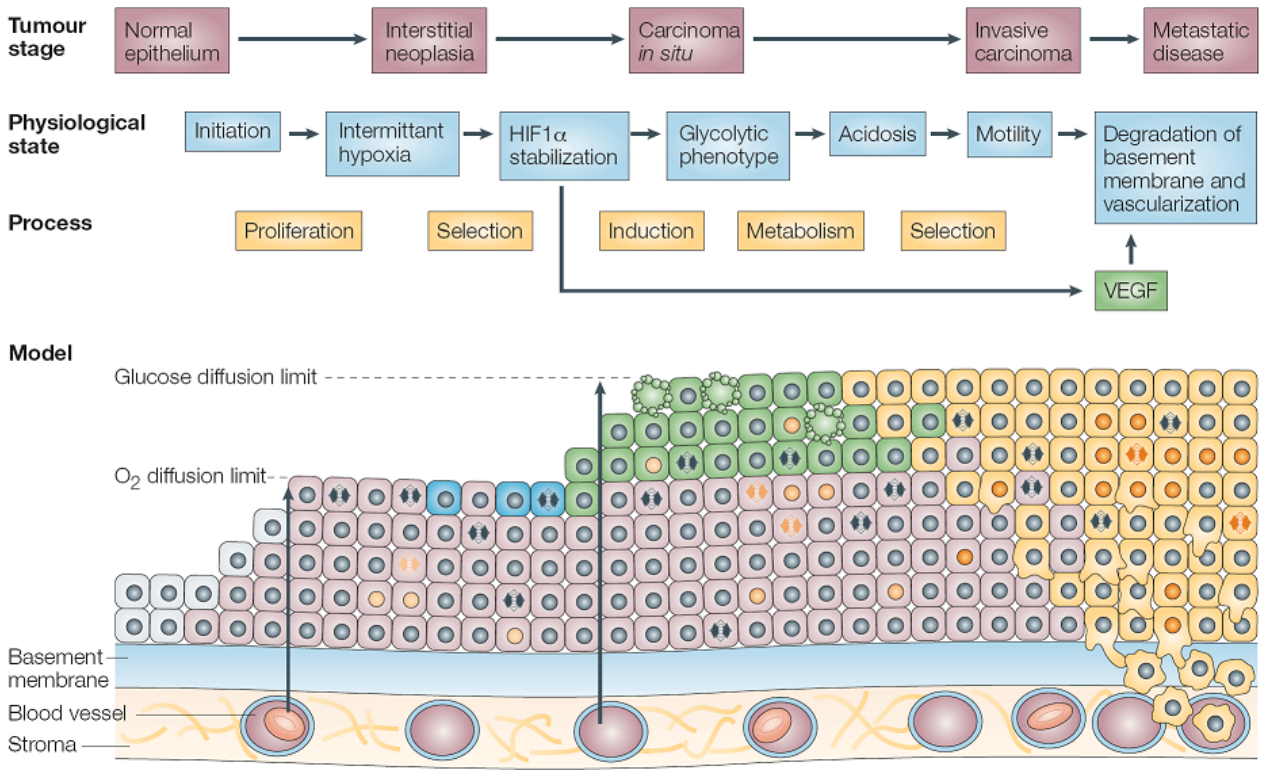

Figure 7. Model for cell-environment interactions in carcinogenesis (adapted from [208]). Cell colours represent different cell types: grey for normal epithelial cells, pink for hyper-proliferative cells, blue for hypoxic cells, green for glycolytic cells and yellow for motile cells. Light orange nuclei represent one mutation while dark orange nuclei represent more than one mutation. Blebbing membranes show apoptotic cells. Abbreviations: HIF-1α, hypoxia-inducible factor 1alpha; VEGF, vascular endothelial growth factor.

Hence, if early hyperplastic lesions develop further than a few layers beyond the basement membrane, regional development of hypoxia will occur, limiting cell growth. This intermittent hypoxia will promote selection for cells with anaerobic glycolysis constitutively up-regulated, allowing further cell growth [208,209,214]. However, how could the cell benefit from entering an energetically less efficient metabolism? Besides being a successful adaptation to hypoxic microenvironment, anaerobic glycolysis allows cancer cells to use the most abundant extracellular nutrient, glucose, to produce ATP, which, thanks to the high glycolytic rates (more than 30-fold higher than normal cells [215]), can easily exceed the ATP yield of oxidative phosphorylation [216]. Additionally, excess pyruvate provides a source for anabolic substrates essential for biosynthetic pathways, including ribose sugars for nucleotides, glycerol and citrate for lipids, and nonessential aminoacids [216,217]. Importantly, aerobic glycolysis may protect DNA from damage by oxygen radicals produced by oxidative phosphorylation [218].

The next limiting step was originally thought to be the glucose diffusion limit [208], however, new evidence demonstrates that the microenvironment acidosis created by the glycolytic phenotype is responsible for further selection, in this case, of an acid-resistant phenotype [196,209,214]. Since prolonged exposure to an acidic microenvironment typically results in necrosis or apoptosis [219,220], additional adaptations of cancer cells will include resistance to acid-induced apoptosis and up-regulation of membrane pH regulators [208]. This potential disadvantage of hyper-glycolytic phenotype, in fact provides further competitive advantage [208] since, on one hand, the significant decrease in local extracellular pH generates a microenvironment that is fatal to the surrounding normal cells, but harmless to cancer cells and, on the other hand, facilitates cancer cell invasion behaviour [221-224] through the acid-induced degradation of the extracellular matrix [225,226] and angiogenesis [227,228].

Therefore, adaptation to hypoxia and acidosis will force the emergence of an adaptive phenotype with constitutive up-regulation of glycolysis and resistance to acid-induced toxicity, that will invade the normoxic regions and ultimately breach the basement membrane towards invasion (Figure 7) [196]. Hence, aerobic glycolysis, a common feature in primary tumours, is pointed as an additional hallmark of cancer, as it is required for evolution of invasive human cancers (Figure 8) [208,229].

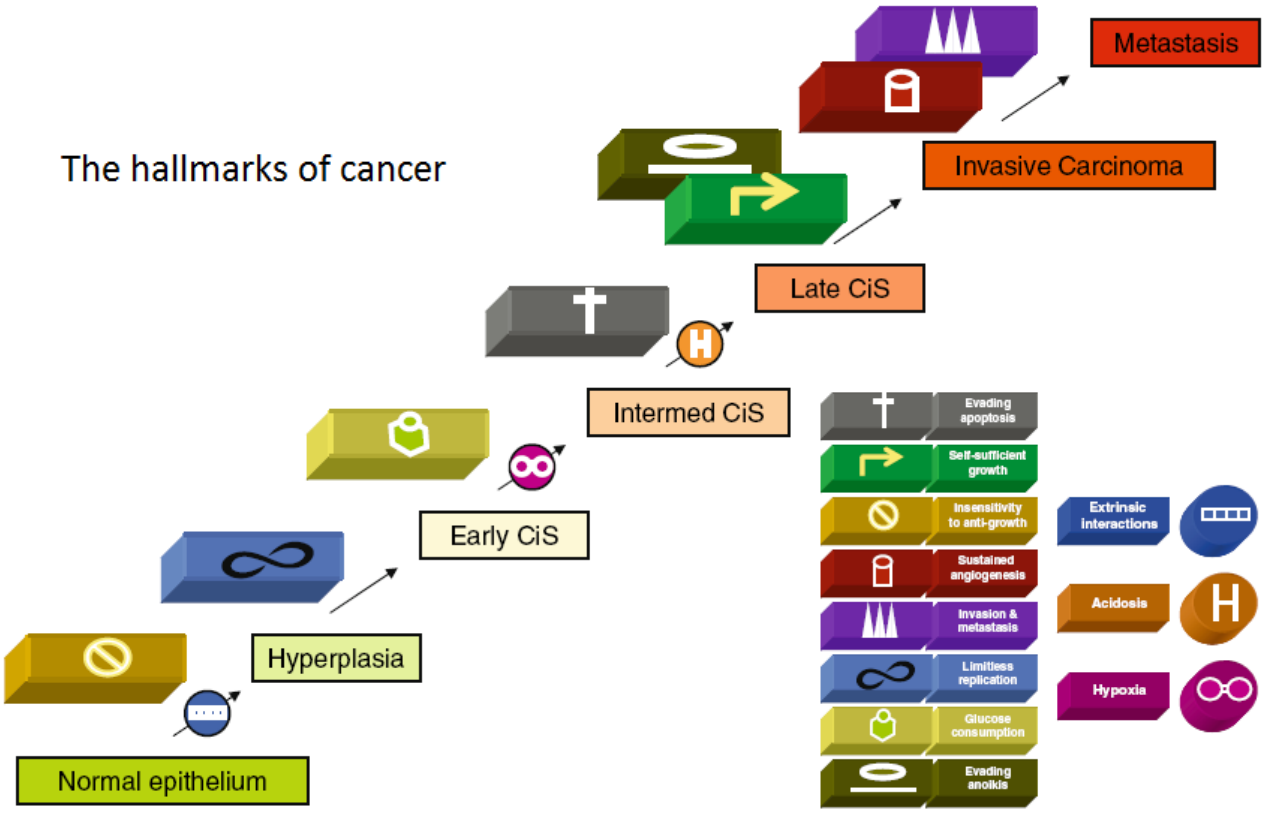

Figure 8. The hallmarks of cancer (adapted from [229]). The eight rectangular icons represent the hallmarks of cancer, while the three circular icons indicate three important components of the physiological microenvironment that must be overcome in cancer progression. Abbreviation: CiS, carcinoma *in situ*.

1.2.1.3. MOLECULAR MECHANISMS: CONTRIBUTION OF HIF-1α

As mentioned above, the glycolytic phenotype arises as an adaptation to the hypoxic environment. It is widely known that the major regulator of adaptation to hypoxic stress is the transcriptional factor **HIF-1α**, which has been widely associated with cancer progression [230-235]. In fact, many enzymes from the glycolytic pathway like the glucose transporter 1 (GLUT1) [236,237], lactate dehydrogenase A (LDH-A) [238], pyruvate dehydrogenase kinase 1 (PDK1) [239,240], among others [235,241,242], are HIF-1α targets (Figure 9). With the increase in glucose uptake into the cell, due to higher amounts of glucose transporters, as well as clearance of pyruvate by rapid conversion of pyruvate into lactate through LDH-A (with regeneration of NAD^+), high glycolytic rates can be maintained. Actually, metastatic cancer cells expressing high levels of HIF-1α, are highly glycolytic, even under normal oxygen conditions, whereas non-metastatic cancer cells consume low amounts of glucose and have low HIF-1α levels [243]. Additionally, by up-regulating PDK1 (which inactivates the tricarboxylic acid (TCA) cycle enzyme pyruvate dehydrogenase (PDH)), HIF-1α will also contribute to suppress the

mitochondrial metabolism, rescuing cells from ROS generation and hypoxia-induced apoptosis [239,240]. Besides contributing to the constitutive glycolytic metabolism, HIF-1α also contributes to the acid-resistant phenotype, by up-regulating, at least, two important pH regulators, MCT4 [135,138] and CAIX [244-246]. As mentioned above, by co-transporting lactate with H^+, MCT4 has an important function as pH regulator. 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. CAIX catalyses the extracellular hydration of CO_2 into HCO_3^- and H^+. Then, HCO_3^- enters the cell through AE1 where, in combination with the accumulating H^+ derived from the glycolytic metabolism, forms CO_2 and H_2O, that will exit the cell by diffusion, causing trapping of the H^+ in the extracellular milieu [174,247]. Therefore, HIF-1α plays a major role in inducing the hyper-glycolytic acid-resistant phenotype.

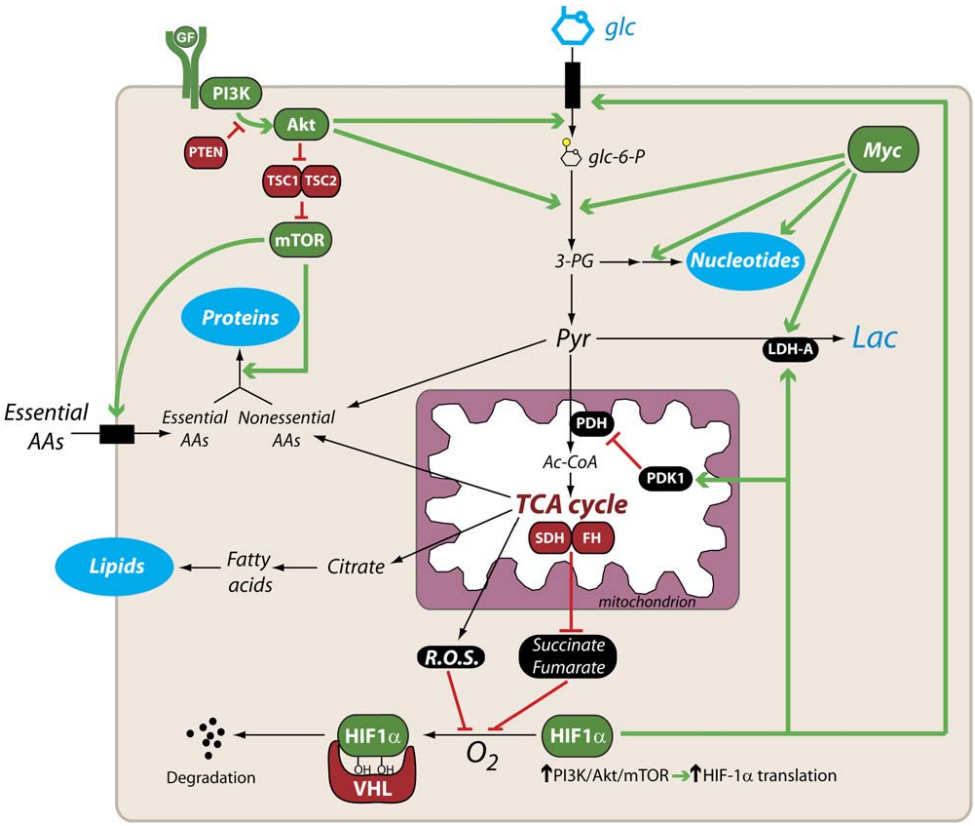

Figure 9. Major signalling network to regulate metabolism in proliferating cells (from [216]). Abbreviations: 3-PG, 3-phosphoglycerate; AAs, aminoacids; Ac-CoA, acetyl-coenzyme A; FH, fumarate hydratase; GF, growth factor; glc, glucose; glc-6-P, glucose-6-phosphate; GLUT1, glucose transporter 1; HIF-1α, hypoxia-inducible factor-1alpha; Lac, lactate; LDH-A, lactate dehydrogenase A; mTOR, mammalian target of rapamycin; ROS, reactive oxygen species; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; Pyr, pyruvate; SDH, succinate dehydrogenase; TCA, tricarboxylic acid; TSC, tuberous sclerosis complex; VHL, von Hippel-Lindau.

Additionally, constitutive up-regulation of the glycolytic phenotype may occur through oncogene activation or tumour suppressor inactivation. Among others, *AKT* [248-250] and *c-myc* [251-253] oncogenes have been described as activating aerobic glycolysis (Figure 9), the last one in cooperation with HIF-1α [254-256]. Loss of the tumour suppressor p53 has also been implicated in the glycolytic phenotype [257,258].

1.2.2. ARE MCTS SUITABLE TARGETS FOR CANCER THERAPY?

The frequency and severity of tumour hypoxia and its association with malignant progression make the hypoxia-induced metabolic adaptations promising targets for cancer therapy [259]. Actually, the development of treatments that target tumour metabolism is receiving renewed attention, with several potential drugs targeting metabolic pathways currently in clinical trials (Table 3, for review see [260]). However, MCTs are not yet included in this list of metabolic targets for cancer therapy.

Table 3. Compounds targeting tumour glycolysis metabolism (adapted from [260]).

Compound	Target	Effect	Stage as anti-tumour therapy	Tumour types targeted	Study number(s)
2-deoxyglucose	Hexokinase	Inhibits glycolysis	Phase I/II	Advanced solid tumours (e.g. lung, breast, prostate and gastric)	• NCT00633087 • NCT00096707 • NCT00247403
Lonidamine	Hexokinase	Inhibits glycolysis	Phase III	Benign prostatic hyperplasia	• NCT00435448 • NCT00237536
3-bromopyruvate	Hexokinase	Inhibits glycolysis	Preclinical	N/A	N/A
TLN-232	Pyruvate kinase	Inhibits glycolysis	Phase II	Metastatic melanoma and renal cell carcinoma	NCT00735332
Dichloroacetate	PDK1	Reactivates PDH	Phase I/II	Metastatic solid tumours, glioma and GBM	• NCT00540176 • NCT00566410 • NCT00703859
Phenylacetate	Glutamine	Reduces plasma glutamine levels	Phase II	Brain tumours (e.g. glioma, astrocytoma and medulloblastoma)	• NCT00003241 • NCT00006450 • NCT00001565
Acetazolamide, Indisulam and other sulfonamides	Carbonic anhydrases	pH regulation	Phase II	Solid tumours (e.g. pancreatic, lung, melanoma and metastatic breast)	• NCT00060567 • NCT00165594 • NCT00165880 • Others
Cariporide	NHE1	pH regulation	Preclinical	N/A	N/A
Perifosine and GSK690693	AKT	Inhibits AKT	Phase I/II	Solid tumours (e.g. renal cancer and NSCLC) and lymphoma	• NCT00399789 • NCT00399152 • NCT00493818
PX-478	HIF1α	Inhibits HIF signalling	Phase I	Advanced solid tumours and lymphoma	NCT00522652
Acriflavine	HIF1α	Inhibits HIF signalling	Preclinical	N/A	N/A
Tirapazamine and other bioreductive compounds	Hypoxia	Resensitizes cells to other treatments	Phase III	Solid tumours (e.g. cervical, SCLC and NSCLC)	• NCT00033410 • NCT00098995 • NCT00017459
Bevacizumab and related compounds	Hypoxia, VEGF and VEGFR	Blocks angiogenesis	US FDA approved	Solid tumours (e.g. malignant glioma, NSCLC, ovarian and colorectal)	>100
MK-0646, BIIB022, AVE1642 and others	IGF1R	Blocks IGF signalling	Phase I/II	Solid tumours (e.g. NSCLC, pancreatic, hepatocellular carcinoma and metastatic breast)	• NCT00799240 • NCT00555724 • NCT00791544 • Others

As mentioned, the acid-resistant phenotype is essential for cancer cell survival. Hence, different pH regulating systems are present in the plasma membrane of cancer cells (Figure 10), including the Na^+/H^+ exchanger 1 (NHE1), CAIX and AE1. Intracellular H^+ ions are primarily extruded by NHE1, while lactic acid together with H^+ are exported by MCTs, in particular MCT1 and MCT4. CAIX, as already described, works in collaboration with AE1 to trap H^+ ions in the extracellular space (for review see [261]). Although MCTs are not the major H^+ transporters, they perform a double role in the adaptation to hypoxia: export of lactate, essential to the hyper-glycolytic phenotype, and pH regulation, important to the acid-resistant phenotype. Thus, MCTs appear as very promising targets in cancer cells with hyper-glycolytic acid-resistant phenotype.

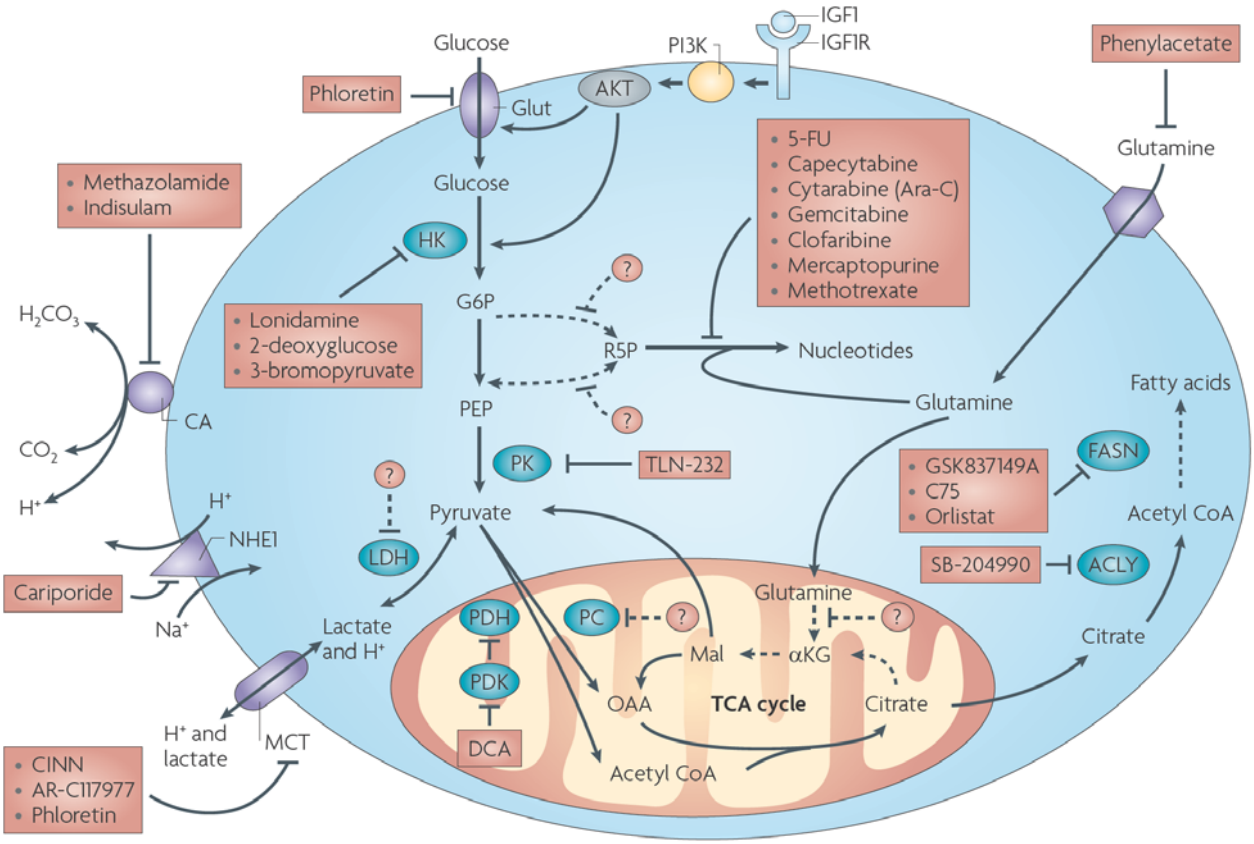

Figure 10. Therapeutic targets for manipulation of metabolism in malignant tumours (from [260]). Abbreviations: 5-FU, 5-fluorouracil; αKG, alpha-ketoglutarate; ACLY, ATP citrate lyase; CA, carbonic anhydrase; CINN, α-cyano-4-hydroxycinnamate; DCA, dichloroacetate; FASN, fatty acid synthase; G6P, glucose-6-phosphate; Glut, glucose transporter; HK, hexokinase; IGF1, insulin-like growth factor 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.

1.2.2.1. LACTATE AS A CONTRIBUTOR FOR THE MALIGNANT PHENOTYPE

As mentioned before, tumour acidity is associated with cancer cell invasion behaviour, i.e. increased migration, invasion and metastases. Additionally, cancer acidosis is also associated with mutagenesis/clastogenesis, radioresistance and resistance to anthracyclines [208]. It has long been known that lactic acid is the main source of tumour acidity, as hyperglycemia acidifies the tumour microenvironment [204,262,263]. Although lactate has been proven to cause acidification of the extracellular milieu [264-268], other sources of acidity, like carbon dioxide, which were described later on, probably have higher contribution to acidification of tumour microenvironment [264-267]. Although sharing its role as tumour acidifier, lactate has other properties which contribute modestly to the malignant behaviour of cancer cells. As mentioned above, T cell activation is dependent on high rates of glycolysis, therefore, dependent on a rapid efflux of lactate from T cells [177]. However, if extracellular concentration of lactate is high, lactate efflux from T cells will be inhibited. This is the case of the tumour micromilieu and, as a consequence, T cell metabolism and function will be disturbed, decreasing the immune response against tumour cells [268]. Also, evidence shows that both lactate and pyruvate regulate hypoxia-inducible gene expression, independently from hypoxia, by stimulating the accumulation of HIF-1α [269]. This indicates that, lactate, *per se*, stimulates the hyper-glycolytic phenotype, providing a positive feed-back. Moreover, exogenous lactate was demonstrated to increase cellular motility [270], vascular endothelial growth factor (VEGF), the major angiogenic factor [271-273], as well as hyaluronan and its receptor CD44, which are molecules involved in the process of cancer invasion and metastisation [274,275]. Altogether, this evidence shows the various biological activities of lactate that can enhance the malignant phenotype of tumour cells, contributing to the association of high tumour lactate concentrations with incidence of metastases [276-279], tumour recurrence, patient survival [278,279] and radioresistance [280].

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 culminate in the production of lactate. This is the case of glutaminolysis and serinolysis [281-284]. Nevertheless, lactate will always be a metabolic end-product, either cancer cells use glycolysis or other energetic pathways for energy and biomass production.

As already mentioned, lactate, besides being an end-product of different metabolic pathways, may also be a substrate for oxidative phosphorylation. Actually, as described in skeletal muscle and in brain, a cell-cell lactate shuttle as been proposed for cancer cells. Therefore, a metabolic symbiosis between glycolytic and oxidative cancer cells was described, in which the

peripheral and oxygenated oxidative cells consume the lactate produced by the central and less oxygenated glycolytic cells [137]. As discussed below, this might have important therapeutical implications.

1.2.2.2. MCT EXPRESSION IN HUMAN SOLID TUMOURS

Giving the increased lactate and acid production by cancer cells, one can anticipate that MCTs, especially MCT1 and MCT4, as the transporters responsible for lactate efflux from cells, with a pH regulation function, are increased in tumours. Also, as already mentioned, MCTs are regulated by HIF-1α [133,135,138] and *c-myc* oncogene [129,131], which are key molecular players in the metabolic adaptations in cancer progression. Although less explored than other proteins involved in the glycolytic pathway or even than other pH regulators, reports on the importance of MCTs in cancer are becoming more frequent with years.

The first report on MCT expression in human tumour samples described a decrease of MCT1 expression (by Western blot) in colonic transition from normality to malignancy [110], which was further supported by a larger study analysing MCT1, MCT2, and MCT4 expressions by Northern blot, Western blot and, only for MCT1, immunohistochemistry, in 25 healthy colon samples, 20 adenomas and 30 carcinomas. MCT1 decrease was confirmed, while MCT2 and MCT4 protein expression was not detected, despite mRNA expression of MCT4 [285]. However, evidence from Koukourakis and collaborators [286] showed a clear and strong membranous expression of MCT1 in cancer cells in all the 70 colorectal carcinomas analysed but not in the 20 normal colonic samples. These contradictory results are probably due to antibody specificity, with special attention to the fact that the first immunohistochemical study failed to show MCT1 expression in the plasma membrane of cancer cells, which is essential for plasma membrane lactate efflux. Koukourakis and collaborators also found MCT1 expression in tumour-associated fibroblasts, favouring absorption of the accumulating lactate from the extracellular matrix, to be used as energy source, as well as lack of endothelial MCT1, to avoid lactate absorption and vascular destruction by acidosis. Additionally, MCT2 was strongly expressed in the cytoplasm of cancer cells and tumour-associated fibroblasts, indicating a possible role of MCT2 in the mitochondrial uptake of pyruvate. Finally, MCT4 was weakly expressed in the tumour micromilieu, suggesting a minimal role in the metabolic intratumoural communication. Besides MCTs, other relevant metabolic proteins were studied and a model of complementary metabolism between cancer cells, tumour-associated fibroblasts and vessels is proposed (Figure 11) [286]. A

similar metabolic cooperation between lung cancer cells and tumour-associated stroma was described, where overexpression of MCT1 was found in all tumours examined, with MCT2 and MCT4 also expressed in cancer cells. Tumour-associated stroma weakly expressed MCTs while no expression of MCTs was found in normal lung [287]. MCT1, in association with its chaperone CD147, was also described in alveolar soft part sarcoma [148].

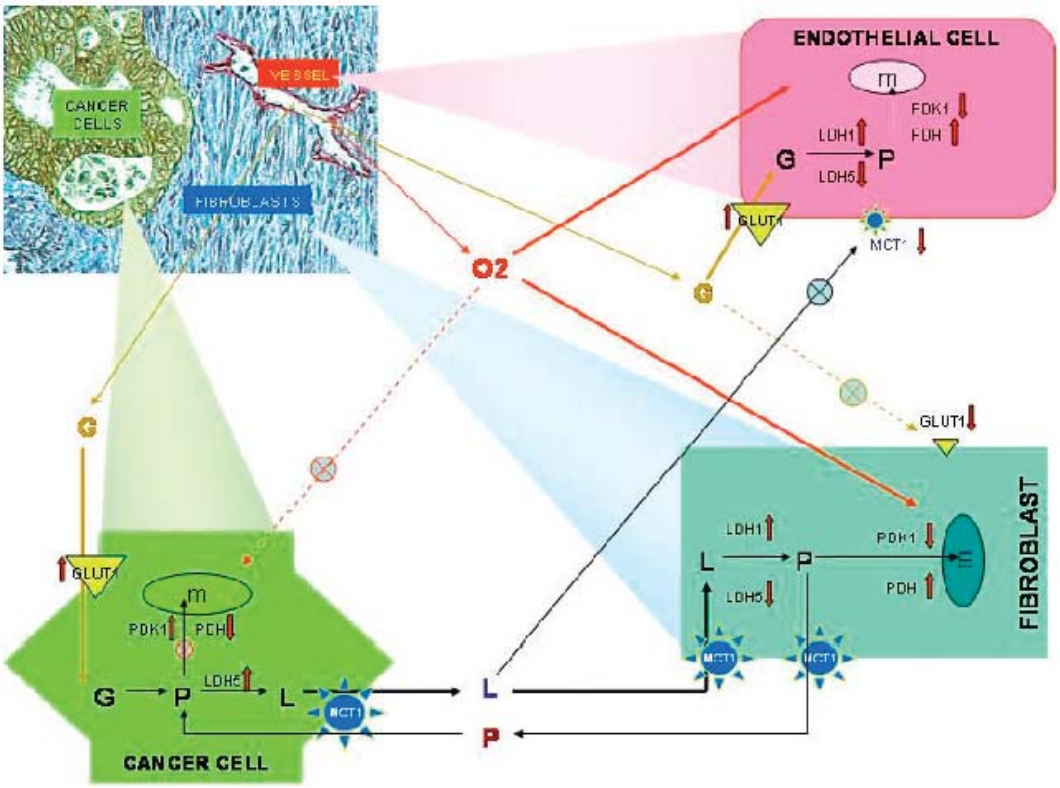

Figure 11. Model of metabolic cooperation between cancer cells, tumour-associated fibroblasts and endothelial cells (from [286]). **Step 1**, glucose (G) reaches cancer cells through the tumour-associated vasculature followed by absorption by glucose transporter 1 (GLUT1). Glucose absorption by stromal fibroblasts is much lower, while, endothelial cells absorb glucose directly from the blood. **Step 2**, in cancer cells, glucose is transformed into pyruvate (P) and subsequently to lactate (L). Cancer cells may, therefore, have minimal requirements for oxygen so that oxygen use is reduced. **Step 3**, the high concentrations of lactate in the cancer cell cytoplasm is rapidly extruded to the extracellular matrix through the intense activity of the monocarboxylate transporter 1 (MCT1). **Step 4**, the high expression of MCT1 in stromal fibroblasts results, under low pH conditions, in intense absorption of lactate that is eventually used as a fuel to acquire energy after its oxidation back to pyruvate. Aerobic metabolism is, therefore, the main source of energy acquired by fibroblasts; thus, the oxygen diffused from the tumour-associated vessels is used mainly by the stroma and not by cancer cells. **Step 5**, excess pyruvate production within fibroblasts creates a gradient between cytoplasm and extracellular matrix, with MCT1 exporting pyruvate that can be used subsequently by cancer cells as a fuel, ending again in lactate production. Abbreviations: LDH, lactate dehydrogenase; m, mitochondria; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase.

In neoplastic human tissues of the central nervous system, strong expression of MCT1 was found in ependymomas, hemangioblastomas and high grade glial neoplasms (anaplastic astrocytomas and glioblastoma multiforme (GBM)), whereas low-grade glial neoplasms (oligodendrogliomas and astrocytomas) were either negative or showed weak MCT1 expression. MCT1 expression was also present in microvessels and ependymocytes of normal tissues [288]. Additionally, Western blot analysis in total protein extracts from normal brain and primary brain tumours (GBMs) demonstrated that normal brain predominantly expressed MCT3, whereas MCT1 and MCT2 were the major isoforms present in GBM tumours. MCT4 was not detected in any of the tumour tissues [289]. A more recent study on a sympathetic nervous system tumour, neuroblastoma, showed, by mRNA quantification, that MCT1 expression in this type of tumour is also high and is associated with age >1 year at diagnosis, stage 4 disease, unfavourable Shimada histopathology, DNA diploid index, *n-myc* amplification and high-risk clinical group (Children's Oncology Group criteria) [130].

As expected, a general up-regulation of MCTs, especially MCT1, is found in solid tumours. However, evidence for MCT down-regulation is not only observed in colon carcinoma [110,285]. In fact, silencing of *SLC16A1* by gene promoter hypermethylation in 4 of 20 breast cases (20%) is suggested, however, the resultant decrease of mRNA and protein were not demonstrated [290].

1.2.2.3. MCT TARGETING IN TUMOUR MODELS

Inhibition of MCTs will have a direct effect on monocarboxylate transport, as well as on pH homeostasis, therefore having an important effect on cancer cell viability. Also, considering the above mentioned cell-cell lactate shuttle in cancer cells, where MCT1 has a crucial role as the gatekeeper of metabolic symbiosis of cancer cells, by importing lactate into oxidative cells, targeting of MCT1 will have important implications in cancer homeostasis (Figure 12). Blocking the capacity of aerobic cells to use lactate will force them to use glucose, depriving hypoxic tumour cells of adequate amounts of glucose and, therefore, favouring hypoxic cell death. Radiotherapy is then particularly suited to eliminate the remaining oxygenated cells in the vicinity of blood cells [137]. Finally, taking into account all the biological activities of lactate that can enhance the malignant phenotype, together with the fact that MCTs' up-regulation has been described in some tumours, MCT inhibition may be a useful therapeutic approach in cancer, counteracting lactate effects and, therefore, among others, increase the immune response against tumour cells and decrease migration capacity of cells.

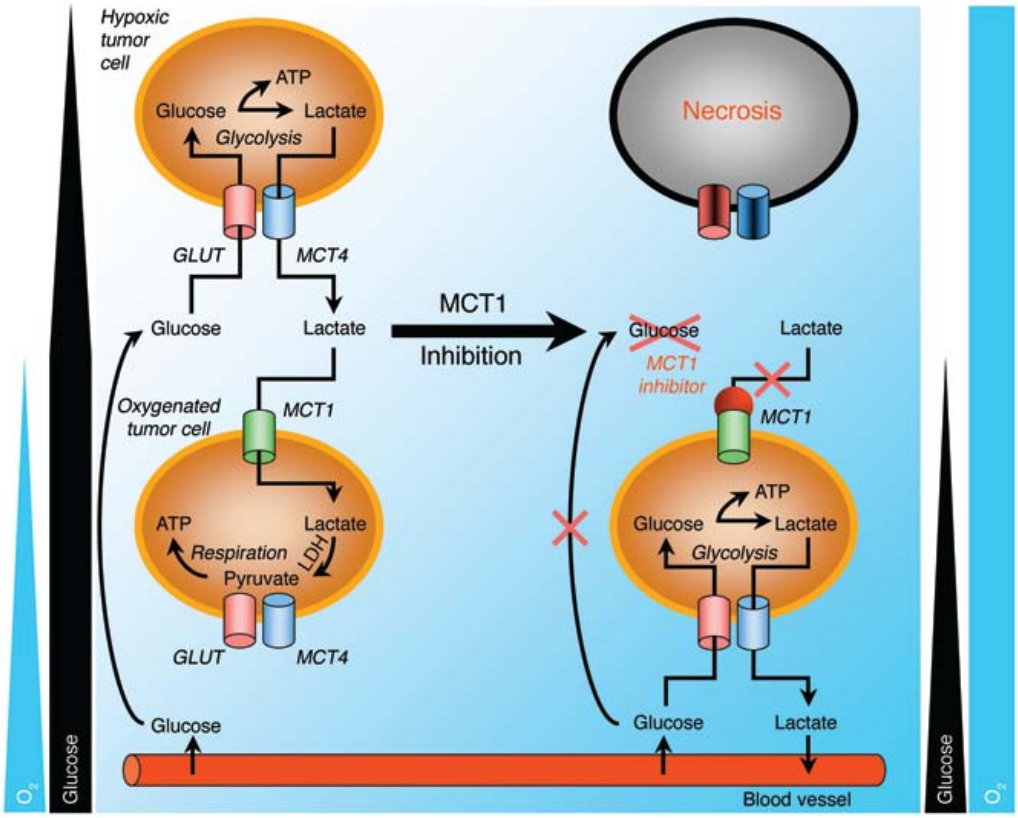

Figure 12. Model for therapeutic targeting of lactate-based symbiosis in tumours (from [137]). Hypoxic tumour cells depend on glucose and glycolysis to produce energy. Lactate, the end-product of glycolysis, diffuses along its concentration gradient towards blood vessels. In contrast, oxygenated tumour cells import lactate through monocarboxylate transporter 1 (MCT1) and oxidise it to produce energy. In the respiration process, lactate is preferred over glucose. As a consequence, glucose freely diffuses through the oxygenated tumour cells to fuel glycolysis of distant, hypoxic tumour cells. This metabolic symbiosis can be disrupted by MCT1 inhibition. Upon MCT1 inhibition, oxidative tumour cells switch from lactate oxidation to glycolysis, thereby preventing adequate glucose delivery to glycolytic cells, which die from glucose starvation. This glycolytic switch is associated with a decrease in oxygen consumption of surviving tumour cells, which is responsible for increased tumour pO_2. MCT1 inhibition is thus a potent antitumour strategy that indirectly eradicates hypoxic/glycolytic tumour cells. Abbreviation: GLUT, glucose transporter.

Actually, it was demonstrated that MCT1 inhibition decreases intracellular pH [130,137,172], leads to *in vitro* cell death [130,137,172,173,289] and enhances cancer cell radiosensitivity [173]. Additionally, silencing of MCT4 results in decreased cancer cell migration [151], by mechanisms that also involve interaction of MCT4 with β_1-integrin [291]. Importantly, promising results using *in vivo* models have also been reported, where administration of CHC retarded tumour growth and rendered tumour cells sensitive to radiation [137].

Over the last few years, different approaches have been used to disrupt the function of tumour MCTs, including the already mentioned CHC [130,137,172,173] and lonidamine [130]. Additionally, since these inhibitors have also other targets rather than MCTs, small-interfering RNAs (siRNAs) have also been used to inhibit MCT activity in a more direct manner [130,137,289]. The use of MCT1 specific inhibitors designed by AstraZeneca may also be an effective strategy to block MCT1 tumour activity both *in vitro* and *in vivo* and, perhaps, may also be adequate compounds to use in the clinical context. As CD147 has an important role in MCT trafficking to the plasma membrane and MCT activity [18,146,149-155], indirect MCT inhibition through CD147 has also proven to have the ability of reducing the malignant potential of cancer cells [152-154].

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Chapter 2. MCTs IN COLORECTAL CARCINOMA

2.1. CHAPTER OVERVIEW

It is known that butyrate plays an important role in the maintenance of colonic homeostasis. This short-chain fatty acid is implicated in the regulation of colonic mucosa growth and *in vitro* cell proliferation and differentiation, involving the activation of several differentiation-specific genes. Additionally, butyrate has a potent effect in inhibiting inflammation and carcinogenesis (for review see [1]). Transport of butyrate into the colonic epithelial cells is mostly done through MCT1 [2], conferring to this MCT isoform a major role in colonic homeostasis.

The multistep colon carcinogenesis is generally known to result from the accumulation of molecular genetic alterations, mainly activation of oncogenes; inactivation of tumour-suppressor genes; and abnormalities in genes involved in DNA mismatch repair [3]. However, other alterations have been described that point to an important metabolic switch from butyrate β-oxidation to glycolysis [4], including up-regulation of GLUT1 [5,6] and production of high levels of glycolytic metabolites, such as lactate [7].

As mentioned previously, data on MCT1 expression in colorectal cancer is quite controversial with reports showing a decrease of MCT1 in the transition from normality to malignancy [5,8], as well as absence of MCT2 and MCT4 expression in colon carcinoma [5], while another describes an increase of MCT1 in colorectal carcinoma, as well as a strong expression of MCT2 in cancer cells cytoplasm, when comparing to normal colonic tissues [6]. In this regard, the evaluation of MCT1, as well as other MCT isoforms in colorectal carcinoma compared to non-neoplastic tissues, will give an important contribution to the understanding of the adaptations that occur toward the metabolic switch occurring in colon carcinogenesis.

In this chapter, MCT1, MCT2 and MCT4 expressions were evaluated in a comprehensive series of 126 colorectal samples. In this study, already published in an international scientific periodical with referees, a significant increase of MCT1, MCT2 and MCT4 expressions was observed. Importantly, while the increase in MCT1 and MCT4 was associated with an increase in cancer cell plasma membrane expression, in the case of MCT2, the increase in cytoplasm expression was accompanied by a loss of plasma membrane expression, pointing to a possible role of MCT2 in an intracellular organelle, possibly mitochondria. Importantly, analysis of MCT expression in regard to the clinic-pathological parameters showed associations of MCT1 plasma membrane expression with vascular invasion as well as a borderline association of MCT4 overall expression with smaller tumours, which will need further confirmation.

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2.2. PUBLISHED RESULTS

The results presented in this chapter were **published** in an international scientific periodical with referees:

Pinheiro C, Longatto-Filho A, Scapulatempo C, Ferreira L, Martins S, Pellerin L, Rodrigues M, Alves VA, Schmitt F, Baltazar F (2008). Increased expression of monocarboxylate transporters 1, 2 and 4 in colorectal carcinomas. *Virchows Arch* 452:139-146.

The results presented in this chapter were also presented as oral communication in the following national scientific meeting:

XLI Congresso Sociedade Portuguesa de Microscopia 2006, Braga, Portugal. ***Pinheiro C****, Longatto A, Ferreira L, Scapulatempo C, Alves VAF, Milanezi F, Schmitt F, Baltazar F. Characterization of monocarboxylate transporters in colorectal carcinomas.*

Additionally, these results were presented as poster in the following international scientific meetings:

Twenty-seventh International Congress of the International-Academy-of-Pathology, 2008, Athens, Greece. Scapulatempo C, Longatto A, Simoes K, ***Pinheiro C****, Schmitt F , Baltazar F, Alves V. Apoptosis detected by cleaved cytokeratin 18 (M30) immunohistochemistry in colorectal cancer is related to acidic tumoral microenviroment. Abstract* ***published*** *in conference proceedings (Histopathology 53 (Sp. Iss. 1):159-160);*

Twenty-first European Congress of Pathology, 2007, Istambul, Turkey. ***Pinheiro C****, Longatto-Filho A, Ferreira L, Scapulatempo C, Alves VAF, Pellerin L, Schmitt F, Baltazar F. Altered expression of monocarboxylate transporters 1, 2 and 4 in colorectal carcinomas. Abstract* ***published*** *in conference proceedings (Virchows Arch 451:325);*

Tenth Cancer Research UK Beatson International Cancer Conference, 2007, Glasgow, United Kingdom. ***Pinheiro C****, Longatto-Filho A, Ferreira L, Scapulatempo C, Pellerin L, Alves VAF, Schmitt F, Baltazar F. Monocarboxylate transporters as new targets for colorectal cancer therapy?*

2.2.1. INCREASED EXPRESSION OF MONOCARBOXYLATE TRANSPORTERS 1, 2 AND 4 IN COLORECTAL CARCINOMAS

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ORIGINAL ARTICLE

Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas

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Abstract Tumour cells are known to be highly glycolytic, thus producing high amounts of lactic acid. Monocarboxylate transporters (MCTs), by promoting the efflux of the accumulating acids, constitute one of the most important mechanisms in the maintenance of tumour intracellular pH. Since data concerning MCT expression in colorectal carcinomas (CRC) are scarce and controversial, the present study aimed to assess the expressions of MCT1, 2, and 4 in a well characterized series of CRC and assess their role in CRC carcinogenesis. CRC samples (126 cases) were analyzed for MCT1, MCT2, and MCT4 immunoexpression and findings correlated with clinico-pathological parameters. Expression of all MCT isoforms in tumour cells was significantly increased when compared to adjacent normal epithelium. Remarkably, there was a significant gain of membrane expression for MCT1 and MCT4 and loss of plasma membrane expression for MCT2 in tumour cells. Plasma membrane expression of MCT1 was directly related to the presence of vascular invasion. This is the larger study on MCT expression in CRC and evaluates for the first time its clinico-pathological significance. The increased expression of these transporters suggests an important role in CRC, which might justify their use, especially MCT1 and MCT4, as targets in CRC drug therapy.

Keywords Monocarboxylate transporter · Colorectal carcinoma

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Introduction

Highly proliferative cancer cells maintain a high rate of glycolysis, producing large amounts of acids, mainly lactic acid. Despite this fact, only the interstitial pH of tumours is low, while the intracellular pH of tumours is either normal or higher than that of normal tissues [3, 10]. In order to prevent apoptosis by cellular acidosis, tumour cells increase proton efflux through pH regulators, such as proton pumps, sodium-proton exchangers, bicarbonate transporters, and

monocarboxylate transporters (MCTs), which are described to be up-regulated in tumour cells [11].

MCTs play a central role in cellular metabolism and are essential for transport of monocarboxylates, such as lactate, across the plasma membrane. Based on sequence homologies, 14 MCT family members were identified, although only a few have been functionally characterized. MCT1–MCT4 isoforms are proton symporters, which have lactate as a common substrate, each isoform exhibiting different biochemical properties [9]. Substrate specificity has been associated with the tissue distribution of each transporter, depending on their metabolic activity: MCT1 has an ubiquitous distribution [6], while MCT2, having a high affinity for the substrate, is found in tissues that use lactate as a substrate (e.g., liver) [2]. MCT4, which is a low-affinity transporter, is present in highly glycolytic tissues (e.g., slow-twitch muscle) [4] and MCT3 is exclusively found in the retinal pigment epithelium and in the choroid plexus epithelium [16].

Using in vitro and ex vivo models, Wahl et al. [19] demonstrated that MCTs are major players in the regulation of pH in melanoma cells and proposed that MCT inhibitors may be particularly effective against malignant melanoma. There are also evidence for the upregulation of MCTs in other tumours such as alveolar sarcoma of soft tissues [13], high grade glial neoplasms [7, 15], and colorectal carcinomas (CRC) [12].

Lactate release from tumour cells is a common endpoint of several metabolic alterations, raising the hypothesis of an association with tumour progression [21]. Some evidence support this hypothesis: low interstitial pH is associated with the upregulation of various angiogenic molecules, which support tumour growth, invasion, and metastasis such as vascular endothelial growth factor [8]. Lactate accumulation has also been associated with metastization and lower disease-free and overall survival in squamous cell carcinomas of the uterine cervix and head and neck carcinomas [1, 20]. Targeting MCT activity would not only induce apoptosis due to cellular acidosis, but would also lead to reduction in tumour angiogenesis, invasion, and metastasis. Thus, MCTs can constitute an attractive target for cancer therapy.

Data concerning expression of MCTs in CRC are scarce and controversial. While Lambert et al. [14] described a decrease in MCT1 expression during transition to malignancy, Koukourakis et al. [12] reported an increase in MCT1 expression in tumour cells, which is supported by the metabolic alterations induced by anaerobic glycolysis. The latter study also assessed the expression of MCT2 and MCT4, finding a strong cytoplasmic expression of MCT2 in cancer cells, but a weak expression of MCT4 in the tumour environment, suggesting a minimal role of this MCT isoform in CRC metabolic homeostasis.

Our study aimed to contribute to the elucidation of the role of MCTs in CRC, by assessing the immunohistochemical expression of the MCT isoforms 1, 2, and 4 in a large and well characterized series of CRC and evaluating its clinico-pathological significance.

Materials and methods

A series of formalin-fixed, paraffin-embedded tissues from 126 CRC was retrieved from the files of the Medical School of the University of São Paulo (Brazil). Tissue samples were analyzed for MCT1, MCT2, and MCT4 immunohistochemical expression using specific antibodies. Comparison of MCT expression in tumour vs normal cells was also possible since, for most cases, the same paraffin section contained both tumour and normal colonic epithelium. MCT immunohistochemical expression was correlated with the available clinico-pathological data, which included: age, gender, tumour size, macroscopic presentation, histological type, tumour localization, depth of invasion, vascular invasion, nodal metastasis, and TNM classification. Normal colorectal mucosa, liver, and skeletal muscle samples were obtained prospectively from patients undergoing surgery: colorectal mucosa and abdominal wall skeletal muscle were obtained from a patient with diagnosis of diverticulosis; liver was from a patient with a diagnosis of metastatic colon carcinoma. Informed written consent was obtained from the individuals prior to removal of the tissue.

Western blotting

The specificity of the antibodies used in immunohistochemistry was evaluated by Western blotting. Tissue samples from human colon, liver, and muscle were used as positive controls for the expression of MCT1, 2, and 4, respectively. Briefly, fresh samples were collected, homogenized in a lysis buffer and then centrifuged at 6,000 rpm, 20 min at 4°C. The supernatants were collected and protein concentrations were determined by the method of Lowry. Protein was separated on a 10% (*w*/*v*) polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked with 1% Tween, 10% milk, and 1% bovine serum albumin in TBS for 1 h. Membranes were then incubated overnight at 4°C with primary polyclonal antibodies for MCT1 (AB3538P, Chemicon International, Temecula, CA, USA), MCT2 (sc-14926, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and MCT4 (AB3316P, Chemicon International), diluted 1:200 for MCT1 and MCT4, and 1:100 for MCT2. Membranes were then incubated with the secondary

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antibody coupled to horseradish peroxidase (SantaCruz Biotechnology) and bound antibodies were visualized by chemiluminescence (Supersignal West Femto kit, Pierce, Rockford, IL, USA).

Immunohistochemistry

Immunohistochemistry was performed according to avidin–biotin–peroxidase complex principle (R.T.U. VECTASTAIN Elite ABC Kit (Universal), Vector Laboratories, Burlingame, CA, USA), with the primary antibodies for MCT1, MCT2, and MCT4, used in Western blotting, diluted 1:200. Briefly, deparaffinized and rehydrated sections were incubated in 0.3% hydrogen peroxide for 30 min, to inactivate endogenous peroxidases, and washed in PBS. Antigen retrieval was performed by immersing slide-mounted sections in 0.01 M citrate buffered solution (pH 6.0) and microwaving (600 W) for 15 min then washed in PBS. Tissue sections were then incubated with a protein blocking solution for 20 min and incubated with the primary antibody overnight at room temperature. Sections were then sequentially washed in PBS and incubated with biotinylated secondary antibody for 30 min, R.T.U. Vectastain® Elite ABC reagent for 45 min at 37°C and developed with 3,3′-diamino-benzidine (DAB+ Substrate System, DakoCytomation, Carpinteria, CA, USA) for 10 min. Negative controls were performed by using the adequate serum controls for the primary antibodies used (N1699 and X0907, DakoCytomation, Carpinteria, CA, USA). Normal colon, kidney, and skeletal muscle tissues were used as positive controls for MCT1, MCT2, and MCT4, respectively. Tissue sections were counterstained with haematoxylin and permanently mounted.

Immunohistochemical evaluation

Sections were scored semi-quantitatively for immunoreaction extension as follows: 0: 0% of immunoreactive cells; 1: <5% of immunoreactive cells; 2: 5–50% of immunoreactive cells; and 3: >50% of immunoreactive cells. Also, intensity of staining was scored semi-qualitatively as 0: negative; 1: weak; 2: intermediate; and 3: strong. Immunoreaction final score was defined as the sum of both parameters (extension and intensity), and grouped as negative (0), weak (2), moderate (3), and strong (4–6). For statistical purposes, only moderate and strong immunoreaction final scores were considered as positive. Finally, positive plasma membrane staining was also assessed. Evaluation of MCT immunohistochemical expression was performed blindly by two independent observers and discordant cases were discussed in a double-head microscope in order to determine a final score.

Fig. 1 Western blotting of human tissue lysates for *MCT1*, *2*, and *4*. Normal colon, liver, and skeletal muscle were used for *MCT1*, *2*, and *4* detection, respectively

Statistical analysis

Data were stored and analyzed using the SPSS statistical software (version 14.0, SPSS Inc., Chicago, IL, USA). The comparison of MCT expression between tumour and normal cells as well as the relationship between MCT expressions and the clinico-pathological parameters were examined for statistical significance using Pearson's chi-square (χ^2) test, with the threshold for significant p values being <0.05.

Results

One hundred and twenty six cases of CRC, corresponding to 56 women and 70 men with a median age of 64 (range 24–95), were analyzed for MCT1, MCT2, and MCT4 immunohistochemical expression. Western blot analyses of normal human tissues, using the antibodies for the three MCT isoforms, revealed protein bands around 50 kDa (Fig. 1), consistent with the molecular weight described for these membrane proteins [7].

Table 1 summarizes the frequency of MCT isoforms 1, 2, and 4 expressions in tumour cells and normal adjacent epithelium. In positive cases, cytoplasmic staining was always present while the frequency of plasma membrane staining differed for each isoform, being more frequent for

Table 1 Pattern of MCT staining in tumour vs normal adjacent epithelium

Isoform	*n*	Immunoreaction		Plasma membrane	
		Positive (%)	*p*	Positive (%)	*p*
MCT1			<0.001		<0.001
Normal	86	34.9		9.3	
Tumour	126	85.7		51.6	
MCT2			0.017		0.031
Normal	84	83.3		14.3	
Tumour	126	93.7		5.6	
MCT4			<0.001		<0.001
Normal	89	55.1		9.0	
Tumour	126	96.0		38.1	

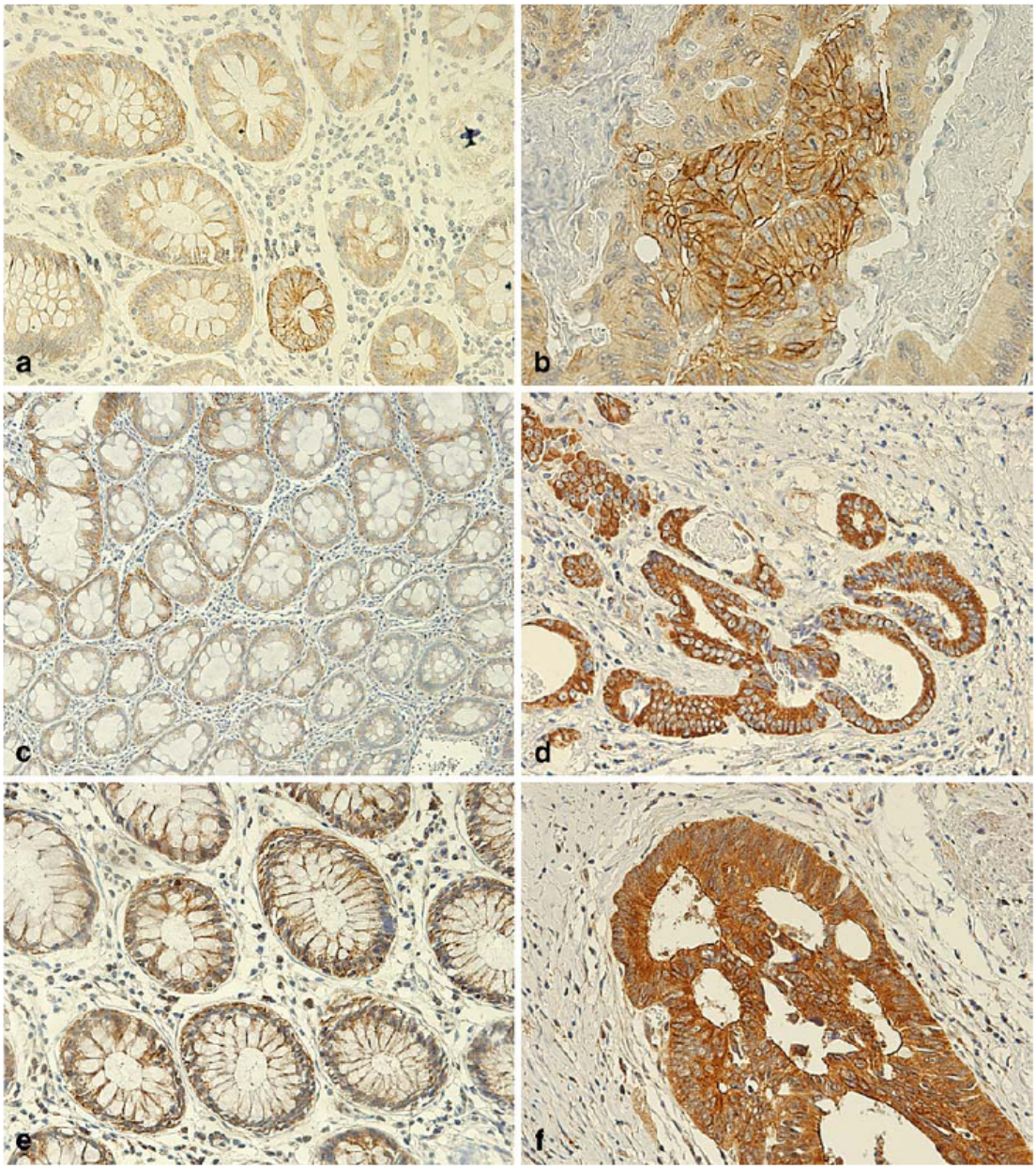

Fig. 2 Immunohistochemical expression of MCT1, 2, and 4 in colon carcinoma samples. Staining in tumour cells (**b**, **d**, and **f**: MCT1, 2, and 4, respectively) was stronger than in the adjacent normal epithelium (**a**, **c**, and **e**: MCT1, 2, and 4, respectively). MCT1 plasma membrane staining (**b**) was frequently observed in tumour cells

MCT1 than for the other isoforms (51.6% vs 38.1% for MCT4 vs 5.6% for MCT2).

MCT1 immunoexpression in tumour cells was significantly increased, when compared to adjacent normal epithelium (85.7% vs 34.9%, $p<0.001$, Table 1). Importantly, plasma membrane staining was also significantly more frequent in tumour than in normal cells (51.6% vs 9.3%, $p<0.001$, Table 1). Plasma membrane expression of MCT1 (Fig. 2) was directly related to the presence of vascular invasion ($p=0.003$, Table 2).

Staining for MCT2 in tumour cells was also more frequent than in the adjacent normal epithelium (93.7% vs 83.3%; $p=0.017$, Table 1). Remarkably, there was a significant loss of plasma membrane staining in tumour cells (14.3% vs 5.6%; $p<0.001$, Table 1). Although it was possible to observe "granule" like cytoplasmic staining for all MCT isoforms, this pattern was much more common for MCT2 in tumour cells (Fig. 2).

MCT4 was the most frequently expressed isoform in tumour cells, with 96% of the cases showing positive

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Table 2 Assessment of correlation between MCTs membrane expression and the clinico-pathological data

Clinical data	Plasma membrane						
	MCT1			MCT2		MCT4	
	n	Positive (%)	*p*	Positive (%)	*p*	Positive (%)	*p*
Tumour size (cm)			0.897		0.117		0.572
<5	53	50.9		1.9		35.8	
≥5	71	52.1		8.5		40.8	
Macroscopic type			0.826		0.360		0.548
Exophytic	26	46.2		0.0		30.8	
Ulcerative	49	55.1		8.2		40.8	
Infiltrative	12	58.3		0.0		25.0	
Sessile-ulcerated	39	48.7		7.7		43.6	
Histological type			0.563		0.551		0.586
Adenocarcinoma	111	50.5		5.4		38.7	
Mucinous	10	60.0		10.0		30.0	
Localization[a]			0.261		0.474		0.923
Right colon	21	42.9		4.8		38.1	
Left colon	40	45.0		2.5		40.0	
Rectum	61	59.0		8.2		36.1	
Invasion			0.251		0.208		0.829
T1+T2	22	40.9		0.0		36.4	
T3+T4	103	54.4		6.8		38.8	
Vascular invasion[b]			0.003		0.090		0.400
Absent	57	36.8		1.8		42.1	
Present	69	63.8		8.7		34.8	
Nodal metastasis			0.212		0.697		1.000
Absent	63	46.0		4.8		38.1	
Present	63	57.1		6.3		38.1	
TNM			0.408		0.722		0.276
Stage I	16	43.8		0.0		31.3	
Stage II	35	42.9		5.7		40.0	
Stage III	38	60.5		7.9		28.9	
Stage IV	36	55.6		5.6		50.0	

[a] Tumour localization was grouped as follows: right colon—cecum and ascendant colon; left colon—splenic flexure, descendant colon, sigmoid and rectosigmoid.
[b] Vascular invasion includes both lymph and blood vessel invasion.

staining, and expression was significantly more frequent than adjacent normal epithelium ($p<0.001$, Table 1). Figure 2 shows representative cases of MCT4 positive staining in tumour cells and in adjacent normal epithelium. As observed for MCT1, MCT4 plasma membrane staining in tumour cells was significantly more frequent than in the adjacent normal epithelium ($p<0.001$, Table 1). Assessment of correlation between MCT4 expression and the clinico-pathological data revealed a positive association between MCT4 positivity and small size tumours ($p=0.049$, Table 3).

Discussion

Experimental evidence point at MCTs as potential targets for cancer therapy [5, 15, 19]. However, the role of these membrane proteins in solid tumour development and survival is not fully understood. In the present study, we showed an evident increase in MCT immunoexpression in CRC cells when compared to adjacent normal epithelium. Most importantly, we evaluated the correlation between MCT expression in CRC and clinico-pathological data for the first time.

Although MCT1 is described as having a ubiquitous distribution, it appears that this MCT isoform plays an important role in the bidirectional transport of lactate and other short-chain fatty acids across the colonic luminal membrane [17]. Unexpectedly, when analysing MCT expression in the available adjacent normal epithelium, we found that MCT1 was the least expressed isoform. However, since these normal tissue areas are in the surroundings of tumour cells, we cannot rule out the possibility that these areas are under the influence of

Table 3 Assessment of correlation between MCTs immunoreaction and the clinico-pathological data

Clinical data	Immunoreaction					
	N	MCT1		MCT2		MCT4	
		Positive (%)	*p*	Positive (%)	*P*	Positive (%)	*p*
Tumour size (cm)			0.383		0.117		0.049
<5	53	88.7		98.1		100.0	
≥5	71	83.1		91.5		93.0	
Macroscopic type			0.267		0.967		0.480
Exophytic	26	76.9		92.3		100.0	
Ulcerative	49	89.8		93.9		93.9	
Infiltrative	12	75.0		91.7		91.7	
Sessile-ulcerated	39	89.7		94.9		97.4	
Histological type			0.509		0.413		0.542
Adenocarcinoma	111	87.4		93.7		96.4	
Mucinous	10	80.0		100.0		100.0	
Localization[a]			0.586		0.714		0.900
Right colon	21	81.0		90.5		95.2	
Left colon	40	82.5		95.0		95.0	
Rectum	61	88.5		95.1		96.7	
Invasion			0.220		0.570		0.292
T1+T2	22	77.3		90.9		100.0	
T3+T4	103	87.4		94.2		95.1	
Vascular invasion[b]			0.942		0.081		0.810
Absent	57	86.0		89.5		96.5	
Present	69	85.5		97.1		95.7	
Nodal metastasis			0.611		1.000		0.648
Absent	63	84.1		93.7		95.2	
Present	63	87.3		93.7		96.8	
TNM			0.610		0.591		0.238
Stage I	16	75.0		87.5		100.0	
Stage II	35	85.7		97.1		91.4	
Stage III	38	86.8		92.1		94.7	
Stage IV	36	88.9		94.4		100.0	

[a] Tumour localization was grouped as follows: right colon—cecum and ascendant colon; left colon—splenic flexure, descendant colon, sigmoid and rectosigmoid.
[b] Vascular invasion includes both lymph and blood vessel invasion.

tumour microenvironment and could show altered MCT expression. It would then be important to further analyze MCT expression in a series of normal colorectal samples. Data concerning MCT1 expression in solid tumours is scarce and there are conflicting data related to CRC [12, 14, 17]. Our immunohistochemical findings are in agreement with the results of Koukourakis et al. [12]. Although using different antibodies from this previous study, we were also able to detect a high percentage of positive cases for MCT1 expression as well as MCT1 plasma membrane expression in a high number of cases. However, these findings diverge from previous reports, where the authors described a decrease in MCT1 expression in CRC during transition from normality to malignancy [14, 17]. Concerning the clinico-pathological data, we found a significant correlation between MCT1 plasma membrane staining and vascular invasion. MCT1 plasma membrane localization is essential for transporter activity and, assuming a role in the efflux of accumulating acids, MCT1 activity would lead to extracellular acidification. Then, the positive correlation between MCT1 plasma membrane staining and vascular invasion would be in harmony with previous reports which stated that extracellular acidification induces invasion [10].

Data concerning MCT2 expression in solid tumour is also limited. Contrasting with the results presented by Lambert et al. [14], we found expression of MCT2 in both adjacent normal epithelium and tumour cells in CRC. The staining pattern was cytoplasmic and, in a low number of cases, associated with the plasma membrane. Notably, we found a significant decrease in plasma membrane staining in tumour cells, when comparing to adjacent normal epithelium. This finding is in agreement with the results of Koukourakis et al. [12], who described lack of plasma membrane expression in tumour cells but a strong cyto-

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plasmic expression. The authors hypothesized that this cytoplasmic expression was probably associated with the presence of MCT2 in mitochondrial membranes. Taking into consideration the physiological role of MCT2 in the transport of pyruvate into the mitochondria, one could hypothesize that the highly glycolytic tumour cell would up-regulate MCT2 in an attempt to oxidize the excess pyruvate produced. This process would also contribute to the "removal" of the acid accumulated in the cytoplasm.

Concerning MCT4, we found it to be the most frequently expressed isoform in tumour cells, an expression which was significantly increased when compared to the adjacent normal epithelium. These findings contrast with the results of Lambert et al. [14], who did not find MCT4 expression in colon carcinoma and with the results of Koukourakis et al. [12], who described weak MCT4 expression in the tumour environment. This discrepancy could be related to the specificity of the antibodies used. In our study, the frequency of MCT4 staining in tumour cells was significantly different from the adjacent normal epithelium, which, together with the Western blot results, led us to believe in the specificity of the antibody used. We also found an increase in MCT4 plasma membrane staining in tumour cells when comparing with the adjacent normal epithelium. This fact supports the hypothesis that MCTs are important in the efflux of accumulated acids in tumour cells, specially MCT4 which, due to its low affinity for lactate, would be important in highly glycolytic cells [4]. We can thus postulate that MCT4 presence in the plasma membrane will allow continuous glycolytic rate (by "removing" the end product), prevention of apoptosis due to intracellular acidification and contribution to cancer cell survival. Evaluation of the correlation between MCT4 expression and clinico-pathological data revealed positive associations with tumour size, however the correlation is weak ($p=0.049$). Nevertheless, assuming that the glycolysis rate in smaller tumours is higher than in bigger tumours [18], and considering the role of MCT4 in lactate efflux, it is understandable that MCT4 activity would not be so crucial in bigger-sized tumours.

One of the most notable findings in this study was the significant gain of MCT1 and MCT4 plasma membrane expression and loss of MCT2 plasma membrane expression in cancer cells. Since MCTs need to be in the plasma membrane in order to export the accumulating acids in highly glycolytic cells, and taking into consideration the substrate affinities of the different isoforms, MCT1 and MCT4 expression in the membrane is in agreement with the metabolic alterations observed in cancer cells. On the other hand, MCT2, which is involved in the uptake of monocarboxylates into the cells in normal cell metabolism [2], does not appear to have an important role in highly glycolytic cancer cells.

This is the most comprehensive study on MCT expression in CRC and the above findings point to MCTs, specially MCT1 and MCT4, as playing an important role in CRC. MCT aberrant expression might justify future studies using these proteins as targets in CRC drug therapy. So far, however, the level of human toxicity and efficacy of the known MCT inhibitors are not currently known [7] and the search for effective and safe inhibitors would be of great value.

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Conflict of interest statement We declare that we have no conflict of interest.

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Chapter 3. MCTs IN CERVICAL CARCINOMA

3.1. CHAPTER OVERVIEW

Cervical carcinogenesis is probably the best defined carcinogenic mechanism. In fact, it is widely accepted that cervical carcinogenesis is triggered by human papillomavirus (HPV) infection, which, present in virtually all cervical tumours, is a necessary cause for cervical cancer development [1]. Although the glycolytic metabolism has been associated with cervical tumours since long time ago [2,3], it was recently demonstrated that HPV-16 E7 oncogene is associated with increased glutaminolysis [4] as well as an increase in transketolase-like enzyme 1, a crucial enzyme of the non-oxidative pathway of the pentose phosphate pathway [5,6]. However, although the relevance of glycolysis in cervical neoplasia is still unknown, lactate, which can result from, besides glycolysis, glutaminolysis and other metabolic pathways [4,7-9], continues being a metabolic end-product that needs to be transported out of the cell.

Despite the important role of lactate in cervical cancer, where high lactate levels have been described as predictor of poor prognosis [10,11], expression of MCTs has not yet been described in human cervical carcinoma samples.

In the following studies, published in international scientific periodicals with referees, the expressions of MCT1, MCT2 and MCT4, as well as the MCT1/MCT4 chaperone CD147, were assessed in a large series of cervical lesions, which included 29 chronic cervicitis (non-neoplastic), 30 low-grade squamous intraepithelial lesions, 32 high-grade squamous intraepithelial lesions, 49 squamous cell carcinomas, 51 adenocarcinomas, and 30 adenosquamous carcinomas of the uterine cervix. In the first study, a significant increase in overall and plasma membrane expression of MCT1 and MCT4 was observed from pre-invasive to invasive squamous lesions and from normal glandular epithelium to adenocarcinomas. For MCT2, the significant alterations in the expression along the progression to the invasive phenotype did not follow a clear increase/decrease pattern. Importantly, it was quite interesting to note that both MCT1 and MCT4 were only expressed in the parabasal and basal cell layers of the normal squamous epithelium, are lost in the upper layers, but ‘‘reappear’’ in altered cells. It seems that these altered cells recover the MCT expression phenotype of the parabasal cell layer, which represents the main proliferative pool of cells. Also, MCT2 was more frequently observed in squamous cell carcinomas, while MCT4 was more frequently observed in adenocarcinomas. Importantly, HPV-positive pre-invasive cases expressed more MCT1 and MCT4 than HPV negative pre-invasive cases, and also presented more MCT1 in plasma membrane. In the second study, CD147 expression was evaluated in the same series of cervical lesions, aiming to evaluate the association between CD147 and MCT expression and support, in human samples, the role of CD147 as a chaperone of MCT1 and MCT4. CD147 was

increased in all cervical lesions, as compared to normal corresponding tissues, and, importantly, CD147 was more frequently expressed in MCT1 and MCT4 positive cases. However, it is important to note that these associations were not between plasma membrane expressions, excluding the role in protein trafficking to the plasma membrane. Moreover, co-expression of MCT1 and CD147 was associated with lymph-node metastasis in adenocarcinomas.

In conclusion, these results point to an important role of both MCT1 and MCT4, as well as their co-expression with CD147, in cervical cancer cell metabolic and microenvironmental adaptations along progression to invasive carcinomas.

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3.2. PUBLISHED RESULTS

The results presented in this chapter were **published** in international scientific periodicals with referees:

Pinheiro C, Longatto-Filho A, Ferreira L, Pereira SM, Etlinger D, Moreira MA, Jubé LF, Queiroz GS, Schmitt F, Baltazar F (2008). Increasing expression of monocarboxylate transporters 1 and 4 along progression to invasive cervical carcinoma. *Int J Gynec Pathol* 27:568-574;

Pinheiro C, Longatto-Filho A, Pereira SM, Etlinger D, Moreira MA, Jubé LF, Queiroz GS, Schmitt F, Baltazar F (2009). Monocarboxylate transporters 1 and 4 are associated with CD147 in cervical carcinoma. *Dis Markers* 26:97-103.

The results presented in this chapter were also presented as poster in the following international scientific meetings:

Eurogin 2007, Monte Carlo, Monaco. ***Pinheiro C****, Longatto-Filho A, Moreira MAR, Queiroz GS, Oton GJB, Júnior AF, Ribeiro LFJ, Schmitt FC, Baltazar F. Altered expression of monocarboxylate transporters in pre-invasive and invasive lesions of the uterine cervix;*

Twentieth EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics, 2008, Geneva, Switzerland. ***Pinheiro C****, Longatto-Filho A, Pereira SM, Etlinger D, Jubé LF, Queiroz GS, Schmitt F, Baltazar F. CD147 expression correlates with monocarboxylate transporters 1 and 4 in cervical carcinoma. Abstract* ***published*** *in conference proceedings (EJC Supplements 6:118-119).*

3.2.1. INCREASING EXPRESSION OF MONOCARBOXYLATE TRANSPORTERS 1 AND 4 ALONG PROGRESSION TO INVASIVE CERVICAL CARCINOMA

International Journal of Gynecological Pathology
00:1–7, Lippincott Williams & Wilkins, Baltimore

Original Article

Increasing Expression of Monocarboxylate Transporters 1 and 4 Along Progression to Invasive Cervical Carcinoma

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Summary: Solid tumor cells are known to be highly glycolytic and, to prevent apoptosis by cellular acidosis, cells increase proton efflux through pH regulators, such as monocarboxylate transporters (MCTs). However, the role of these membrane proteins in solid tumor development and survival is not fully understood. We aimed to evaluate the expression of the MCT isoforms 1, 2, and 4 in a large series of cervical lesions (neoplastic and non-neoplastic) and assess its clinical-pathologic significance. The series analyzed included 29 chronic cervicitis, 30 low-grade squamous intraepithelial lesions, 32 high-grade squamous intraepithelial lesions, 49 squamous cell carcinomas, 51 adenocarcinomas, and 30 adenosquamous carcinomas of the uterine cervix. Analysis of the expression of MCT isoforms 1, 2, and 4 was performed by immunohistochemistry with specific antibodies. Immunoreactions were evaluated both qualitatively and semiquantitatively. We found a significant increase in MCT expression from preinvasive to invasive squamous lesions and from normal glandular epithelium to adenocarcinomas. This is the first study evaluating the significance of MCT expression in lesions of the uterine cervix, including invasive carcinomas, and the results found herein led us to believe that these membrane proteins are involved in the progression to invasiveness in uterine cervix carcinoma. **Key Words:** Monocarboxylate transporters—Cervical carcinoma—HPV.

Cancer cells are highly proliferative and in a hypoxic microenvironment are dependent on anaerobic glycolysis for energy. This leads to production of large quantities of lactate, which is widely believed to be the main cause of tumor acidity (1,2). As lactate release from tumor cells is a common end point downstream of several metabolic alterations, it could be hypothesized that this event is associated with tumor progression. Actually, lactate content within cervical cancer was associated with increased incidence of metastasis (3,4) and with a shorter disease-free and overall survival (4). Similar results were found in head and neck cancer (5,6). Also, low interstitial pH is associated with upregulation of various angiogenic molecules, such as vascular endothelial growth factor (7), which support tumor growth, invasion, and metastasis.

The transport of lactate across the plasma membrane is mediated by a family of proton-coupled

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monocarboxylate transporters (MCTs), which comprises 14 members. The isoforms MCT1 to MCT4 are proton symporters that exhibit different affinities for lactate, leading to different tissue expression: MCT1 has an ubiquitous distribution (8), whereas MCT2, having high affinity for the substrate, is found in tissues that use lactate for gluconeogenesis (eg, liver) (9). MCT3 is exclusively found in the retinal pigment epithelium and in the choroid plexus epithelium (10) and MCT4, which is a low-affinity transporter, is present in highly glycolytic tissues (eg, skeletal muscle) (11).

As MCTs are vital for intracellular pH homeostasis, by exporting the accumulating lactic acid (12), it is reasonable to hypothesize that these proteins may be upregulated in cancer, where high levels of lactate are produced. There is evidence for the upregulation of MCTs in tumors, such as soft tissue sarcoma (13), high-grade gliomas (14,15), colorectal carcinomas (16,17), neuroblastomas (18), and lung cancer (19), however, only one of these studies investigated the clinico-pathologic significance of MCT expression.

In cervical cancer, as already mentioned, lactate was associated with poor prognosis, however, to the best of our knowledge, no study evaluating the role of MCT expression has been performed. Therefore, the aim of this work was to study the role of MCTs in cervical cancer and its precursor lesions, by evaluating the immunohistochemical expression of the MCT isoforms 1, 2, and 4 in a large series of cervical lesions (neoplastic and non-neoplastic) and assessing its clinico-pathologic value.

MATERIALS AND METHODS

The material studied was comprised of 91 formalin-fixed paraffin-embedded samples selected from the files of Pathology Division of Adolfo Lutz Institute, São Paulo, Brazil, which included 29 cases of chronic cervicitis (herein designated as "negative" for human papillomavirus [HPV]-induced lesion), 30 cases of cervical intraepithelial neoplasia grade I (CIN 1, herein designated as low-grade squamous intraepithelial lesion [LSIL), and 32 cases of cervical intraepithelial neoplasia grades II and III (CIN2/3, herein designated as high-grade squamous intraepithelial lesion [HSIL]). Liquid-based cytology samples (DNACitoliq system, Digene Brasil, São Paulo, Brazil) of cases categorized as intraepithelial lesions were tested for high-risk human papillomavirus with Hybrid Capture II (HC2) System (Digene Brasil, São Paulo, Brazil). The protocol was performed according to the manufacturer's instructions, using the residual material of cytologic examination. We also analyzed a series of formalin-fixed, paraffin-embedded tissue samples from 130 patients with squamous cell carcinoma (SCC, n = 49), adenocarcinoma (AC, n = 51), and adenosquamous cell carcinoma (ASC, n = 30) of the uterine cervix, examined and treated at 2 Institutions: Araújo Jorge Hospital and the Pathology Department of the School of Medicine of the Federal University of Goiás, Goiania, in Goias State, Brazil. All histopathologic diagnoses were revised and categorized according to the World Health Organization classification (20). Clinico-pathologic data of the patients included age at diagnosis, lymph node and/or distant metastasis, recurrence, and overall survival.

Immunohistochemistry

Immunohistochemistry was performed according to avidin-biotin-peroxidase complex principle (R.T.U. Vectastain Elite ABC Kit [Universal], Vector Laboratories, Burlingame, CA), with the primary antibodies for MCT1 (AB3538P, Chemicon International, Temecula, CA), MCT2 (sc-14926, Santa Cruz Biotechnology, Santa Cruz, CA), and MCT4 (AB3316P, Chemicon International, Temecula, CA), diluted 1:200, as previously described (17). Briefly, deparaffinized and rehydrated slide-mounted sections were incubated in 0.3% hydrogen peroxide for 30 minutes, to inactivate endogenous peroxidases, and washed in phosphate-buffered saline. Antigen retrieval was performed by immersing sections in 0.01 mol/L of citrate-buffered solution (pH 6.0), and microwaving (600 W) for 15 minutes, and then washed in phosphate-buffered saline. Tissue sections were then incubated with protein blocking solution for 20 minutes and incubated with the primary antibody overnight at room temperature. Sections were then sequentially washed in phosphate-buffered saline and incubated with biotinylated secondary antibody for 30 minutes, R.T.U. Vectastain Elite ABC reagent for 45 minutes at 37°C, and developed with 3,3′-diaminobenzidine (DAB+ Substrate System, Dako, Carpinteria, CA) for 10 minutes. Negative controls were performed by using adequate negative controls for the primary antibodies (X0907 and N1699, Dako, Carpinteria, CA). Colon carcinoma was used as positive control for MCT1 and MCT4, whereas for MCT2 kidney was used. Tissue sections were

counterstained with hematoxylin and permanently mounted.

Immunohistochemical Evaluation

Sections were evaluated for immunoreaction, which included both cytoplasmic and membrane-positive staining. Immunoreaction extent was scored semiquantitatively as follows: 0—0% of immunoreactive cells; 1—<5% of immunoreactive cells; 2—5% to 50% of immunoreactive cells; and 3—>50% of immunoreactive cells. Also, intensity of staining was scored semiqualitatively as 0—negative; 1—weak; 2—intermediate; and 3—strong. Immunoreaction final score was defined as the sum of both parameters (extent and intensity), and grouped as negative (0), weak (2), moderate (3), and strong (4–6). For statistical purposes, only moderate and strong immunoreaction final scores were considered as positive. Finally, positive plasma membrane staining was also assessed and positive cases included all plasma membrane positive cases, with or without cytoplasmic expression. Evaluation of MCT immunohistochemical expression was performed blindly by 2 independent observers. Discordant results were discussed in a double-head microscope and a final score was agreed.

Statistical Analysis

Data were stored and analyzed using the SPSS statistical software (version 14.0, SPSS Inc, Chicago, Illinois). The comparison of MCT expression between tumor and normal cells as well as the relationship between MCT expression and the clinico-pathologic parameters were examined for statistical significance using Pearson's χ^2 test, being threshold for significance P values <0.05. Survival curve was plotted using the method of Kaplan and Meier and data compared using the logrank test, using a cut-off of 24 months. Owing to lack of information, 18 cases of SCC, 8 cases of AC, and 1 case of ASC were not included in the statistical analysis regarding the clinic-pathologic parameters.

RESULTS

In general, MCT expression was predominantly found in the cytoplasm, with some cases presenting also plasma membrane staining (Figs. 1, 2). In addition, MCT2 expression was frequently found in fibroblasts and blood vessels, whereas MCT1 and MCT4 expressions were seldom found in these structures. In the normal squamous epithelium, we observed the following expression pattern: both MCT1 and MCT4 were limited to the parabasal and basal cell layers, whereas MCT2 was observed not only in the parabasal and basal cell layers, but also in the upper layers.

Figure 2 compares the frequency of MCT isoforms 1, 2, and 4 expression in all the squamous epithelial lesions studied, as well as non-neoplastic and neoplastic glandular tissues. For statistical purposes, positive MCT immunoreaction in cervicitis (negative for HPV-induced lesions) was only considered positive if located in the squamous epithelial area. A significant increase in MCT1 and MCT4 expression in the squamous epithelium was observed in the transition from normality to malignancy ($P<0.001$, Figs. 1A–D, 2A, C). However, plasma membrane expression, essential for transporter activity, maintained this progressive increase only for MCT4 ($P<0.001$, Fig. 2C), whereas for MCT1 a loss in membrane expression was observed in SCC ($P<0.001$, Fig. 2A). Regarding MCT2 expression in the squamous epithelium, the differences observed did not follow the same pattern as MCT1 and MCT4, with cervicitis and HSIL presenting a more frequent expression than low-grade squamous intraepithelial lesion and SCC ($P<0.001$, Fig. 2B). Also, membrane expression of MCT2 was low, when compared with MCT1 and MCT4. Concerning the glandular epithelium, as we do not have intermediate lesions, it was only possible to compare neoplastic tissues (AC) with normal glandular tissue of the biopsy material. MCT4 was the most frequently expressed isoform in normal glands (Figs. 1E, 2C), followed by MCT2. Interestingly, MCT1 expression was not found in normal glandular tissue. In adenocarcinomas, all MCT isoforms presented a significantly increased expression in tumor cells, when compared with normal glandular tissue ($P<0.001$ for MCT1 and MCT2 and $P=0.002$ for MCT4, Fig. 2), whereas membrane expression was absent in the normal gland but present in tumor cells for MCT1 and MCT4 ($P<0.001$). MCT2 membrane expression was absent in both normal and tumor glandular cells. Figures 1D, F–H show representative cases of MCT expression in the different tumor histologic types studied.

Clinical outcome was available for 103 carcinoma cases (31 SCC, 43 AC, and 29 ASC) enabling the assessment of correlations with MCT expression (Table 1). The only correlations found were with

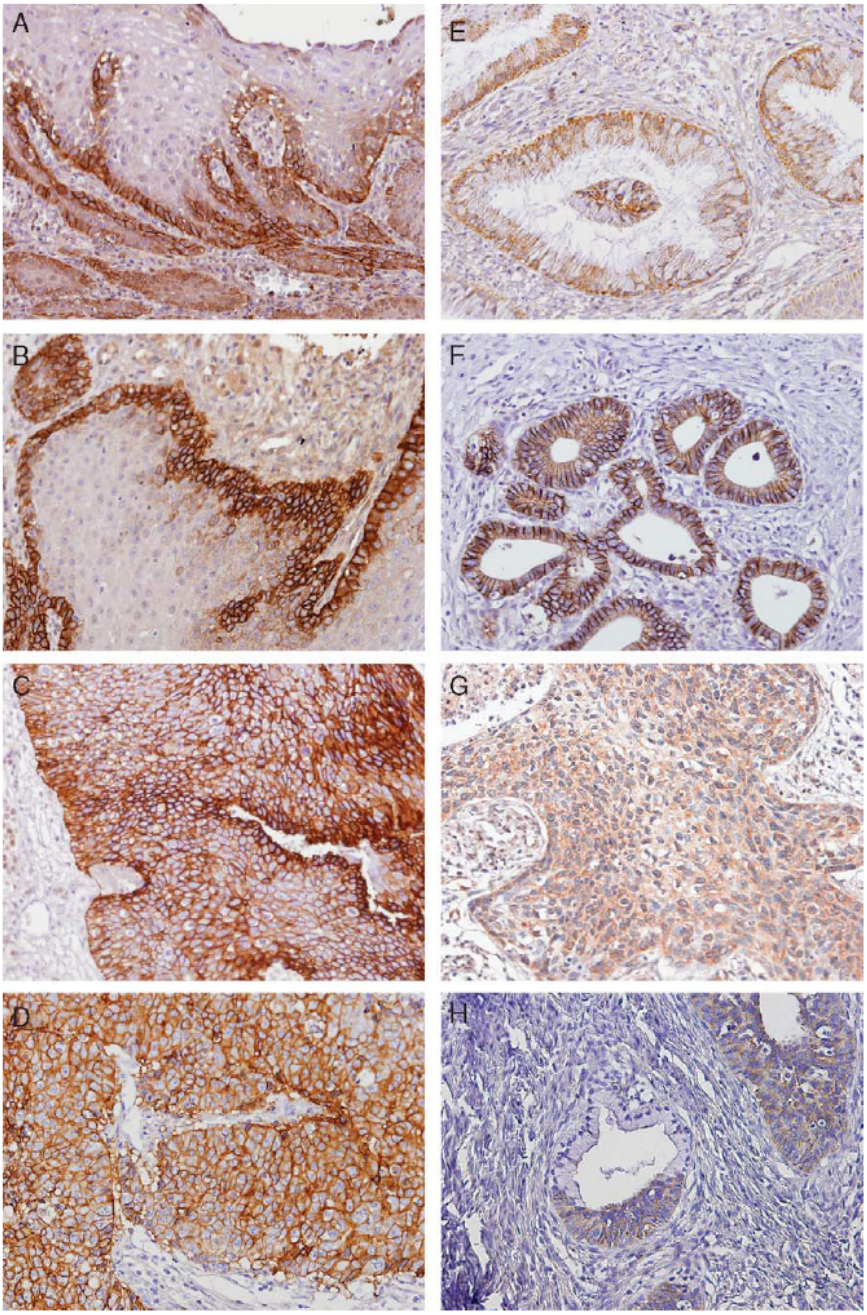

FIG. 1. Immunohistochemical expression of monocarboxylate transporter (MCT) 1, 2, and 4 in cervical lesions. A to D, Progressive increase of MCT1 expression in the squamous epithelium, in progression to invasive phenotype (A, negative for HPV-induced lesion; B, low-grade squamous intraepithelial lesion; C, high-grade squamous intraepithelial lesion; and D, squamous cell carcinoma); E, MCT4 expression in the normal glandular; F to H, representative cases of MCT expression in the different tumor histologic types studied (F, MCT1 expression in adenocarcinoma; G, MCT2 expression in squamous cell carcinoma; and H, MCT4 expression in adenosquamous carcinoma). All pictures are at a $\times$ 200 magnification.

the histologic type, being MCT2 less frequently expressed in SCC ($P = 0.001$) and MCT4 more frequently expressed in AC ($P < 0.001$). No associations were found between MCT plasma membrane expression and clinico-pathologic data (data not shown). As expression of MCT2 and MCT4 was significantly distinct among histologic types, association with age, lymph node/distant metastasis, and

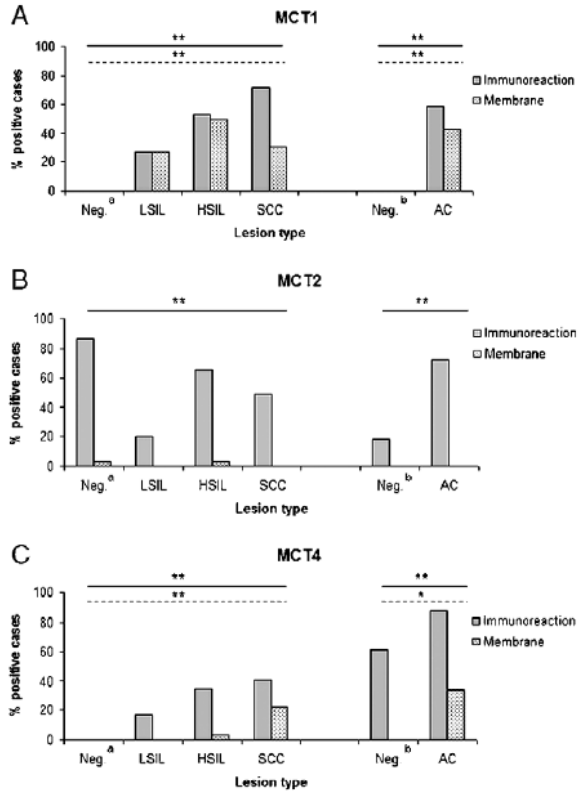

FIG. 2. Frequency of monocarboxylate transporter (MCT) staining in all squamous lesions studied, as well as in non-neoplastic and neoplastic glandular tissues. "Immunoreaction" includes both cytoplasmic and membrane-positive staining; "membrane" includes all plasma membrane-positive cases, with or without cytoplasmic expression. A, MCT1; B, MCT2; and C, MCT4; [a]negative for HPV-induced cervical lesions; [b]normal glandular epithelium; continuous line: statistical significance for immunoreaction results; interrupted line: statistical significance for membrane staining results; * $P=0.002$; ** $P<0.001$. AC indicates adenocarcinoma; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma.

recurrence was assessed by histologic type; however, no correlations were found (data not shown). Survival data of the patients were also available and, although not statistically significant, we found a tendency for patients with MCT4 expressing ASC to present a shorter overall survival ($P=0.099$, Fig. 3).

HPV status of the biopsy material was determined, and samples included 54/91 HPV-positive cases (7/29 cervicitis, 16/30 low-grade squamous intraepithelial lesion, and 31/32 HSIL). Table 2 depicts the correlation between MCT expression and HPV status. HPV positivity correlated with both MCT1 expression ($P=0.003$) and membrane localization ($P=0.005$) and also with MCT4 expression ($P=0.005$).

DISCUSSION

It was recently described that lactate is associated with poor prognosis in cervical cancer (3,4); however, no study evaluating the role of MCT expression in this type of tumors has been performed. So, the present work is an attempt to bring some light into the understanding of the metabolic alterations that occur during cervical cancer progression, with especial focus on lactate transport in tumor cells.

On the whole, we found a significant increase in MCT expression from preinvasive to invasive squamous lesions and from normal glandular epithelium to adenocarcinomas. It was quite interesting to note that both MCT1 and MCT4 were only expressed in the parabasal and basal cell layers of the normal squamous epithelium, lost in the upper layers, but "reappear" in altered cells. It seems that these altered

TABLE 1. *Correlation between MCT immunoreaction and the clinico-pathologic data in carcinoma cases*

Immunoreaction		MCT1		MCT2		MCT4	
Clinical data	n	Positive (%)	*P*	Positive (%)	*P*	Positive (%)	*P*
Age*			0.518		0.612		0.924
>49	43	60.5		69.8		67.4	
≤49	60	66.7		65.0		68.3	
Histologic type			0.284		**0.001**		**<0.001**
SCC	49	71.4		49.0		40.8	
AC	51	58.8		72.5		88.0	
ASC	30	73.3		86.7		63.3	
Lymph node/ metastasis†			0.755		0.779		0.873
Absent	77	64.9		66.2		67.5	
Present	26	61.5		69.2		69.2	
Recurrence			0.675		0.275		0.839
Negative	96	63.5		65.6		67.7	
Positive	7	71.4		85.7		71.4	

* Mean value was used for age cut-off.
†Lymph node/metastasis includes both lymph node and distant metastasis.
AC indicates adenocarcinoma; ASC, adenosquamous carcinoma; MCT, monocarboxylate transporter; SCC, squamous cell carcinoma.

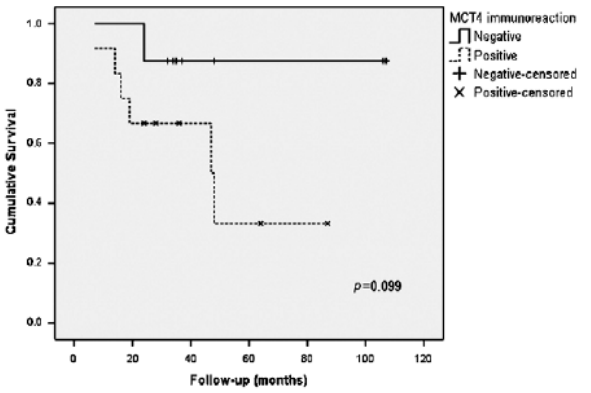

FIG. 3. The illustration represents the survival curve related to monocarboxylate transporter (MCT) 4 immunoreaction in adenosquamous cell carcinoma. Patients with tumor negative for the expression of MCT4 show shorter survival (interrupted line), whereas longer survival values were obtained for patients with tumor positive for MCT4 expression (continuous line) ($P = 0.099$).

cells recover the MCT expression phenotype of the parabasal cell layer, which represents the main proliferative pool of cells. However, it is important to note that the membrane expression of these 2 isoforms does not follow a similar pattern: MCT1 membrane expression decreases in the transition from HSIL to SCC, whereas an evident increase in MCT4 membrane expression is observed. As membrane localization of the transporter is essential for efflux of accumulating acids, it is reasonable to assume that highly proliferative cancer cells would express increased levels of MCT4, the low-affinity isoform, in the membrane, to allow continuous glycolytic rate, prevent apoptosis owing to intracellular acidification, and contribute to cancer cell survival. The reason why MCT1 is not overexpressed in the plasma membrane of SCC cells, despite the fact of being more expressed in SCC than in the other lesions, is not evident; however, this event may be associated with the already described dependence of MCT1 and MCT4 on the expression of the same ancillary protein CD147 for correct expression in the plasma membrane (21,22). We can then speculate that the levels of CD147 might, in some way, favor MCT4 membrane expression instead of MCT1 membrane expression, the isoform most prone to export the high levels of lactate resultant from the high glycolytic rates of cancer cells. As MCT2 does not follow a regular pattern of expression throughout squamous lesions in evolution to malignancy, one can hypothesize that this isoform does not play, at first sight, an important role in the metabolism of these cells. This observation is in agreement with the main role of MCT2, which is the uptake of monocarboxylates into lactate consuming cells (9). In fact, MCT2 expression is barely found in the plasma membrane of cervical lesions, being even absent in the plasma membrane of tumor cells.

TABLE 2. *Correlation between MCT expression and HPV status in preinvasive lesions*

		Immunoreaction		Plasma membrane	
MCT isoform	n	Positive (%)	*P*	Positive (%)	*P*
MCT1			**0.003**		**0.005**
HPV-negative	37	10.8		10.8	
HPV-positive	54	38.9		37.0	
MCT2			0.951		0.786
HPV-negative	37	56.8		2.7	
HPV-positive	54	57.4		1.9	
MCT4			**0.037**		0.412
HPV-negative	36	8.3		0.0	
HPV-positive	54	25.9		1.9	

HPV indicates human papillomavirus; MCT, monocarboxylate transporter.

In glandular neoplastic tissue, a significant gain in all isoforms was observed, when compared with normal tissue. This increase in expression was accompanied by a significant gain of plasma membrane expression for MCT1 and MCT4, which is in accordance with the cancer metabolic alterations already mentioned. However, we observed a significant gain of MCT2 in the cytoplasm of tumor cells, but lack of membrane expression. This event was already observed by Koukourakis et al. (16), in colorectal adenocarcinomas, who hypothesized that this cytoplasmic expression could be associated with the presence of MCT2 in mitochondrial membranes. Assuming the physiologic role of MCT2 in the transport of pyruvate into the mitochondria, one could suppose that the highly glycolytic tumor cell would upregulate MCT2 in an attempt to oxidize the excess pyruvate produced. This event would also contribute to the "removal" of the acid accumulated in the cytoplasm.

Despite the lack of significant associations between MCT expression and the clinico-pathologic data available for the patients, one cannot ignore the trend for patients with MCT4 expressing ASC to present a worse overall survival. ASCs are a rare type of cervical carcinoma, described to be very aggressive (23). As the number of patients with ASC and with a minimum follow-up of 24 months was low ($n = 21$), an increase in the number of cases would be crucial to improve statistical power. If confirmed, this assumption would complement the knowledge on cervical carcinoma prognosis, where the presence of lactate has a poor prognostic value (3,4).

HPV is responsible for one of the most common sexually transmitted infections worldwide and infection with high-risk HPV types is a necessary cause of cervical cancer (24). The frequency of high-risk HPV infection increases with the progression of the lesion, being present in virtually all cancer cases (25). Interestingly, we found that HPV-positive preinvasive cases express more MCT1 and MCT4 than HPV-negative cases, and also present more MCT1 in plasma membrane. Thus, we can speculate that HPV infection may, by some mechanism, stimulate MCT1 and MCT4 expressions as well as trafficking of MCT1 to the plasma membrane, but not MCT2 expression, probably with the purpose of favoring higher glycolytic rates during tumorigenesis, as HPV stimulates cell proliferation (26,27). However, functional studies will be needed to confirm this hypothesis.

To the best of our knowledge, this is the first study evaluating the significance of MCT expression in cervical cancer. All the above findings point at MCT1 and MCT4, especially MCT4, as having an important role in cervical cancer development and survival. If confirmed by further studies, these metabolic alterations may be explored to develop new directed therapeutic interventions in cervical cancer.

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3.2.2. MONOCARBOXYLATE TRANSPORTERS 1 AND 4 ARE ASSOCIATED WITH CD147 IN CERVICAL CARCINOMA

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Monocarboxylate transporters 1 and 4 are associated with CD147 in cervical carcinoma

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Abstract. Due to the highly glycolytic metabolism of solid tumours, there is an increased acid production, however, cells are able to maintain physiological pH through plasma membrane efflux of the accumulating protons. Acid efflux through MCTs (monocarboxylate transporters) constitutes one of the most important mechanisms involved in tumour intracellular pH maintenance. Still, the molecular mechanisms underlying the regulation of these proteins are not fully understood. We aimed to evaluate the association between CD147 (MCT1 and MCT4 chaperone) and MCT expression in cervical cancer lesions and the clinico-pathological significance of CD147 expression, alone and in combination with MCTs. The series included 83 biopsy samples of precursor lesions and surgical specimens of 126 invasive carcinomas. Analysis of CD147 expression was performed by immunohistochemistry. CD147 expression was higher in squamous and adenocarcinoma tissues than in the non-neoplastic counterparts and, importantly, both MCT1 and MCT4 were more frequently expressed in CD147 positive cases. Additionally, co-expression of CD147 with MCT1 was associated with lymph-node and/or distant metastases in adenocarcinomas. Our results show a close association between CD147 and MCT1 and MCT4 expressions in human cervical cancer and provided evidence for a prognostic value of CD147 and MCT1 co-expression.

Keywords: CD147, monocarboxylate transporters, cervical carcinoma

1. Introduction

In order to maintain high growth rates in hypoxic environment, cancer cells switch to anaerobic glycolysis to obtain energy. Actually, this metabolic change is maintained even in the presence of oxygen, as described by Warburg [1]. One consequence of cytosolic glucose metabolism is the increase in intracellular lactic acid concentration, which has to be tightly regulated to allow tumour cell survival and proliferation. Acid efflux through Monocarboxylate Transporters (MCTs) constitutes one of the most important mechanisms involved in the maintenance of tumour intracellular pH [2]. Indeed, MCT upregulation has been recently reported in some tumours, including brain [3–5], colorectal [6,7], lung [8] and, more recently, we described upregulation of MCT1 and 4 in cervical cancer [9].

MCT expression appears to be influenced by altered physiologic conditions, however, the underlying molecular events involved in MCT regulation are poorly understood. Recently, it was demonstrated that prop-

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er expression and activity of MCT1 and MCT4 requires an ancillary protein known as CD147 or EMMPRIN [10–12]. On the other hand, silencing studies showed that maturation and cell surface expression of CD147 depends on MCT1 and MCT4 expressions [13, 14]. CD147 has already been described as a key element in tumour growth and metastasis by stimulating the synthesis of several matrix metalloproteinases, leading to enhanced tumour cell invasion [15,16], and also by stimulating angiogenesis [17]. This protein is described to be up-regulated in several human cancers [16,18,19], including cervical squamous cell carcinoma [20], where it was found to correlate with pelvic lymph-node metastasis and resistance to radiotherapy [21].

Given that CD147 is described as an MCT regulator, we aimed to assess the association between CD147 and MCT1, MCT2 and MCT4 expressions, in a large and complete series of cervical lesions. Also, and since CD147 is poorly explored in cervical carcinoma, we intended to unveil the prognostic value of CD147 expression, alone and in combination with MCTs.

2. Materials and methods

2.1. Case selection

The material studied comprised 83 formalin-fixed paraffin embedded samples selected from the files of Pathology Division of Adolfo Lutz Institute, São Paulo, Brazil, which included biopsies of 28 chronic cervicitis (herein designated as "negative" for HPV-induced lesion), 26 Cervical Intraepithelial Neoplasia grade I (CIN 1, herein designated as low-grade squamous intraepithelial lesion – LSIL) and 29 Cervical Intraepithelial Neoplasia grades II and III (CIN2/3, herein designated as high-grade squamous intraepithelial lesions - HSIL). We also analyzed a series of formalin-fixed, paraffin-embedded tissue samples from 126 patients with squamous cells carcinoma (SCC, $n = 49$), adenocarcinoma (AC, $n = 50$) and adenosquamous carcinoma (ASC, $n = 27$) of the uterine cervix, examined and treated at two Institutions: Araújo Jorge Hospital and the Pathology Department of the School of Medicine of the Federal University of Goiás, Goiania, in Goiás State, Brazil. All histopathological diagnoses were revised and categorized according to the WHO classification [22]. Clinico-pathological data of the patients included age at diagnosis, HPV status, lymph-node and/or distant metastasis, recurrence and overall survival.

2.2. MCT immunohistochemistry

Data on MCT1, MCT2 and MCT4 expressions was available for all the 209 samples [9]. MCT immunohistochemistry was performed according to avidin-biotin-peroxidase complex principle (R.T.U. VECTASTAIN Elite ABC Kit (Universal), Vector Laboratories, Burlingame, CA, USA), with the primary antibodies for MCT1 (AB3538P, Chemicon International, Temecula, CA, USA), MCT2 (sc-14926, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and MCT4 (AB3316P, Chemicon International, Temecula, CA, USA), diluted 1:200, as previously described [7].

2.3. CD147 immunohistochemistry

CD147 immunohistochemistry was performed based on the streptavidin–biotin–peroxidase complex principle (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA), using a primary antibody raised against CD147 (18-7344, ZYMED Laboratories Inc., South San Francisco, CA, diluted 1:750). Briefly, deparaffinized and rehydrated sections were immersed in EDTA (pH 8.0), heated up to 98°C in a water bath for 15 minutes and washed in PBS. Endogenous peroxidases were inactivated with 3% hydrogen peroxide in methanol for 10 minutes, followed by washing in PBS. Tissue sections were incubated with blocking solution for 10 minutes and incubated at room temperature with the primary antibody for 2 hours. Sections were then sequentially washed in PBS and incubated with biotinylated goat anti-polyvalent antibody for 10 minutes, streptavidin peroxidase for 10 minutes, and developed with 3,3'-diamino-benzidine (DAB+ Substrate System, Dako, Carpinteria, CA) for 10 minutes. Negative controls were performed by using the adequate serum control (N1698, Dako, Carpinteria, CA) and cervical squamous carcinoma was used as positive control. Tissue sections were counterstained with haematoxylin and permanently mounted.

2.4. Immunohistochemical evaluation

Sections were evaluated for immunoreaction, which included cytoplasmic and/or membrane positive staining. Immunoreaction extent was scored semi-quantitatively as follows: 0: 0% of immunoreactive cells; 1: $< 5\%$ of immunoreactive cells; 2: 5–50% of immunoreactive cells; and 3: $> 50\%$ of immunoreactive cells. Also, intensity of staining was scored semi-

qualitatively as 0: negative; 1: weak; 2: intermediate; and 3: strong. Immunoreaction final score was defined as the sum of both parameters (extent and intensity), and grouped as negative (score 0 and 2) and positive (3–6), as previously described [7]. Finally, since plasma membrane location of CD147 is essential for MCT1 and MCT4 membrane localization and activity [10–12], we also grouped the plasma membrane positive cases, including all the positive cases, with or without cytoplasmic expression. Immunohistochemical evaluation was performed blindly by two independent observers and discordant results were discussed in a double-head microscope and a final score was agreed.

2.5. Statistical analysis

Data were stored and analyzed using the SPSS statistical software (version 14.0, SPSS Inc., Chicago, IL, USA). The comparison of CD147 expression between tumor and normal cells as well as the relationship between CD147 expression and the clinico-pathological parameters were examined for statistical significance using Pearson's chi-square (χ^2) test and Fisher's exact test (when $n < 5$), being threshold for significance p values < 0.05. The same analysis was performed in order to compare CD147 expression with MCTs. Survival curves were plotted using the method of Kaplan and Meier and data compared using the log-rank test, using a cut-off of 24 months. Due to lack of information, 18 cases of SCC and 1 case of ASC were not evaluated for clinico-pathological significance.

3. Results

We analyzed the expression of CD147 in a series of cervical samples which included 83 biopsies of cervix intraepithelial lesions and 126 surgical specimens of invasive cervical carcinomas. CD147 expression was mainly found in the plasma membrane (Fig. 1), with cases presenting both membrane and cytoplasmic staining and some only cytoplasmic expression. Figure 2 compares the frequency of CD147 expression in all the squamous epithelial lesions studied, as well as non-neoplastic and neoplastic glandular tissues. CD147 expression was significantly different in the various squamous epithelial lesions ($p < 0.001$, Fig. 2), showing a more frequent expression in squamous neoplasias than in their non-neoplastic counterparts. Concerning the glandular epithelium, since our series does not include adenocarcinoma precursor lesions, it was only possible

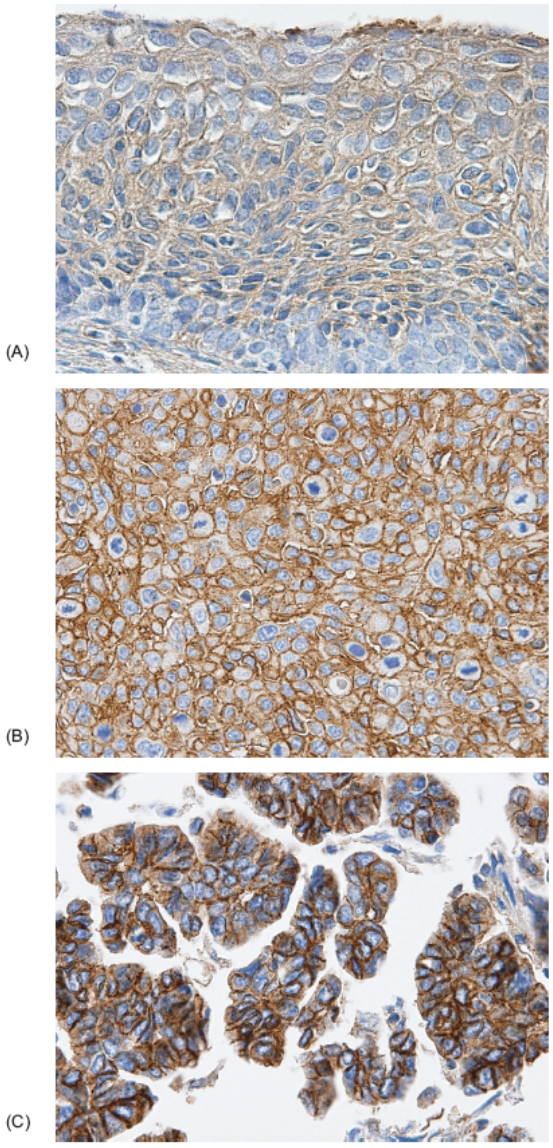

Fig. 1. Immunohistochemical expression of CD147 in cervical lesions. Representative cases of CD147 expression in: high-grade squamous intraepithelial lesion (**A**); squamous cell carcinoma (**B**); adenocarcinoma (**C**). All pictures are at 400X magnification.

to compare neoplastic lesions (AC) with non-neplastic glandular tissue from the biopsy material. Here, an evident gain in CD147 expression was observed in adenocarcinoma tumour cells (Fig. 1C), when compared to normal glandular tissue ($p < 0.001$, Fig. 2).

HPV status of the biopsy material was known and samples included 50/88 high risk HPV positive cases (6/28 cervicitis, 16/26 LSIL and 28/29 HSIL); however, no association was found between HPV infection and CD147 expression (data not shown). The clinico-pathological significance of CD147 expression, as well

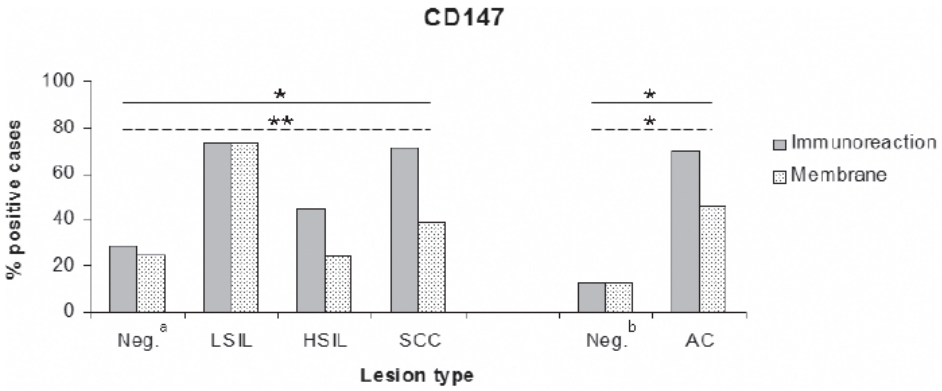

Fig. 2. Frequency of CD147 staining in all squamous lesions studied, including non-neoplastic and neoplastic glandular tissues. "Immunoreaction" refers to both cytoplasmic and membrane positive staining; "membrane" refers to plasma membrane positive cases, with or without cytoplasmic expression; [a]negative for HPV-induced cervical lesions; [b]non-neoplastic glandular epithelium; continuous line: statistical significance for immunoreaction results; interrupted line: statistical significance for membrane staining results; $*p < 0.001$; $**p = 0.001$. LSIL – low-grade squamous intraepithelial lesion; HSIL – high-grade squamous intraepithelial lesion; SCC – squamous cell carcinoma; AC – adenocarcinoma.

as co-expression of CD147 and both MCT1 and MCT4 was assessed for 99 carcinomas (Table 1). Considering all primary tumours, no association was found with the clinico-pathological data; however, since the 3 histological types behave differently and probably present different metabolic activity, associations were assessed by histological type. Regarding individual expression of CD147, no significant associations were observed, but co-expression of CD147 with MCT1 was significantly associated with lymph-node and/or distant metastases in adenocarcinomas ($p = 0.033$). No statistically significant associations were found with survival data (data not shown).

Consistent with the assumption that CD147 functions as a regulator of MCT1 and MCT4 expressions, we observed that both MCT1 and MCT4 correlated with CD147 immunoreaction ($p = 0.001$ for MCT1 immunoreaction and MCT4 plasma membrane expression; $p = 0.002$ for MCT1 plasma membrane expression and MCT4 immunoreaction, Table 2), while only MCT4 immunoreaction was associated with plasma membrane expression of CD147 ($p = 0.014$, Table 2). As anticipated, no association was found between CD147 and MCT2 expressions.

4. Discussion

MCTs were recently described as crucial proteins in cancer cell pH homeostasis [2]. Although regulation of MCT expression is still far from being well understood, one of the best characterized MCT regulation mechanisms is through the co-expression with CD147 at the plasma membrane [10–13].

The important contribution of CD147 in promoting tumour growth, invasion and metastasis has been widely explored in past years [16,19], however, in what concerns cervical cancer, this issue is far from being clarified. Here, we describe the expression of CD147 in a complete series of cervical carcinoma but also look at an important feature of this protein, little explored in cancer: the association with MCTs. In the present study, we observed an increase in CD147 expression in both squamous and glandular tumours, when compared to non-neoplastic corresponding tissues. Nonetheless, in opposition to previous results from Sier and collaborators [20], who described no staining in normal epithelial cells, we observed a considerable expression of CD147 in the non-neoplastic epithelium. However, the non-neoplastic epithelium herein described was in fact biopsy material from cervicitis, which may explain the presence of CD147, knowing that this protein has a role in inflammation [23]. The frequency of CD147 expression in our tumour series was around 70%, which is in accordance with previous results in cervical squamous cell carcinomas [21]. In opposition to what we previously observed for MCT2 and MCT4 [9], CD147, like MCT1, was not differently expressed among the three histological types studied and no association was observed with the clinico-pathological data, namely with lymph-node metastases, contrasting with what has been recently described [21].

Taking into consideration the close association between CD147 and MCTs [10–14], we sought for correlations between these proteins in a series of cervical lesions. We observed that both MCT1 and MCT4 overexpressions correlated with CD147, while only MCT4 was associated with CD147 plasma membrane stain-

Table 1
Correlation of CD147 and combined CD147 and MCT expressions with the clinico-pathological data of carcinoma cases

	Expression		CD147 immunoreaction		CD147 membrane		CD147+MCT1		CD147+MCT4	
Clinical data		n	Positive (%)	p	Positive (%)	p	Positive (%)	p	Positive (%)	p
All carcinomas										
Age[a]				0.204		0.941		0.598		0.200
	>49	**48**	64.6		45.8		16.7		19.1	
	$\leqslant 49$	**58**	75.9		46.6		20.7		10.3	
Histological type				0.734		0.755		0.819		0.683
	SCC	**49**	71.4		38.8		16.3		10.2	
	AC	**50**	70.0		46.0		20.0		16.0	
	ASC	**27**	63.0		44.4		14.8		14.8	
Lymph node/metastases[b]				0.539		0.254		0.149		0.376
	Absent	**79**	69.6		43.0		15.2		12.8	
	Present	**25**	76.0		56.0		28.0		20.0	
Recurrence				1.000		0.729		0.667		0.495
	Negative	**95**	70.5		45.3		17.9		13.8	
	Positive	**9**	77.8		55.6		22.2		22.2	
SCC										
Age[a]				1.000		0.576		0.664		0.607
	>49	**14**	78.6		42.9		14.3		7.1	
	$\leqslant 49$	**17**	82.4		52.9		23.5		17.6	
Lymph node/metastases[b]				0.553		0.172		1.000		0.112
	Absent	**26**	76.9		42.3		19.2		7.7	
	Present	**5**	100.0		80.0		20.0		40.0	
Recurrence										
	Negative	**31**	80.6		48.4		19.4		12.9	
	Positive	**0**								
AC										
Age[a]				0.305		0.674		0.942		0.130
	>49	**24**	62.5		50.0		20.8		26.1	
	$\leqslant 49$	**25**	76.0		44.0		20.0		8.0	
Lymph node/metastases[b]				0.405		0.446		**0.033**		0.613
	Absent	**39**	66.7		43.6		12.8		15.8	
	Present	**8**	87.5		62.5		50.0		25.0	
Recurrence				0.657		0.690		1.000		0.587
	Negative	**40**	67.5		45.0		20.0		15.4	
	Positive	**7**	85.7		57.1		14.3		28.6	
ASC										
Age[a]				0.339		1.000		1.000		0.538
	>49	**10**	50.0		40.0		10.0		20.0	
	$\leqslant 49$	**16**	68.8		43.8		18.8		6.3	
Lymph node/metastasis[b]				0.756		0.951		1.000		1.000
	Absent	**14**	64.3		42.9		14.3		14.3	
	Present	**12**	58.3		41.7		16.7		8.3	
Recurrence				1.000		1.000		0.289		1.000
	Negative	**24**	62.5		41.7		12.5		12.5	
	Positive	**2**	50.0		50.0		50.0		0.0	

[a]The age cut off was considered as the mean age of the patients at time of diagnosis; [b]Lymph node/metastasis includes both lymph-node and distant metastases. "CD147+MCT isoform" refers to co-expression of CD147 and MCT in the plasma membrane. SCC – squamous cell carcinoma; AC – adenocarcinoma; ASC – adenosquamous carcinoma.
"Immunoreaction" refers to both cytoplasmic and membrane positive staining; "membrane" refers to plasma membrane positive cases, with or without cytoplasmic expression.

ing. Although it was expectable to found correlations between membrane expressions, since part of the regulation involves membrane co-localization of the proteins [10,11], absence of CD147 is also responsible for lower levels of MCT1 and MCT4 whole protein expression [12] and for endolysosomal degradation of MCT4 [13], explaining the association between overall expressions. As anticipated, since the MCT2 chaperone is not CD147 but gp70 [11] and lack of CD147 does not affect MCT2 protein levels [12], we found no association between CD147 and MCT2 expression. Knowing that MCTs need to interact with CD147 to be

Table 2
Correlation between MCT and CD147 expressions in all cervical lesions

MCT isoform			CD147 Immunoreaction		CD147 Plasma membrane	
		n	Positive (%)	*p*	Positive (%)	*p*
MCT1	immunoreaction			**0.001**		0.110
	Negative	**142**	43.7		32.4	
	Positive	**109**	64.2		42.2	
	plasma membrane			**0.002**		0.182
	Negative	**179**	46.4		34.1	
	Positive	**72**	68.1		43.1	
MCT2	immunoreaction			0.322		0.389
	Negative	**106**	50.0		40.6	
	Positive	**142**	56.3		35.2	
MCT4	immunoreaction			**0.002**		**0.014**
	Negative	**126**	44.4		30.2	
	Positive	**119**	63.9		45.4	
	plasma membrane			**0.001**		0.146
	Negative	**210**	49.5		35.7	
	Positive	**35**	80.0		48.6	

"Immunoreaction" refers to both cytoplasmic and membrane positive staining; "membrane" refers to plasma membrane positive cases, with or without cytoplasmic expression.

functionally active [10,11], we hypothesized that co-expression of the transporters with the chaperone could favor the malignant potential of cancer cells. In fact, we observed that co-expression of CD147 and MCT1 in adenocarcinomas was more frequent in patients with lymph-node and/or distant metastases, being in accordance with the synergistic activity between MCTs and CD147, leading to an enhanced metastatic potential of cancer cells, through acidification of the tumor microenvironment [13]. Some other possibly relevant associations did not achieve significance, due to the number of cases representing each histological type; thus, larger studies are needed to confirm or decline these associations.

In sum, we evaluated the clinico-pathological significance of CD147 in cervical cancer, alone and in combination with MCTs, and provided evidence for the association between CD147 and both MCT1 and 4 expressions in human samples. We also showed that CD147 and MCT1 co-expression could have a prognostic value. Nevertheless, further studies are needed to explore the possible synergistic effect of these molecules in the metastatic potential of cervical carcinoma cells.

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Chapter 4. MCTs IN GASTRIC CARCINOMA

4.1. CHAPTER OVERVIEW

The primary epithelial tumour of the stomach is the adenocarcinoma, which develops from the stomach mucosa, usually maintaining glandular differentiation [1]. Clinical and epidemiological studies have suggested a link between gastric cancer and concurrent or previous infection with a bacterium or virus. In this context, *Helicobacter pylori* emerges as a contributor for gastric cancer in around 60% of cases and multiple studies have shown that *H. pylori* infection is associated with a 2.7- to 12-fold increase in risk of gastric cancer development [2].

It is widely known that the normal gastric environment is very acidic and many pH regulators are involved in cellular homeostasis, including AE, NHE and Na^+/HCO_3^- co-transporter [3]. Gastric mucosa is protected from the acidic environment by different mechanisms, including the mucus-bicarbonate-phospholipid "barrier" [4]; however, this barrier may be impaired in different clinical conditions, such as *H. pylori* infection [5,6], contributing to perpetuation of chronic inflammation and gastric carcinogenesis [5].

Little is known on the metabolic features of gastric cancer cells, however, recent studies point at an enhanced glycolytic metabolism in gastric carcinoma [7,8].

In the following study, already published in an international periodical with referees, aiming to determine if MCTs may have an important role in gastric cancer, MCT1, MCT4 and CD147 expressions were evaluated in a series of 190 gastric primary tumours, as well as in 71 non-neoplastic tissues and 42 lymph-node metastases. In contrast to what was found in the previous types of tumours, neither MCT1 nor MCT4 were up-regulated in gastric adenocarcinomas. Actually, MCT4 expression was more frequently observed in normal gastric mucosa than in gastric cancer cells and even less frequently observed in lymph-node metastasis, indicating a progressive loss of this MCT isoform with disease progression. Also, MCT4 expression was associated with Lauren's classification of intestinal-type carcinoma. MCT1 and CD147 were expressed similarly in normal gastric mucosa, primary tumours and lymph-node metastasis; however, MCT1 was present in the majority of samples (around 80%) while CD147 was present in less than a half of the samples. This may indicate that MCT1 has a major contribution in gastric homeostasis, which is maintained along carcinogenesis, while CD147 should have a more modest role in this type of cancer. Both MCT1 and MCT4 expressions were positively associated with CD147 and, importantly, the prognostic value of CD147 was associated with co-expression with MCT1, but not with MCT4.

In conclusion, these results suggest that, although not up-regulated in gastric carcinoma, the complex MCT1/CD147 may have an important prognostic value in this type of tumour.

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4.2. PUBLISHED RESULTS

The results presented in this chapter were **published** in an international scientific periodical with referees:

Pinheiro C, Longatto-Filho A, Simoes K, Jacob CE, Bresciani CJC, Zilberstein B, Cecconello I, Alves VAF, Schmitt F, Baltazar F (2009). The prognostic value of CD147/EMMPRIN is associated with monocarboxylate transporter 1 co-expression in gastric cancer. *Eur J Cancer* 45:2418-2424.

The results presented in this chapter were also presented as poster in the following international scientific meetings/course:

Third Intercontinental Congress of Pathology, 2008, Barcelona, Spain. ***Pinheiro C****, Longatto-Filho A, Ferreira L, Simões K, Alves V, Schmitt F, Baltazar F. Monocarboxylate transporter expression correlates with CD147 in gastric cancer. Abstract* ***published*** *in conference proceedings (Virchows Arch 452 (Suppl. 1):S202-S203);*

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FEBS Advanced Lecture Course "Matrix Pathobiology, Signaling and Molecular Targets", 2009, Patras, Greece. ***Pinheiro C****, Longatto-Filho A, Simoes K, Jacob CE, Bresciani CJC, Zilberstein B, Cecconello I, Alves VAF, Schmitt F, Baltazar F. The prognostic value of CD147/EMMPRIN is associated with monocarboxylate transporter 1 co-expression in gastric cancer.*

4.2.1. THE PROGNOSTIC VALUE OF CD147/EMMPRIN IS ASSOCIATED WITH MONOCARBOXYLATE TRANSPORTER 1 CO-EXPRESSION IN GASTRIC CANCER

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The prognostic value of CD147/EMMPRIN is associated with monocarboxylate transporter 1 co-expression in gastric cancer

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ABSTRACT

The aim of the present work was to assess the role of monocarboxylate transporters (MCTs), namely MCT1 and MCT4 as well as MCT/CD147 co-expression in gastric tissues and evaluate their clinico-pathological significance in gastric carcinoma. For that, we analysed the immunohistochemical expression of MCT1, MCT4 and CD147, in a large series of gastric samples, including non-neoplastic, tumour and metastatic tissues. A significant decrease in MCT4 plasma membrane expression was observed from non-neoplastic to gastric primary malignant tissues and to lymph-node metastasis and both MCT1 and MCT4 correlated with CD147. Importantly, both MCT4 and CD147 were more frequently expressed in Lauren's intestinal-type tumours and MCT1/CD147 co-expression was associated with advanced gastric carcinoma, Lauren's intestinal type, TNM staging and lymph-node metastasis. Our results showed that the prognostic value of CD147 was associated with MCT1 co-expression in gastric cancer cells, supporting the view that CD147 plasma membrane activity is dependent on MCT co-expression.

1. Introduction

Despite the worldwide decline in incidence and mortality of gastric carcinoma along the second half of the 20th century, it is still the 4th most common cancer and the 2nd leading cause of cancer-related deaths in the world.[1] Prognosis of gastric carcinoma patients depends on several pathological variables. Among them, histological typing seems to have some prognostic relevance. The most prestigious histological classifications include: Lauren's and World Health Organization (WHO). Lauren's histological classification identifies two major patterns: intestinal-type (which principally includes papillary, well-differentiated adenocarcinomas, moderately differentiated or mucinous adenocarcinomas without signet

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ring cell carcinoma (SRC) cells of WHO classification) and diffuse-type carcinomas (usually corresponding to poorly differentiated adenocarcinomas, SRC and undifferentiated adenocarcinomas of WHO classification). This classification distinguishes, by microscopic morphology, two main cancer entities with different clinical and epidemiological features (for review see [2]). The prognosis of gastric carcinoma depends also largely on tumour classification in early gastric cancer, which presents a better prognosis, or advanced gastric carcinoma, with an unfavourable prognosis.[3] Although this nomenclature may suggest a progression from one entity to the other, it is still not clear whether these are different stages of the same tumour or independent entities.[4]

Early epithelial carcinogenesis occurs under hypoxic conditions, since altered cells are separated from the vascularised stroma that supplies oxygen and nutrients. To maintain ATP levels, cancer cells increase their rates of glycolysis, developing a significant proliferative advantage. However, this phenotype leads to an overload of lactic acid, which must be expelled out of the cell, causing a decrease in the extracellular pH. Constitutive up-regulation of glycolysis requires additional adaptations, namely, resistance to apoptosis and up-regulation of membrane transporters to maintain normal intracellular pH.[5] Besides being an adaptation to high glycolytic phenotype, acidic environment represents *per se* a significant advantage for tumour cells since it is associated with increased migration, invasion and metastases, among others.[5–7] One of the most important groups of proteins involved in intracellular pH regulation is monocarboxylate transporters (MCTs), which are also responsible for transmembrane transport of lactate.[8] Indeed, there are evidences for the up-regulation of MCTs in tumours, such as high grade glial neoplasms,[9,10] colorectal,[11,12] cervical[13] and lung carcinomas,[14] but only our studies[12,13] evaluated the clinico-pathological significance of MCT altered expression. Besides an increased MCT expression in colorectal[12] and cervical carcinomas,[13] we found an association between MCT1 positivity and vascular invasion in colorectal carcinomas[12] and both MCT1 and MCT4 expressions with high risk HPV infection in uterine cervix carcinomas.[13]

MCT expression appears to be influenced by altered physiologic conditions, however, the underlying molecular events involved in MCT regulation are poorly understood. Recently, it was demonstrated that proper expression and activity of MCT1 and MCT4 require co-expression of CD147, also known as EMMPRIN or Basigin.[15–19] On the other hand, silencing studies showed that maturation and cell surface expression of CD147 depend on MCT1 and MCT4 expressions.[18,19] Recently, our group described a close association of both MCT1 and MCT4 with CD147, in cervical cancer.[20] CD147 alone has already been described as a key element in oncogenesis by stimulating the synthesis of several matrix metalloproteinases, leading to enhanced tumour cell invasion.[21,22] This protein is described to be up-regulated in human cancers,[21–23] including gastric carcinomas,[24] however if its role in cancer is associated with MCTs is not known.

One of the aims of the present study is to assess the role of MCTs in gastric cancer, by analysing the immunohistochemical expression of the MCT isoforms 1 and 4 in a large series of gastric samples, including non-neoplastic, tumour and metastatic tissues, and evaluate its clinico-pathological value. We also intend to infer about the significance of MCT and CD147 co-expression.

2. Materials and methods

2.1. Case selection

Gastric tissues were obtained from 190 patients with gastric carcinoma (including 71 non-neoplastic tissues, 190 primary tumours and 42 lymph-node metastases). Samples were retrieved from the files of the Department of Pathology, Hospital das Clinicas, University of São Paulo, School of Medicine, São Paulo, Brazil, and organised in 10 tissue microarrays (TMAs). To achieve representative sampling and minimise sample loss, sample duplicates were included in the TMAs. Even so, some cases were lost during the immunohistochemical procedure. Relevant data available included patient's age and gender as well as tumour size and location, macroscopic classification, Lauren's classification, TNM staging, depth of invasion, lymph-node metastases and both lymphatic and vascular invasions.

2.2. Immunohistochemistry

2.2.1. MCT and CD147 detections

MCT immunohistochemistry was performed according to the avidin–biotin-peroxidase complex method (R.T.U. VECTASTAIN Elite ABC Kit (Universal), Vector Laboratories, Burlingame, CA), with primary antibodies for MCT1 (AB3538P, Chemicon International, Temecula, CA) and MCT4 (AB3316P, Chemicon International, Temecula, CA), diluted 1:200, as previously described.[12] Immunohistochemistry for CD147 was performed according to the streptavidin–biotin-peroxidase complex principle (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA), using a primary antibody raised against CD147 (18-7344, ZYMED Laboratories Inc., South San Francisco, CA) diluted 1:750, as previously described.[20] Negative controls were performed by using appropriate serum controls for the primary antibodies (N1699, Dako, Carpinteria, CA), colon carcinoma tissue was used as positive control for both MCT1 and MCT4 and cervical squamous carcinoma for CD147. Tissue sections were counterstained with haematoxylin and permanently mounted.

2.2.2. Immunohistochemical evaluation

Since plasma membrane location is essential for protein activity, immunoreactions for MCTs and CD147 were only considered positive when plasma membrane staining was present. Sections were scored semi-quantitatively for plasma membrane immunoreaction as follows: 0: 0% of immunoreactive cells; 1: <5% of immunoreactive cells; 2: 5–50% of immunoreactive cells; and 3: >50% of immunoreactive cells. Also, intensity of staining was scored semi-qualitatively as follows: 0: negative; 1: weak; 2: intermediate; and 3: strong. The final score was defined as the sum of both parameters (extent and intensity), and grouped as negative (scores 0 and 2) and positive (scores 3–6), as previously described.[12] Immunohistochemical evaluation was performed blindly

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by two independent observers and discordant cases were discussed in a double-head microscope in order to determine the final score.

2.3. Statistical analysis

Data were stored and analysed using the SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL). All comparisons were examined for statistical significance using Pearson's chi-square (χ^2) test and Fisher's exact test (when $n < 5$), being threshold for significance p values <0.05.

3. Results

A total of 303 gastric samples organised into TMAs (Tissue Microarrays), including 71 non-neoplastic mucosas, 190 primary tumours and 42 metastatic tissues, were assessed for MCT1 and MCT4 immunohistochemical expressions. We also evaluated CD147 as putative regulator of MCT expression in gastric cells.

In general, positive MCT1 expression was observed in both plasma membrane and cytoplasm (Fig. 1A), while MCT4 expression was mainly observed in the cytoplasm, with few cases presenting plasma membrane staining (Fig. 1B). Regarding CD147, expression was mainly found in the plasma membrane (Fig. 1C). Table 1 summarises MCT and CD147 expressions in non-neoplastic and primary tumour tissues, as well as in lymph-node metastasis. As can be seen, no significant differences were observed for MCT1 and CD147 expressions. On the other hand, a significant decrease was observed for MCT4 plasma membrane expression in the transition from non-neoplastic to gastric primary malignant tissues and to lymph-node metastasis ($p < 0.001$).

In order to assess the role of CD147 in MCT regulation, as described in the literature,[15–19] we searched for associations between the expression of this regulator and the expressions of MCT1 and MCT4 in gastric carcinoma (Table 2). This analysis showed that both MCT1 and MCT4 correlated with CD147 ($p = 0.021$ and $p = 0.001$, respectively). Fig. 1A and C shows sequential tumour sections stained for MCT1 and CD147, in which positive cells for both proteins can be seen.

The clinico-pathological data available allowed assessment of correlations with MCTs (Table 3) and CD147 expressions (Table 4). Importantly, both MCT4 and CD147 were more frequently expressed in Lauren's intestinal-type tumours ($p < 0.001$ and $p = 0.010$, respectively). Additionally, although not significant ($p = 0.065$), MCT4 was more frequently expressed in early gastric cancer. CD147 expression was also associated with male gender ($p = 0.031$), advanced gastric carcinoma ($p = 0.001$), TNM stages III/IV ($p = 0.006$), depth of invasion ($p = 0.002$) and presence of lymph-node metastasis ($p = 0.003$). As proper membrane expression and activity of both MCT1 and MCT4 require association with CD147,[15–19] we also analysed the clinico-pathological significance of MCT/CD147 co-expression (Table 4). Simultaneous expression of MCT1 and CD147 was associated with advanced gastric carcinoma ($p = 0.030$), Lauren's intestinal-type ($p = 0.020$), TNM stages III/IV ($p = 0.004$) and presence of lymph-node metastasis ($p = 0.018$), and, although not significant, a tendency for invading tumours to co-express MCT1 and CD147 was also observed ($p = 0.073$). Co-expression of MCT4 and CD147 was only associated with Lauren's intestinal type ($p = 0.023$). Since MCT4 and CD147 were more frequently expressed in Lauren's intestinal type, we also evaluated the clinico-pathological significance of the co-expression of these molecules, in the tumours included in this group. Similar associations were found when analysing all primary tumours, with the addition of an association between MCT4 and younger patients ($p = 0.044$) and between CD147 and bigger tumours ($p = 0.024$) (data not shown).

4. Discussion

Up-regulation of glycolysis and adaptation to acidosis are key events in the transition from *in situ* to invasive cancer.[5] Owing to their essential function in exporting lactate, the end-product of glycolysis, MCTs are likely to be key elements in the regulation of tumour intracellular pH and induction of extracellular acidosis. Thus, the role of these transporters in tumours must be clarified in order to understand their contribution to the glycolytic and acidic phenotypes of tumours. Few reports lay on this matter and none tackled

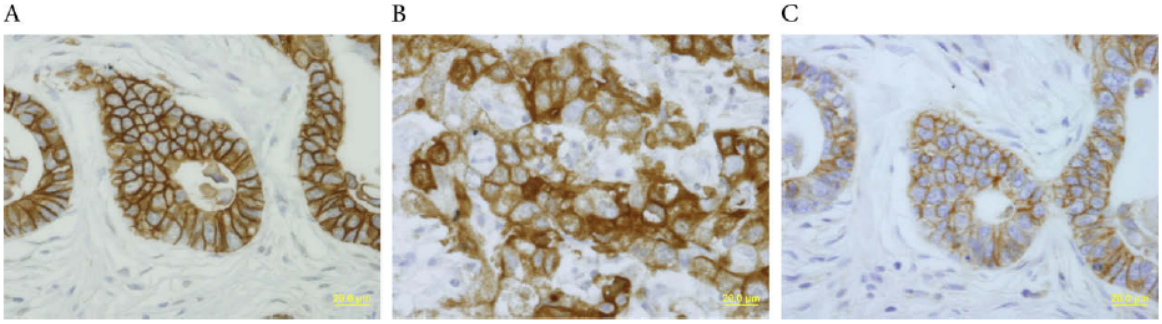

Fig. 1 – Immunohistochemical expression of monocarboxylate transporter 1 (MCT1), monocarboxylate transporter 4 (MCT4) and CD147 in gastric cancer samples. MCT1 expression was observed in both plasma membrane and cytoplasm (A), MCT4 expression was mainly observed in the cytoplasm, although few cases presented plasma membrane staining (B), while CD147 expression was clearly found in the plasma membrane (C). Tumour cells positive for both MCT1 and CD147 can be seen in the sequential sections A and C.

Table 1 – Frequency of monocarboxylate transporters (MCTs) and CD147 expressions in gastric samples.

	MCT1			MCT4			CD147		
	n	Positive (%)	*p*	*n*	Positive (%)	*p*	*n*	Positive (%)	*p*
			0.654			<0.001			0.624
Non-neoplastic	71	84.5		61	50.8		66	39.4	
Primary tumour	177	79.7		175	18.3		160	41.2	
Lymph-node metastasis	35	82.9		35	8.6		39	48.7	

Table 2 – Association between monocarboxylate transporters (MCTs) and CD147 expressions in gastric primary tumours.

	MCT1			MCT4		
	n	Positive (%)	*p*	*n*	Positive (%)	*p*
CD147			0.021			0.001
Negative	89	73.0		84	10.7	
Positive	61	88.5		61	32.8	

Table 3 – Correlations between monocarboxylate transporter 1 (MCT1) and 4 (MCT4) membrane expressions in primary tumours and the clinico-pathological data.

Clinico-pathological data	MCT1			MCT4		
	n	Positive (%)	*p*	*n*	Positive (%)	*p*
Age			0.788			0.380
>61	86	81.4		85	15.3	
⩽61	84	79.8		83	20.5	
Gender			0.333			0.208
Female	56	83.9		56	12.5	
Male	116	77.6		113	20.4	
Tumour size (cm)			0.664			0.223
<4	69	81.2		67	22.4	
⩾4	102	78.4		100	15.0	
Tumour localisation			0.402			0.503
Body	42	76.2		40	17.5	
Antrum	114	83.3		112	18.8	
Others	6	66.7		6	0.0	
Macroscopic classification			0.397			0.065
Early gastric cancer	54	75.9		50	26.0	
Advanced gastric cancer	119	81.5		120	14.2	
Lauren's classification			0.269			<0.001
Intestinal-type	105	82.9		107	26.2	
Diffuse-type	62	75.8		59	3.4	
TNM			0.604			0.789
IB + II	145	78.6		142	18.3	
III + IV	27	85.2		27	14.8	
pT			0.405			0.120
Mucosa	54	75.9		50	26.0	
Muscular propria/subserosa	111	82.9		111	13.5	
Adjacent structures	6	66.7		7	28.6	
Lymph-node metastasis			0.189			0.440
Absent	76	75.0		76	19.7	
Present	95	83.2		92	15.2	
Lymphatic invasion			0.557			0.288
Absent	97	81.4		94	20.2	
Present	72	77.8		72	13.9	
Vascular invasion			0.950			0.194
Absent	143	79.7		140	19.3	
Present	24	79.2		24	8.3	

Median was used for age and tumour size cut-off.

Table 4 – Correlations between CD147 and MCT (monocarboxylate transporter)/CD147 expressions in primary tumours and the clinico-pathological data.

Clinico-pathological data	Plasma membrane								
	CD147			MCT1/CD147			MCT4/CD147		
	n	Positive (%)	*p*	*n*	Positive (%)	*p*	*n*	Positive (%)	*p*
Age			0.894			0.634			0.355
>61	83	41.0		80	35.0		78	11.5	
⩽61	69	42.0		67	38.8		65	16.9	
Gender			0.031			0.109			0.209
Female	51	29.4		48	27.1		48	8.3	
Male	103	47.6		101	40.6		96	16.7	
Tumour size (cm)			0.055			0.456			0.517
<4	60	31.7		59	32.2		55	16.4	
⩾4	93	47.3		89	38.2		88	12.5	
Tumour localisation			0.891			0.983			0.649
Body	36	38.9		35	37.1		33	15.2	
Antrum	103	41.7		100	37.0		97	13.4	
Others	6	33.3		6	33.3		5	0.0	
Macroscopic classification			0.001			0.030			0.626
Early gastric cancer	52	23.1		50	24.0		44	15.9	
Advanced gastric cancer	103	50.5		100	42.0		101	12.9	
Lauren's classification			0.010			0.020			0.023
Intestinal-type	101	49.5		97	43.3		96	18.8	
Diffuse-type	48	27.1		47	23.4		45	4.4	
TNM			0.006			0.004			0.304
IB + II	134	37.3		129	31.8		125	12.8	
III + IV	14	70.0		20	65.0		19	21.1	
pT			0.002			0.073			0.307
Mucosa	52	23.1		50	24.0		44	15.9	
Muscular propria/subserosa	95	50.5		92	42.4		93	11.8	
Adjacent structures	6	66.7		6	50.0		6	33.3	
Lymph-node metastasis			0.003			0.018			0.815
Absent	74	29.7		71	26.8		68	14.7	
Present	79	53.2		77	45.5		75	13.3	
Lymphatic invasion			0.474			0.864			0.913
Absent	89	39.3		84	35.7		80	13.8	
Present	62	45.2		62	37.1		61	13.1	
Vascular invasion			0.692			0.649			0.738
Absent	127	40.9		122	36.9		117	14.5	
Present	22	45.5		22	31.8		22	9.1	

Median was used for age and tumour size cut-off.

gastric tumours. Therefore, the present study is an attempt to shed light on the involvement of MCTs in tumours arising from the gastric mucosa. With this purpose, we analysed MCT immunohistochemical expression in gastric samples organised into TMAs. The TMA technology allows simultaneous screening of large series of samples without losing tumour representativity.[25]

Although MCTs have been little explored in cancer, there are evidences for the up-regulation of these proteins in a variety of tumours[9–14] but also reports of down-regulation.[26,27] Interestingly, in the present study, no alteration in expression frequency was identified for MCT1, whereas a significant decrease in MCT4 expression was observed. It is important to highlight that the gastric milieu is not comparable to the majority of body tissues, since gastric mucosa is permanently under acidic conditions. This particular condition demands a very high metabolic conversion which may involve high MCT4 activity in homeostasis. Considering the acidic environment of gastric cells, the role of MCTs in cellular pH regulation,[8] as well as the capacity of MCT4 to export lactate through a proton symport, it is, perhaps, not surprising to find important levels of MCT4 in normal gastric cells. Similarly to what we observed for MCT4, carbonic anhydrase IX, also involved in the maintenance of intracellular pH and commonly up-regulated in cancer, is less frequently expressed in gastric carcinoma than in normal gastric epithelium.[28] To note that both MCT4 and carbonic anhydrase IX are HIF-1α (hypoxia-inducible factor 1) target proteins,[28,29] which may give some clues about the metabolic and molecular alterations occurring in the transition from normal gastric epithelium to gastric carcinoma. However, the reason why these two molecules are down-regulated in gastric cancer cells, as well as the mechanism by which these events take place, remains to be unveiled.

Despite the fact that MCT4 was down-regulated in our series of gastric tumour samples, a significant association with Lauren's intestinal-type tumours was found. This group of tumours includes well-differentiated carcinomas in opposition to the diffuse-type group, which are poorly-differentiated carcinomas with worse prognosis.[2,3] Therefore, intestinal-type

tumours are more alike normal gastric epithelium and the increased expression of MCT4 in this type of tumours is in agreement with our observation that the non-neoplastic epithelium expresses more MCT4.

CD147 is described as an important key element in oncogenesis[21,22] and there are some published data regarding its significance in gastric carcinoma.[24,30] In the present work, we found a higher CD147 expression in intestinal-type carcinomas, which is in agreement with the results reported by others.[30] Additionally, CD147 has been pointed as a good marker for local invasion and prognosis, being associated with tumour size, depth of invasion and both lymphatic and blood vessel invasions.[24] We confirmed some of these associations, namely tumour size (only for intestinal tumour type) and depth of invasion, and also found positive associations with higher TNM staging and lymph-node metastasis, supporting the role of CD147 as a good indicator of prognosis. Although the last associations were not described by Zheng and collaborators,[24] our results are in agreement with the enhanced metastatic capacity of CD147 expressing tumour cells, due to its role as a matrix metalloproteinase-inducing factor as well as inducer of VEGF and hyaluronan production.[21,22] Importantly, we assessed the correlation between MCTs and CD147, as this molecule is described as an important regulator of MCT activity and expression.[15–19] Here, we observed a close association between the expressions of CD147 and both MCT1 and MCT4, which provides further evidence for the regulation of MCT1/MCT4 by CD147, specifically in gastric tumour cells. On the other hand, there are a significant number of MCT positive cases, negative for CD147, which led us to speculate that MCT plasma membrane expression may depend on a yet non-identified regulation mechanism. Nevertheless, most clinico-pathological associations found for CD147 were maintained for MCT1/CD147 co-expression, which supports previous data showing that CD147 activity is dependent on MCT1 co-expression.[19] Although CD147 expression was also associated with MCT4, the co-expression of MCT4/CD147 gave no additional information than the expression of MCT4 alone, showing that, in opposition to MCT1, MCT4 associated to CD147, is not contributing to the aggressive phenotype of gastric cancer.

In the present study, we analysed the expression of MCT1 and MCT4 in non-neoplastic gastric mucosa, primary gastric tumours and lymph-node metastases, as well as CD147 as MCT regulator, in gastric tissues. Importantly, we evaluated for the first time the correlation between MCT expression and clinico-pathological data in gastric cancer and found important associations, especially with intestinal-type carcinomas. Notably, our data confirm the prognostic value of CD147 in cancer and show for the first time that this value is associated with MCT1 co-expression in gastric cancer cells.

Ethics

The present study has been approved by the local Ethic Committees.

Conflict of interest statement

None declared.

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Chapter 5. MCTs IN BREAST CARCINOMA

5.1. CHAPTER OVERVIEW

As the majority of carcinogenesis processes, the progression of normal mammary epithelium into invasive carcinoma is widely accepted as a multistep process, where different events like loss of tumour-suppressor function, abnormal oncogene activation, abnormal response to growth factors, among others, take place [1].

FdG-PET is considered the most reliable imaging technique for the diagnosis and prognosis of breast cancer, especially regarding the detection of distant metastases, recurrent disease as well as monitoring response to therapy [1]. As increased glucose uptake by cancer cells is on the basis of this imaging technique, breast cancer cells have to exhibit enhanced glycolytic rates, which has been confirmed by some studies [2,3].

Although MCT expression has been evaluated in some breast cancer cell lines, no data on the frequency or prognostic value of MCT expression in human breast carcinoma was available.

In this chapter, three distinct studies in breast carcinoma are presented. Firstly, results already published in an international periodical with referees showed an increase of MCT1 expression in breast carcinoma, in a series comprising 249 samples. Also, both MCT1 and MCT4 expressions were associated with CD147. Importantly, MCT1 and CD147, alone or in co-expression, were associated with basal-like subtype (a more aggressive breast cancer group) and other poor prognostic variables, pointing at a role of MCT1/CD147 in breast carcinoma aggressiveness. In the second study, already submitted for publication, GLUT1 and CAIX were evaluated as indicators of the hypoxia-induced adaptations towards a glycolytic phenotype. Importantly, both MCT1 and CD147, but not MCT4, were significantly associated with GLUT1 and CAIX. Also, additional data on the prognostic value of GLUT1 and CAIX was provided, showing the association of these two proteins with poor prognostic variables, and supporting the association of CAIX with shorter disease-free survival. Finally, *in vitro* studies were performed to shed some light into the contribution of MCT1 to breast cancer cell metabolism and viability. For that, MCT1 inhibition studies were performed in a variety of human breast cancer cells. In this work, after analysing the expression of MCT1, MCT4, CD147, as well as GLUT1 and CAIX in the human breast cancer cell lines MCF-7/AZ, SkBr3, MDA-MB-468, BT-20, MDA-MB-231 and Hs578T, MCT1 inhibition studies were carried out using the MCT1 classical inhibitor CHC. This inhibitor induced a significant decrease in total biomass of almost all cell lines, though at different magnitudes, which did not correlate with MCT1 expression. Importantly, MDA-MB-468, a basal-like subtype breast cancer line, showed a CHC-induced inhibition of total biomass, which was accompanied with a decrease in glucose consumption and lactate production, evidencing the role

of MCT1 in this group of breast carcinomas. However, the most sensitive cell line, MDA-MB-231, was negative for MCT1 expression and CHC-induced inhibition was not accompanied by extracellular decrease in lactate amounts, suggesting an off-target effect of CHC.

Overall, the results presented in this chapter point at MCT1 as a potential therapeutic target in breast cancer, with special emphasis on basal-like subtype, which so far does not have a specific molecular therapy.

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5.2. PUBLISHED RESULTS

This chapter includes the following results, **published** in an international scientific periodical with referees:

Pinheiro C, Albergaria A, Paredes J, Sousa B, Dufloth R, Vieira D, Schmitt F, Baltazar F. Monocarboxylate transporter 1 is upregulated in basal-like breast carcinoma. *Histopathology* In press.

The following results were also presented as poster in the following international scientific meetings:

CNIO-ONCOTRAIN Meeting: "New battlefields in human cancer- Attacking in many fronts", 2008, Madrid, Spain. ***Pinheiro C****, Sousa B, Ferreira L, Dufloth R, Vieira D, Schmitt F, Baltazar F. Monocarboxylate transporter 1 is upregulated in ER negative breast cancer;*

Eleventh Cancer Research UK Beatson International Cancer Conference, 2008, Glasgow, United Kingdom. ***Pinheiro C****, Sousa B, Ferreira L, Dufloth R, Vieira D, Schmitt F, Baltazar F. Monocarboxylate transporter 1 (MCT1) overexpression is associated with poor prognosis in breast carcinomas.*

5.2.1. MONOCARBOXYLATE TRANSPORTER 1 IS UP-REGULATED IN BASAL-LIKE BREAST CARCINOMA

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Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma

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Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma

Aims: Monocarboxylate transporters (MCTs) have been considered promising targets for cancer therapy, since they facilitate lactate efflux in glycolytic tumours. However, their role in solid tumours is still poorly understood. Thus, the present work aimed to contribute to understanding the involvement of MCT1 and MCT4 in breast cancer progression as well as MCT regulation by CD147.

Methods and results: The expression of the membrane transporters MCT1 and MCT4 was analysed in a series of breast carcinomas (249 cases) and their clinicopathological significance investigated. Additionally, we analysed the significance of CD147 co-expression, as an important regulator of MCT expression and activity. MCT1 was significantly increased in breast carcinomas when compared with normal breast tissue and, importantly, both MCT1 and CD147 were associated with poor prognostic variables such as basal-like subtype and high grade tumours.

Conclusions: These results provide evidence for a prognostic value of MCT1 in breast carcinoma and support the exploitation of the complex MCT1/CD147 as a promising target for cancer therapy, especially in basal-like breast carcinoma.

Keywords: breast carcinoma, CD147, immunohistochemistry, monocarboxylate transporter

Abbreviations: CK, cytokeratin; DAB, 3,3′-diaminobenzidine; DCIS, ductal carcinoma *in situ;* EGFR, epidermal growth factor receptor; ER, oestrogen receptor; MCTs, monocarboxylate transporters; PR, progesterone receptor; TMA, tissue microarray

Introduction

Despite advances in cancer therapy, breast cancer is still the leading cause of cancer-related death in women worldwide.[1] Breast cancer can be classified according to gene expression profiles into four main groups: basal-like, luminal (A and B), HER2+ and normal-like breast carcinomas,[2–7] which have important prognostic implications: basal-like and HER2+ tumours have more aggressive clinical behaviour when compared with luminal and normal-like breast carcinomas.[2–4,6,7] Basal-like breast carcinomas, in contrast to the other groups, do not have a specific molecular therapy, necessitating the search for new molecular targets in this aggressive group of tumours.[8,9]

The extracellular pH of solid tumours is generally low, whereas cytosolic pH is normal or higher than that of normal tissues.[10,11] In breast cancer cells, this lower extracellular pH is explained by, *inter alia*, two complementary mechanisms: increased lactate production through glycolysis and proton-pump activity in the plasma membrane. The former, which is quantitatively more important, is consistent with the

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known high glycolytic rates of cancer cells, especially under anaerobic conditions, which results in production of large amounts of lactate.[12] Acidification of the extracellular microenvironment has several implications in tumour progression, e.g. up-regulation of various angiogenic molecules including vascular endothelial growth factor,[13,14] which support tumour growth, invasion and metastasis, and up-regulation of proteases implicated in cancer cell invasiveness, through digestion of the extracellular matrix.[14]

One of the most important mechanisms involved in intracellular pH regulation is the co-transport of lactate and a proton, which is mediated by a family of membrane proteins known as monocarboxylate transporters (MCTs).[15] These proteins are also responsible for the transport of pyruvate and ketone bodies, being critical for metabolic communication between cells.[16] Taking into consideration the high glycolytic rates of cancer cells, it is to be expected that MCTs would be up-regulated, to allow maintenance of glycolysis rates and prevent apoptosis by intracellular acidosis. Indeed, there is evidence for the up-regulation of MCTs in several tumours.[17–24] However, only a few studies have evaluated the clinicopathological significance of MCT overexpression.[23–25] We have recently described MCT1 and MCT4 overexpression in colorectal carcinomas, as well as an association between plasma membrane expression of MCT1 and vascular invasion.[24] Furthermore, we observed an increase in MCT1 and MCT4 during progression to invasive cervical carcinoma and an association between both MCT1 and MCT4 expression and high-risk human papillomavirus infection.[23] Moreover, we observed preferential expression of MCT4 in intestinal-type gastric carcinoma, although with a decrease in MCT4 expression from normal to malignant gastric mucosa.[25]

To the best of our knowledge, MCT analysis in breast cancer samples has been performed in only one study, where silencing of MCT1 by methylation of the 5′ region of the gene was suggested, in four of 19 breast cancer samples.[26] However, this result is far from clarifying the role of MCTs in breast cancer development and/or progression.

It was recently demonstrated that CD147, a known key regulator of oncogenesis,[27–29] is an ancillary protein required for cell surface expression and activity of MCT1 and MCT4.[30–32] On the other hand, silencing studies have shown that maturation and cell surface expression of CD147 depends on both MCT1 and MCT4 expression.[33,34] Recently, and supporting this evidence, we described a close association between both MCT1 and MCT4 and CD147 in cervical cancer.[35]

In the present work, we sought to assess the involvement of MCT1 and MCT4 in breast cancer progression by analysing the expression of these membrane transporters in a series of breast carcinomas, and to investigate its clinicopathological significance. We also aimed to contribute to the understanding of MCT regulation by CD147 in tumours, by analysing the significance of MCT and CD147 co-expression in breast carcinoma.

Materials and methods

CASE SELECTION

Case selection was based on availability of follow-up information and amount of material, ensuring adequate numbers for statistical analysis. Thus, a series of 249 formalin-fixed paraffin-embedded primary breast carcinomas was retrieved from the files of the Department of Pathology, Hospital do Divino Espírito Santo (Azores, Portugal) and from the Federal University of Santa Catarina (Florianópolis-SC, Brazil). Samples were obtained from patients aged 30–89 years. Haematoxylin and eosin-stained sections of all cases were reviewed by three pathologists (R.D., D.V. and F.S.) and the diagnoses were confirmed as follows: 208 invasive ductal carcinomas, seven invasive lobular carcinomas, three mixed lobular-ductal carcinomas, three tubular, eight medullary and 20 invasive breast carcinomas of other special histological types. Representative areas of tumour samples, as well as 53 samples of normal breast tissue, mostly from the same breast with tumour, were carefully selected and organized into tissue microarrays (TMAs), with cores of 2 mm diameter. Each case was represented in the TMA by at least two cores. In 45 cases, areas of ductal carcinoma *in situ* (DCIS) were present and were also analysed. Relevant clinicopathological data from these tumours included tumour size, molecular classification, histological grade and lymph node metastasis. The distribution of prognostic factors with a high number of cases with lymph node metastasis and oestrogen receptor (ER)-negative status, reflects a large number of advanced cases in the series.

The molecular classification was carried out based on immunohistochemical results for ER, progesterone receptor (PR), HER2, epidermal growth factor receptor, cytokeratin (CK) 5, CK14, vimentin and Ki67. Tumours positive for ER and/or PR were classified as luminal. Cases positive for ER/PR and for HER2 and/or high Ki67 index were subclassified as luminal B. Cases classified as HER2 overexpressing were characterized by HER2 overexpression and negativity for ER/PR, and cases defined as 'basal-like' were negative

for ER/PR and HER2 and positive for at least one of the 'basal markers' tested.

Follow-up information was available for 218 cases, ranging from a minimum of two to a maximum of 129 months (median 32 months). Disease-free survival interval data were evaluated and defined as the time from the date of surgery to the date of breast cancer-derived relapse/metastasis. Due to the short follow-up of the studied series and consequent limited number of death events, overall survival was not analysed.

This study was conducted under the relevant national law regulating the usage of biological specimens from tumour banks, where the samples are exclusively available for research purposes in the case of retrospective studies.

IMMUNOHISTOCHEMISTRY

MCT detection

Immunohistochemistry was performed according to the avidin–biotin–peroxidase complex principle [R.T.U. VECTASTAIN Elite ABC Kit (Universal); Vector Laboratories, Burlingame, CA, USA], with the primary antibodies for MCT1 (AB3538P; Chemicon International, Temecula, CA, USA) and MCT4 (AB3316P; Chemicon International), diluted 1:200 for MCT1 and 1:100 for MCT4, as previously described.[23–25] Briefly, deparaffinized and rehydrated tissue sections were submitted to antigen retrieval by immersion in citrate buffer (0.01 M, pH 6.0) and heated to 98°C for 20 min, in a water bath. Tissues were then incubated with the primary antibody overnight at room temperature and immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB+ Substrate System; Dako, Carpinteria, CA, USA). Colonic carcinoma was used as positive control for both MCT1 and MCT4.

CD147 detection

Immunohistochemistry was performed according to the avidin–biotin–peroxidase complex principle (Ultravision Detection System Anti-polyvalent, Horseradish peroxidase; Lab Vision Corp., Fremont, CA, USA), using a primary antibody raised against CD147 (18-7344; Zymed Laboratories Inc., South San Francisco, CA, USA) diluted 1:750, as previously described.[35] Briefly, deparaffinized and rehydrated tissue sections were submitted to antigen retrieval by immersion in ethylenediamine tetraaceticacid (pH 8.0) heated to 98°C for 15 min, in a water bath. Tissues were then incubated with the primary antibody, 2 h at room temperature, and immunoreactivity was visualized with DAB (DAB+ Substrate System; Dako). Cervical squamous carcinoma was used as positive control.

Immunohistochemical evaluation

Since plasma membrane location is essential for protein activity, immunoreactions for MCTs and CD147 were considered positive only when plasma membrane reactivity was present. Sections were scored semiquantitatively in relation to the positive control as previously described:[23–25,35] 0, 0% of immunoreactive cells; 1, <5% of immunoreactive cells; 2, 5–50% of immunoreactive cells; and 3, >50% of immunoreactive cells. Also, intensity of staining was scored as: 0, negative; 1, weak; 2, intermediate; and 3, strong. Final immunoreactivity score was defined as the sum of both parameters (extent and intensity), and grouped as negative (score 0 and 2) and positive (3–6). Evaluation was performed blindly by two independent observers and discordant results were discussed at a double-head microscope to reach the final score.

STATISTICAL ANALYSIS

Data were stored and analysed using Statview statistical software (SAS Institute Inc., Cary, NC, USA). All comparisons were examined for statistical significance using Pearson's chi-squared test or Fisher's exact test, as appropriate; the threshold for significance was set at $P < 0.05$. Disease-free survival curves were estimated by the method of Kaplan–Meier and data compared using the log rank test. A cut-off of 60 months (5 years) was considered, since in the first 5 years following primary therapy recurrence rates are expected to be highest, especially in series with high numbers of ER– cases like ours, where the hazard of recurrence is higher.[36] Cases lacking one or more of the clinicopathological variables were not included in the specific statistical analysis.

Results

A total of 249 breast carcinoma samples for MCT1, MCT4 and CD147 immunohistochemical expression were analysed. Due to technical problems (mainly tissue loss during immunohistochemistry), some cases are missing in the expression analysis.

MCT1 expression was mainly found at the plasma membrane whereas MCT4 was observed both in the cytoplasm and plasma membrane (Figure 1A,B). CD147 was mainly detected at the plasma membrane (Figure 1C), with some cases also showing cytoplasmic immunoreactivity. Table 1 summarizes the frequency of MCT and CD147 expression in non-neoplastic mammary epithelium and tumour samples. A significant increase in MCT1 expression was observed in tumours when compared with normal tissues

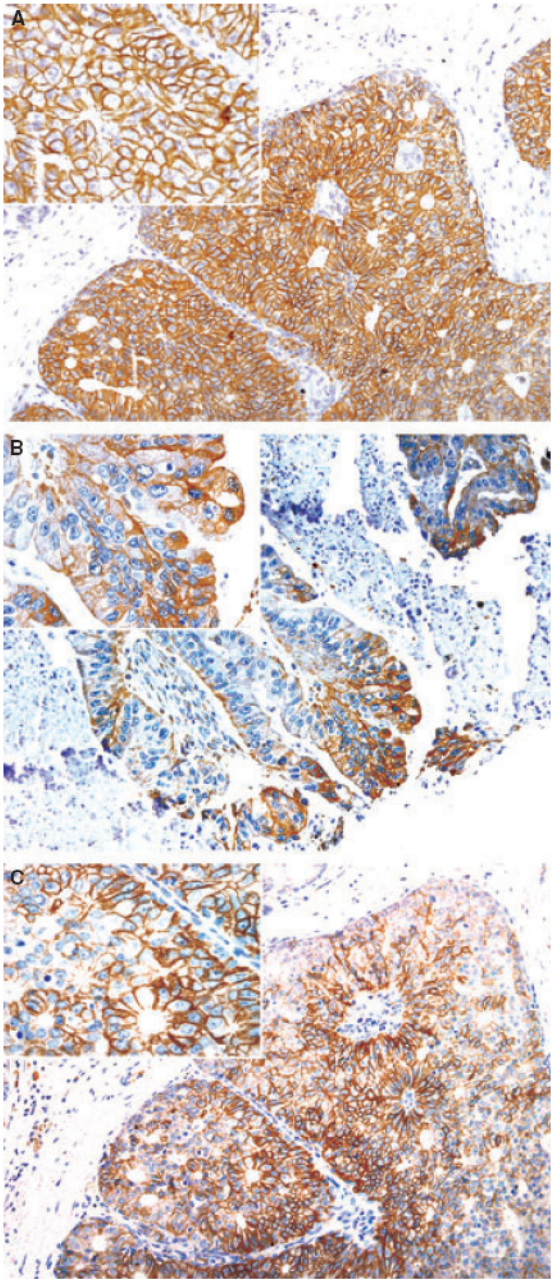

Figure 1. Immunohistochemical expression of monocarboxylate transporter (MCT) 1, MCT4 and CD147 in breast carcinoma samples. MCT1 expression is mainly found on the plasma membrane of tumour cells (A), whereas MCT4 reactivity is found both in the cytoplasm and plasma membrane (B). CD147 is mainly observed on the plasma membrane of tumour cells (C).

($P = 0.0138$), with 19.5% of tumours showing MCT1 expression, while no significant difference was observed for MCT4 and CD147.

Analysis of CD147 and MCT plasma membrane expression showed an association between CD147 and both MCT1 and MCT4 ($P < 0.0001$ and $P = 0.0083$, respectively, Table 2). DCIS, when present in tissue sections, was also evaluated for MCT and CD147 expression. DCIS was found in 45 sections, and results for MCT and CD147 expression were always concordant with the corresponding invasive lesion.

Biological and clinical data available allowed the assessment of correlations with MCT and CD147 expression (Table 3). Importantly, we found significant correlations between prognostic parameters and both MCT and CD147 membranous expression. Correlations between MCT1 expression and basal-like subtype ($P < 0.0001$), high histological grade ($P = 0.0003$), absence of ER and PR expression ($P < 0.0001$ and $P = 0.0001$, respectively), CK5 and CK14 expression ($P = 0.0362$ and $P = 0.0006$, respectively), vimentin expression ($P < 0.0001$) and Ki67 expression ($P = 0.0297$) were observed. No association was observed between MCT4 expression and clinicopathological

Table 1. Frequency of monocarboxylate transporter (MCT) 1, MCT4 and CD147 expression in breast carcinoma tissues compared with normal breast epithelium

	n	Positive (%)	*P*
MCT1			
Normal breast epithelium	53	3 (5.7)	**0.0138**
Breast carcinoma	221	43 (19.4)	
MCT4			
Normal breast epithelium	45	0 (0.0)	0.0823
Breast carcinoma	219	16 (7.3)	
CD147			
Normal breast epithelium	47	2 (4.2)	0.2570
Breast carcinoma	219	24 (11.0)	

Table 2. Association between CD147 and monocarboxylate transporter (MCT) expression in breast carcinoma samples

	n	CD147 Positive (%)	*P*
MCT1			
Negative	172	5 (2.9)	<0.0001
Positive	42	19 (45.2)	
MCT4			
Negative	200	19 (9.5)	0.0083
Positive	16	5 (31.2)	

Table 3. Associations of monocarboxylate transporter (MCT) 1, MCT4 and CD147 expression with clinicopathological data from breast cancer cases

Clinicopathological data	MCT1			MCT4			CD147		
	n	Positive (%)	*P*	*n*	Positive (%)	*P*	*n*	Positive (%)	*P*
Tumour size, mm									
<20	73	10 (13.7)	0.2544	72	6 (8.3)	0.3152	73	7 (9.6)	0.7334
20–50	105	21 (20.0)		104	9 (8.6)		104	13 (12.5)	
>50	25	7 (28.0)		25	0 (0.0)		25	2 (8.0)	
Subtype									
Luminal	125	13 (10.4)	**<0.0001**	122	10 (8.2)	0.9529	123	5 (4.1)	**<0.0001**
Basal-like	42	21 (50.0)		42	3 (7.1)		42	14 (33.3)	
HER2 overexpressing	32	5 (15.6)		33	3 (9.1)		32	4 (12.5)	
Histological grade									
I	45	5 (11.1)	**0.0003**	43	5 (11.6)	0.1637	44	0 (0.0)	**<0.0001**
II	104	13 (12.5)		103	4 (3.9)		102	6 (5.9)	
III	71	25 (35.2)		71	7 (9.8)		71	18 (25.4)	
Oestrogen receptor									
Negative	95	30 (31.6)	**<0.0001**	95	6 (6.3)	0.5989	93	19 (20.4)	**0.0002**
Positive	125	13 (10.4)		122	10 (8.2)		123	5 (4.1)	
Progesterone receptor									
Negative	137	38 (27.7)	**0.0001**	139	11 (7.9)	0.6843	136	22 (16.2)	**0.0014**
Positive	80	5 (6.2)		78	5 (6.4)		80	2 (2.5)	
HER2 overexpression									
Negative	175	37 (21.1)	0.2135	172	11 (6.4)	0.4959	174	19 (10.9)	>0.9999
Positive	40	5 (12.5)		41	4 (9.8)		39	4 (10.2)	
EGFR									
Negative	206	39 (18.9)	0.2343	204	14 (6.9)	0.1925	204	21 (10.3)	0.1108
Positive	11	4 (36.4)		11	2 (18.2)		11	3 (27.3)	
CK5									
Negative	174	29 (16.7)	**0.0362**	171	10 (5.8)	0.0974	170	14 (8.2)	**0.0116**
Positive	46	14 (30.4)		46	6 (13.0)		47	10 (21.3)	
CK14									
Negative	201	34 (16.9)	**0.0006**	199	15 (7.5)	>0.9999	199	17 (8.5)	**<0.0001**
Positive	15	8 (53.3)		15	1 (6.7)		15	7 (46.7)	
Vimentin									
Negative	175	26 (14.8)	**<0.0001**	173	12 (6.9)	0.4869	172	9 (5.2)	**<0.0001**
Positive	36	17 (47.2)		36	4 (11.1)		36	15 (41.7)	

 Histopathology

Table 3. (Continued)

Clinicopathological data	MCT1			MCT4			CD147		
	n	Positive (%)	*P*	*n*	Positive (%)	*P*	*n*	Positive (%)	*P*
Ki67									
<20%	139	21 (15.1)	**0.0297**	138	9 (6.5)	0.5258	138	10 (7.2)	**0.0179**
>20%	81	22 (27.2)		79	7 (8.9)		79	14 (17.7)	
Lymph node metastasis									
Absent	95	21 (22.1)	0.2771	92	7 (7.6)	0.8866	95	15 (15.8)	0.1566
Present	100	16 (16.0)		99	7 (7.1)		99	9 (9.1)	

EGFR, Epidermal growth factor receptor.

factors. CD147 expression associations were very similar to those of MCT1: CD147 correlated with basal-like subtype ($P < 0.0001$), high histological grade ($P < 0.0001$), absence of ER and PR expression ($P = 0.0002$ and $P = 0.0014$, respectively), CK5 and CK14 expression ($P = 0.0116$ and $P < 0.0001$, respectively), vimentin expression ($P < 0.0001$) and Ki67 expression ($P = 0.0179$).

When analysing MCT1/CD147 co-expression (data not shown) as an indicator of transporter activity, we observed the same associations as for MCT1 and CD147 alone. The clinicopathological significance of MCT4/CD147 co-expression was not assessed since the number of cases co-expressing these two molecules was very low ($n = 5$).

Kaplan–Meier survival curves were estimated for each marker (MCTs and CD147), but no significant correlations were found (data not shown).

Discussion

Some evidence points to MCTs as potential targets for cancer therapy.[19,22,37] However, the role of these proteins in solid tumour development and survival is still unclear, especially in breast cancer, where there is a very limited number of studies.

A previous report on breast cancer has suggested a possible silencing of MCT1 expression.[26] However, in the present study we showed a significant gain in MCT1 plasma membrane expression and no significant alteration in MCT4 expression in tumours when compared with non-neoplastic tissue. Considering the metabolic alterations of cancer, where high glycolytic rates lead to high levels of lactate, an increase in MCT1 expression would allow continuous glycolysis, avoiding intracellular acidosis and subsequent apoptosis. Also, and importantly, this would give a powerful growth advantage to cancer cells, necessary for progression to invasiveness.[38] Notably, and besides the increased expression in tumour cells, the presence of MCT1 in the plasma membrane was associated with various clinical and biological parameters. Importantly, MCT1 expression correlated with a basal-like phenotype, and, as expected, was associated with most of the basal markers studied, such as CK5, CK14 and vimentin, and inversely associated with the expression of ER and PR. These results are supported by data available in expression array datasets (ONCOMINE),[39] where increased expression in MCT1 can be seen in basal-like breast carcinoma.[40,41] Other correlations, such as the association with Ki67 proliferative index and high histological grade, suggest that MCT1 is present in more aggressive tumours.

Taking into consideration the need to export lactate in cancer cells and the fact that MCT4 is a low-affinity transporter found in highly glycolytic tissues,[42] an increase in MCT4 expression would be expected in breast tumours, similar to that observed in both colorectal[24] and cervical carcinomas.[23] However, in the present work, although MCT4 was not expressed in normal tissue and was detected in the plasma membrane of some tumour samples, this increase was not significant. Thus, our results point to preferential expression of the MCT isoform 1, as an adaptation to a malignant phenotype. Actually, despite the similar physiological function of MCT1 and MCT4, they are regulated by different mechanisms; for example, it has recently been described that hypoxia-inducible factor 1α, a major regulator of tumour cell adaptation to hypoxic stress, up-regulates MCT4, but not MCT1.[43]

CD147, a protein that, among many other functions,[27–29] stimulates synthesis of matrix metalloproteinases, is up-regulated in many human

cancers,[27,28,44,45] including breast carcinomas,[44,45] where it is described as a prognostic factor.[44] In the present work, this documented increase in CD147 was not observed, but the close association with both MCT1 and MCT4 previously demonstrated[25,30–35] was confirmed in the breast tumour samples analysed here. As observed for MCT1, CD147 expression was more frequently found in basal-like, ER– and PR– and high histological grade tumours as well as in tumours expressing CK5, CK14, vimentin and Ki67, which is in accordance with previous results on CD147 expression in breast carcinoma.[44] MCT1/CD147 co-expression showed similar associations with MCT1 and CD147 alone. A similar importance for MCT1/CD147 co-expression in gastric cancer has already been described by our group in gastric cancer, where we observed an association of MCT1/CD147 co-expression with indicators of worse prognosis, such as advanced gastric cancer, higher TNM staging and presence of lymph node metastasis.[25] However, although these associations may be mostly attributed to CD147 and its many functions in cancer,[27–29] MCT1 alone also appears to have an important role in tumours with worse prognosis, as evidenced by the significant results obtained. Importantly, it should be noted that less than half of cases expressing MCT1 and MCT4 on the plasma membrane co-express CD147. This suggests that MCT trafficking to the plasma membrane might be dependent on another ancillary protein, as pointed out by recent *in vitro* evidence.[46]

In the present study, we have investigated the expression of MCT isoforms 1 and 4, and their chaperone CD147. Most importantly, we have evaluated for the first time the correlation between MCT expression in breast cancer and the clinicopathological data. It is important to highlight that MCT1 is up-regulated in a subset of aggressive breast carcinomas (basal-like) and, since these tumours do not have a specific molecular therapy,[8] the development of therapeutic approaches targeting MCT1 could be a promising strategy to treat such tumours. However, other studies, including *in vitro* approaches are warranted to confirm these observations.

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5.3. SUBMITTED RESULTS

This chapter also comprises the following manuscript, **submitted** for publication to an international scientific periodical with referees:

Pinheiro C, Sousa B, Albergaria A, Paredes J, Dufloth R, Vieira D, Schmitt F, Baltazar F. GLUT1 and CAIX expression profiles in breast cancer correlate with MCT1 overexpression and adverse prognostic factors.

The following results were also presented as poster in the following international scientific meeting:

EACR-21, 2010, Oslo, Sweden. ***Pinheiro C****, Sousa B, Albergaria A, Paredes J, Dufloth R, Vieira D, Schmitt F, Baltazar F. GLUT1 and CAIX expression profiles in breast cancer correlate with MCT1 overexpression.*

5.3.1. GLUT1 AND CAIX EXPRESSION PROFILES IN BREAST CANCER CORRELATE WITH MCT1 OVEREXPRESSION AND ADVERSE PROGNOSTIC FACTORS

GLUT1 and CAIX expression profiles in breast cancer correlate with MCT1 overexpression and adverse prognostic factors

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ABSTRACT

The goal of the present work was to evaluate the correlation of glucose transporter 1 (GLUT1) and carbonic anhydrase IX (CAIX) with the monocarboxylate transporters 1 (MCT1) and 4 (MCT4) and their chaperone, CD147, in breast cancer. The clinico-pathological value of GLUT1 and CAIX was also evaluated. For that, we analysed the immunohistochemical expression of GLUT1 and CAIX, in a large series of invasive breast carcinoma samples (n=124), previously characterised for MCT1, MCT4 and CD147 expression. GLUT1 expression was found in 46% of the cases (57/124), while CAIX expression was found in 18% of the cases (22/122). Importantly, both MCT1 and CD147, but not MCT4, were associated with GLUT1 and CAIX expression. Also, GLUT1 and CAIX correlated with each other. Concerning the clinico-pathological values, GLUT1 was associated with high grade tumours, basal-like subtype, absence of progesterone receptor and presence of vimentin and Ki67 expression. Additionally, CAIX was associated with high tumour size, high histological grade, basal-like subtype, absence of estrogen and progesterone receptors and presence of basal cytokeratins and vimentin expression. Finally, patients with CAIX positive tumours had a significant shorter disease-free survival.

The association between MCT1 and both GLUT1 and CAIX may result from the hypoxia-mediated metabolic adaptations, which will confer a glycolytic, acid-resistant and more aggressive phenotype to cancer cells.

Keywords: GLUT1; CAIX; monocarboxylate transporters (MCT); CD147/EMMPRIN; breast carcinoma; immunohistochemistry.
Abbreviations: CA (carbonic anhydrase); ER (estrogen receptor); GLUT (glucose transporter); HIF-1α (hypoxia inducible-factor 1 alpha); MCT (monocarboxylate transporter); PR (progesterone receptor).

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INTRODUCTION

Early epithelial carcinogenesis occurs under hypoxic conditions, since altered cells are separated from the vascularised stroma, source of oxygen and nutrients. To maintain the needed ATP levels, cancer cells increase their rates of glycolysis, acquiring a significant proliferative advantage. However, this phenotype leads to an overload of lactic acid, which must be exported from the cell, causing a decrease in the extracellular pH.

Constitutive up-regulation of glycolysis requires additional adaptations, namely, resistance to apoptosis

and up-regulation of membrane transporters to maintain normal intracellular pH [1]. The need to increase glucose uptake, to allow high glucose consumption rates, is achieved by up-regulation of glucose transporters (GLUT) in the plasma membrane of cancer cells, especially the hypoxia-responsive GLUT1 [2;3]. The role of GLUT1 in breast cancer remains poorly elucidated [4;5].

Besides being an adaptation to high glycolytic phenotype, acidic environment represents *per se* a significant advantage for tumour cells since it is associated with increased migration, invasion and metastases, among others [1;6;7]. Although lactate produced by glycolysis under hypoxic conditions is a significant contributor to the acidic extracellular pH, there is also a substantial contribution from carbonic acid [8]. In this context, the hypoxia-responsive carbonic anhydrase isoforms, CAIX and CAXII, emerge as important contributors to the regulation of cancer cell intracellular pH [9-11], with CAIX, in particular, being associated with poor prognosis in breast cancer [12-15].

Another important group of proteins involved in intracellular pH regulation are monocarboxylate transporters (MCTs), which are also responsible for transmembrane transport of lactate [16]. By performing these two inter-dependent activities (lactate transport coupled with a proton), MCTs appear as strong potential targets for cancer therapy. Indeed, there are evidences for the up-regulation of MCTs in tumours, such as high grade glial neoplasms [17;18], colorectal [19;20], lung [21], cervical [22], and breast carcinomas [23]. Besides analysing MCT expression in tumours, our group also assessed for the first time the clinico-pathological value of their overexpression [20;22-24]. MCT expression appears to be influenced by altered physiologic conditions; however, the underlying molecular events involved in MCT regulation are poorly understood. Recently, it was demonstrated that proper expression and activity of MCT1 and MCT4 requires co-expression of CD147, also known as EMMPRIN or Basigin [25-27]. Based on this, we described the association between CD147 and both MCT1 and MCT4 in human cervical [28], gastric [24] and breast cancer [23]. Furthermore, the hypoxia inducible-factor 1 alpha (HIF-1α), which regulates many genes codifying proteins involved in the glycolytic pathway (like GLUT1) and pH regulation (like the Na^+/H^+ exchanger NHE1 and both CAIX and CAXII) [29], also regulates MCT1 [30] and MCT4 [30;31]. However, there is some controversy around MCT1 regulation by hypoxia, with some studies reporting MCT1 repression by hypoxia [31;32].

One of the goals of the present study was to evaluate the association between the HIF-1α downstream targets GLUT1 and CAIX and both MCT1 and MCT4, as well as their chaperone, CD147, in invasive breast carcinomas. We also intended to strengthen the clinico-pathological value of GLUT1 and CAIX in breast cancer.

MATERIALS AND METHODS

Case selection

Case selection was based on availability of follow up information and amount of material, ensuring adequate numbers for statistical analysis. Thus, a series of 124 formalin-fixed paraffin embedded breast carcinoma tissues was retrieved from the files of the Department of Pathology, Hospital do Divino Espírito Santo, Azores, Portugal, and from the Federal University of Santa Catarina, Florianopolis-SC, Brazil. Haematoxylin/eosin stained sections of all cases were reviewed by three pathologists (R.D., D.V. and F.S.). Tumour samples were organised into 14 tissue microarrays (TMAs), with 20 tumour cores (2 mm diameter) each, also including several samples of normal breast tissue. Each case was represented in the TMA by at least two cores. Relevant clinico-pathological data included tumour size (TNM), molecular subtype, histological grade, estrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), epidermal growth

factor receptor 1 (EGFR), basal cytokeratins (CK5 and CK14), vimentin and Ki67 expression status. Information on lymph-node metastasis, disease-free survival and overall survival was also available. These tumour samples were previously analysed by our group for MCT1, MCT4 and CD147 expressions [23].

Immunohistochemistry

GLUT1 and CAIX detection

Immunohistochemistry was performed based on the streptavidin–biotin–peroxidase complex principle (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA), using rabbit polyclonal primary antibodies raised against GLUT1 (ab15309, AbCam, Cambridge, UK, diluted 1:500) and CAIX (ab15086, AbCam, Cambridge, UK, diluted 1:2000). Briefly, deparaffinised and rehydrated sections were immersed in citrate buffer (0.01M, pH 6.0) heated up to 98ºC, in a water bath, for 10 minutes (GLUT1) or 20 minutes (CAIX) and washed in PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes, followed by washing with PBS. Tissue sections were incubated with blocking solution for 10 minutes and incubated at room temperature with the primary antibody for 2 hours. Sections were then sequentially washed in PBS and incubated with biotinylated goat anti-polyvalent antibody for 10 minutes, streptavidin peroxidase for 10 minutes, and developed with 3,3'-diamino-benzidine (DAB+ Substrate System, Dako, Carpinteria, CA) for 10 minutes. Negative controls were performed by using an adequate serum controls for the primary antibodies (N1699, Dako, Carpinteria, CA) and skin and gastric mucosa were used as positive controls for GLUT1 and CAIX, respectively. Tissue sections were counterstained with haematoxylin and permanently mounted.

Immunohistochemical evaluation

As described for MCT and CD147 [23], GLUT1 and CAIX immunohistochemical reactions were scored semi-quantitatively for plasma membrane staining as follows: 0: 0% of immunoreactive cells; 1: <5% of immunoreactive cells; 2: 5-50% of immunoreactive cells; and 3: >50% of immunoreactive cells. Also, intensity of staining was scored semi-qualitatively as follows: 0: negative; 1: weak; 2: intermediate; and 3: strong. The final score was defined as the sum of both parameters (extension and intensity), and grouped as negative (score 0 and 2) and positive (score 3-6), as previously described.

Statistical analysis

Data were stored and analysed using the Statview statistical software (SAS Institute Inc., Cary, NC). All comparisons were examined for statistical significance using Pearson's chi-square (χ^2) test or Fisher's exact test, as adequate, being the threshold for significance p values <0.05. Disease-free and overall survival curves were plotted using the method of Kaplan-Meier and data compared using the log-rank test. A cut-off of 60 months (5 years) was considered, since in the first 5 years following primary therapy recurrence rates are expected to be highest, especially in series with high number of ER negative cases like ours, where the hazard of recurrence is higher [33]. Cases lacking one or more of the clinico-pathological variables were not included in the specific statistical analysis.

RESULTS

A total of 124 breast carcinoma samples, organised into TMAs (Tissue Microarrays), were assessed for GLUT1 and CAIX immunohistochemical expressions.

In general, positive GLUT1 expression was observed in both plasma membrane and cytoplasm (Figure 1A), while CAIX expression was mainly observed in the plasma membrane, with some cases presenting also cytoplasm staining (Figure 1B). GLUT1 expression was observed only in the epithelium cells of 1 out 20 normal samples evaluated (5%), with a

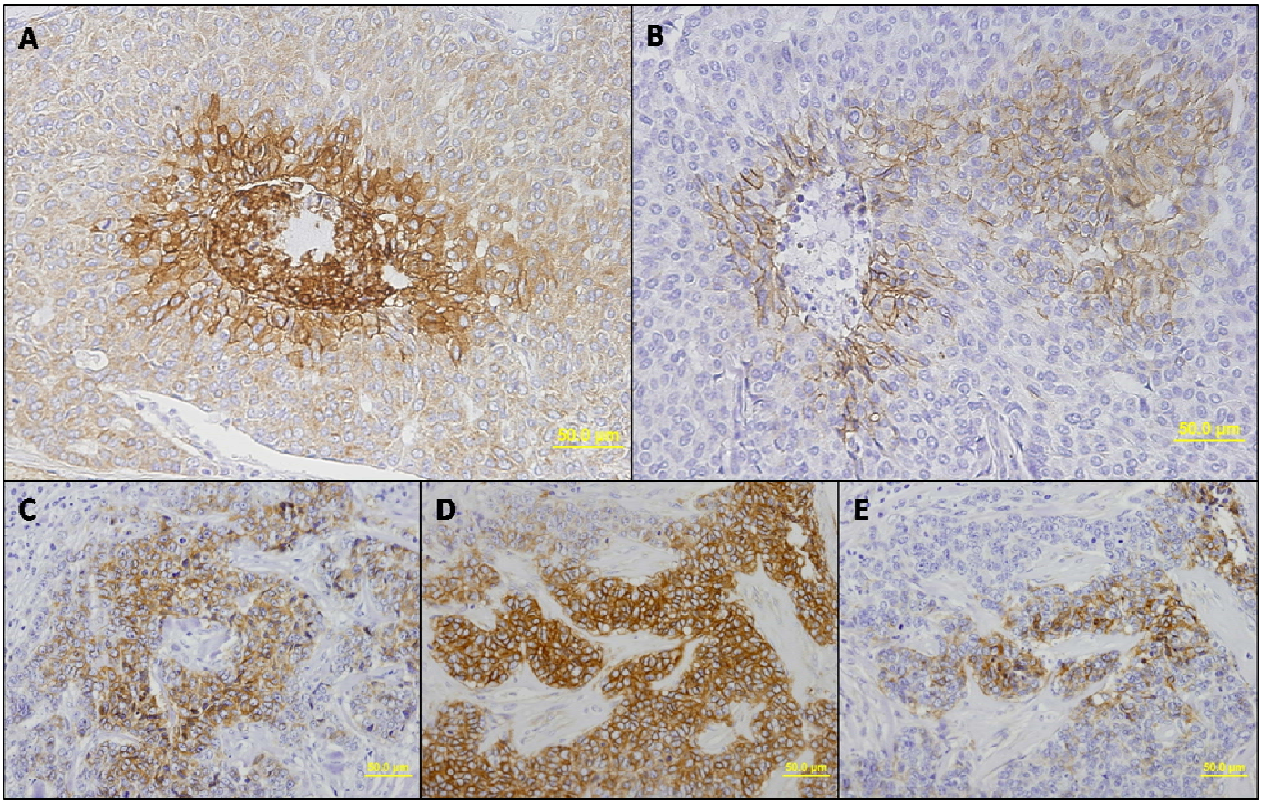

Figure 1. Immunohistochemical expression of glucose transporter 1 (GLUT1), carbonic anhydrase IX (CAIX) and monocarboxylate transporter 1 (MCT1), in breast carcinoma samples. GLUT1 and CAIX expression was frequently observed in perinecrotic regions (A and B, respectively). CAIX expression was usually focal and mainly restricted to tumour cells. Lower panel shows a breast cancer case simultaneously positive for MCT1 (C), GLUT1 (D) and CAIX (E), with staining in the same tumour region.

significantly higher frequency (46%) in tumour samples (57/124, p=0.0005), while CAIX expression was absent in normal tissue but was found in 18% (22/122) of breast cancer cases (p=0.0388). Importantly, the expression of both molecules was frequently observed in peri-necrotic regions, as can be observed in Figure 1 (A and B), especially CAIX which was usually focal and mainly restricted to tumour cells, adjacent to areas of necrosis.

Importantly, when comparing the expression of the previously analysed MCT1, MCT4 and CD147 [23] with GLUT1 and CAIX, we found that both MCT1 and CD147, but not MCT4, were more frequently expressed in GLUT1 and CAIX positive tumour samples (Table 1). Also, as expected, GLUT1 and CAIX were significantly co-expressed, with 81.8% (18/22) of CAIX positive cases also positive for GLUT1 *versus* 37.5% (36/96) GLUT1 positive in the CAIX negative group, p=0.0002. Figure 1 (C, D, E) shows a breast cancer case simultaneously positive for MCT1, GLUT1 and CAIX, in the same tumour area.

Assessment of the clinico-pathological value of GLUT1 and CAIX also retrieved important results (Table 2). We found significant associations between

Table 1. Association of CAIX and GLUT1 with MCT1, MCT4 and CD147 expression in breast carcinoma samples.

	n	MCT1 Positive (%)	*p*	MCT4 Positive (%)	*p*	CD147 Positive (%)	*p*
GLUT1	**106**		**<0.0001**		0.3473		**0.0032**
Negative	55	1 (1.8)		4 (7.3)		2 (3.6)	
Positive	51	15 (29.4)		7 (13.7)		12 (23.5)	
CAIX	**105**		**<0.0001**		0.6897		**0.0005**
Negative	84	6 (7.1)		8 (9.5)		7 (8.3)	
Positive	21	10 (47.6)		3 (14.3)		8 (38.0)	

GLUT1 expression and high grade tumours (p=0.0014), basal-like subtype (p=0.0008), absence of progesterone receptor (p=0.0162), presence of vimentin (p=0.0033) and high Ki67 expression (p=0.0339). Additionally, CAIX was associated with the majority of the clinico-pathological parameters analysed, including tumour size (p=0.0034), histological grade (p=0.0263), molecular subtype (p=0.0050), ER and PR negativity (p=0.0014 and p=0.0292, respectively), expression of CK5 (p=0.0002), CK14 (p=0.0102) and vimentin (p=0.0004). Analysis of GLUT1 expression and patient's survival (disease-free survival and overall survival) showed no significant differences between negative and positive groups (data not shown), but, importantly, patients with CAIX positive tumours had a lower disease free-survival than patients with CAIX negative tumours (43.2 versus 52.4 months, respectively, p=0.045) (Figure 2). No significant differences in overall survival were observed between the CAIX negative group and the CAIX positive group.

Table 2. Associations of CAIX and GLUT1 expression with clinico-pathological data from breast cancer cases.

Clinico-pathological data		*GLUT1*			*CAIX*		
		n	**Positive (%)**	***p***	**n**	**Positive (%)**	***p***
T size (TNM)		**121**		0.5218	**119**		**0.0034**
	T1	**46**	23 (50.0)		**45**	6 (13.3)	
	T2	**63**	25 (39.7)		**63**	9 (14.3)	
	T3	**12**	6 (50.0)		**11**	6 (54.5)	
Histological grade		**124**		**0.0014**	**122**		**0.0263**
	I	**23**	5 (21.7)		**24**	2 (8.3)	
	II	**55**	22 (40.0)		**51**	6 (11.8)	
	III	**46**	30 (65.2)		**47**	14 (29.8)	
Subtype		**114**		**0.0008**	**113**		**0.0050**
	Luminal	**78**	31 (39.7)		**79**	8 (10.1)	
	Basal-like	**25**	20 (80.0)		**24**	9 (37.5)	
	HER2 overexpressing	**11**	3 (27.3)		**10**	3 (30.0)	
Estrogen receptor		**124**		0.1059	**122**		**0.0014**
	Negative	**45**	25 (55.6)		**42**	14 (33.3)	
	Positive	**79**	32 (40.5)		**80**	8 (10.0)	
Progesterone receptor		**124**		**0.0162**	**122**		**0.0292**
	Negative	**75**	41 (54.7)		**73**	18 (24.6)	
	Positive	**49**	16 (32.6)		**49**	4 (8.2)	
HER2 overexpression		**123**		0.5885	**121**		0.4556
	Negative	**110**	51 (46.4)		**109**	19 (17.4)	
	Positive	**13**	5 (38.5)		**12**	3 (25.0)	
EGFR		**124**		>0.9999	**122**		0.6643
	Negative	**115**	53 (46.1)		**113**	20 (17.7)	
	Positive	**9**	4 (44.4)		**9**	2 (22.2)	
CK5		**124**		0.1188	**122**		**0.0002**
	Negative	**98**	42 (42.8)		**97**	11 (11.3)	
	Positive	**26**	15 (57.7)		**25**	11 (44.0)	
CK14		**121**		0.0508	**121**		**0.0102**
	Negative	**114**	51 (44.7)		**115**	18 (15.6)	
	Positive	**7**	6 (85.7)		**6**	4 (66.7)	
Vimentin		**106**		**0.0033**	**106**		**0.0004**
	Negative	**89**	38 (42.7)		**88**	12 (13.6)	
	Positive	**17**	14 (82.4)		**18**	9 (50.0)	
Ki67		**124**		**0.0339**	**122**		0.5214
	< 20%	**65**	24 (36.9)		**63**	10 (15.9)	
	> 20%	**59**	33 (55.9)		**59**	12 (20.3)	
Lymph-node metastasis		**117**		0.6326	**115**		0.5840
	Absent	**58**	24 (41.4)		**55**	8 (14.5)	
	Present	**59**	27 (45.8)		**60**	11 (18.3)	

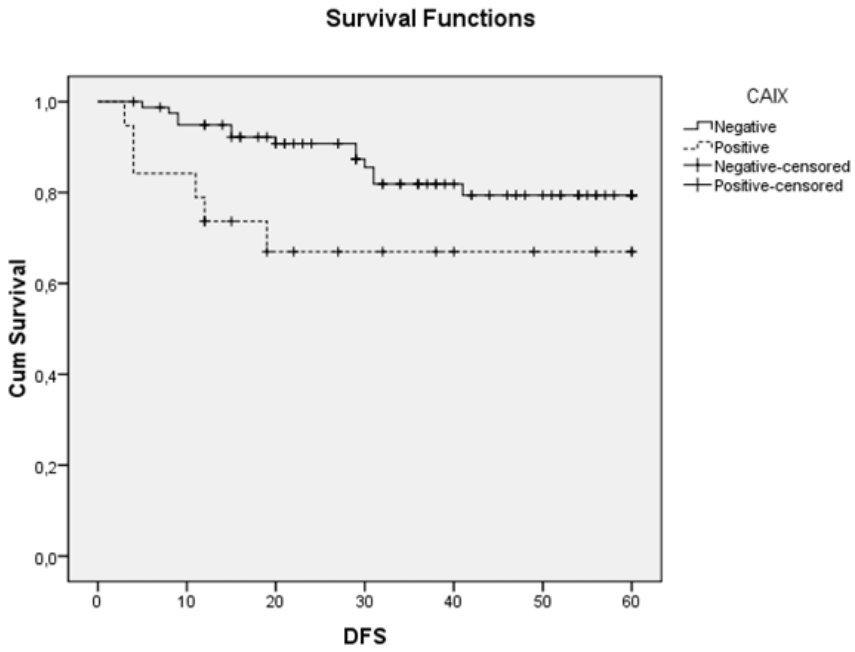

Figure 2. Disease-free survival (DFS) curve regarding CAIX immunoreaction in breast cancer patients. Patients with positive tumours for CAIX expression show shorter disease-free survival (interrupted line) than patients with CAIX negative tumours (continuous line) ($p = 0.045$).

DISCUSSION

Up-regulation of glucose conversion into lactate, even in the presence of oxygen (Warburg effect), has been described as a possible adaptive mechanism to overcome intermittent hypoxia in pre-malignant lesions. This metabolic switch leads to an increase in acid production by cancer cells and, therefore, the need for further adaptation by means of intracellular pH regulation and resistance to extracellular acidity [1]. In this perspective, MCTs emerge as important contributors to cancer cell adaptation due to their function, on one hand, of lactate export, allowing continuous glycolysis, and, on the other hand, of tumour intracellular pH regulation and induction of extracellular acidosis, by co-transporting lactate and a proton. Although some literature review the contribution of MCTs to the glycolytic and acidic phenotype of tumours [34;35], the significance of tumour MCT expression in this context is still not clear [19;21]. Thus, the main aim of the present work was to determine if glycolytic and acid-resistant tumours, with up-regulation of GLUT1 and CAIX, present a higher expression of MCTs, supporting the involvement of these transporters in the metabolic adaptations of cancer cells.

GLUT1, involved in glucose uptake, is up-regulated in a variety of tumours (for review see [36]), being the hypoxia transcription factor HIF-1α the major regulator of its expression in cancer cells [37;38]. HIF-1α, the master transcriptional regulator of tumour cell adaptation to hypoxic stress, activates a number of genes, many of which code for protein involved in O_2 delivery, angiogenesis, energy preservation (including glucose transporters and glycolytic enzymes), and other processes essential to tumour cell survival, proliferation, and spread [29]. Moreover, GLUT1, CAIX and MCT4 are downstream targets of HIF-1α [30;31;39] and both GLUT1 and CAIX are also recognised as tumour hypoxia markers [40]. However, in our study, MCT4 expression was not increased in GLUT1 or CAIX positive tumours. In contrast, MCT1 was more frequently expressed in both GLUT1 and CAIX positive tumours, pointing to a hypoxia dependent up-regulation of this MCT isoform, which is accompanied by co-expression of its chaperone CD147, essential for plasma membrane localisation and transporter activity [25-27;41;42]. This finding is of major importance since it reinforces the induction of MCT1

expression by hypoxia [30], which is contested by some groups [31;32]. With these associations, one can support the role of functional MCT1 as the lactate transporter responsible for lactate efflux in highly glycolytic breast cancer cells, especially in basal-like tumours. The transport activity of MCT1 is considered one of the most important mechanisms of intracellular pH regulation [16]. Besides MCTs, carbonic anhydrases, especially CAIX, play a major role in maintenance of intracellular (and extracellular) pH levels, by contributing to the extrusion of the protons generated by the high metabolic rates of glycolytic cancer cells. At the tumour cell surface, CAIX catalyses the extracellular trapping of acid by hydrating the cell-generated CO_2 into HCO_3^- and H^+ [11]. Thus, it is not surprising to see an association between MCT1 and both GLUT1 and CAIX, which is likely a result of the overall HIF-1α-mediated metabolic adaptations, conferring a glycolytic, acid-resistant phenotype to cancer cells.

According to the previously described [14;43;44], in our tumour series, GLUT1 and CAIX were mainly observed in the vicinity of necrotic areas (a consequence of tumour hypoxia), which supports the hypoxia-mediated regulation of the expression of these proteins. Both hypoxia markers were absent in the normal breast tissue, but up-regulated in breast tumour tissues, with expression frequencies concordant with previous reports [4;5;12;13;15;45]. Although the clinico-pathological value of GLUT1 and CAIX in breast cancer has already been studied by others [5;12-15], our data strengthens the importance of these proteins as prognostic markers, especially GLUT1, which has been few explored in breast cancer [4;5]. So far, GLUT1 has been associated with lower disease-free survival, loss of ER and PR [4] and both higher grade [4;5] and proliferative activity (through Ki67 expression) [5]. In the present work, although we did not find associations of GLUT1 with disease-free or overall survival, GLUT1 was more frequently expressed in high grade tumours, negative for PR and with high proliferative index (Ki67). Importantly, we found GLUT1 to be more frequently expressed in basal-like tumours, as well as in vimentin positive tumours. In respect to CAIX, more data has been published in breast carcinomas, which identifies CAIX as a good marker of aggressive tumour behaviour. This protein was positively correlated with higher tumour size, basal-like [15] and high grade tumours, loss of ER [12;14;15;46] and PR [46] as well as with shorter disease-free survival [13-15]. Here, we support all the previous findings described by other groups, by associating CAIX expression with high histological grade, loss of ER and PR and, importantly, basal-like subtype and disease-free survival. These results suggest that the basal-like subtype tumours may be more representative of the glycolytic, acid-resistant phenotype proposed for cancer cells and this hypoxia mediated phenotype may explain, at least partly, the more aggressive phenotype of this breast carcinoma subtype.

In the present study, we investigated the expression of the key hypoxia regulated proteins GLUT1 and CAIX. Importantly, they were positively associated with the major lactate transporter, MCT1, especially in a subset of aggressive breast carcinomas (basal-like), where these proteins are more frequently expressed. Since this subtype of tumours does not have a specific molecular therapy [47], the development of therapeutic approaches targeting these particular metabolic features could be a promising strategy to be explored in the treatment of basal-like breast tumours.

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CONFLICT OF INTEREST STATEMENT

These authors declare no conflict of interest.

ETHICS

The present study has been approved by the local Ethic Committees.

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5.4. UNPUBLISHED RESULTS

Finally, this chapter also comprises the following unpublished results, entitled "Sensitivity of breast cancers to CHC, a monocarboxyate transporter 1 inhibitor", **to be submitted** for publication in an international scientific periodical with referees.

The following results were also presented as poster in the following national scientific meeting:

XIX Porto Cancer Meeting, 2010, Porto, Portugal. ***Pinheiro C****, Pinheiro S, Gonçalves V, Vieira A, Paredes J, Schmitt F, Baltazar F. Sensitivity of breast cancer cells to CHC, an inhibitor of monocarboxylate transporter 1.*

5.4.1. SENSITIVITY OF BREAST CANCER CELLS TO CHC, A MONOCARBOXYLATE TRANSPORTER 1 INHIBITOR

Sensitivity of breast cancer cells to CHC, a monocarboxylate transporter 1 inhibitor

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ABSTRACT

Tumour microenvironment is known to be acidic due to the high glycolytic rates of tumour cells. Monocarboxylate transporters (MCTs) are one of the major players responsible for the maintenance of tumour intracellular pH, which is achieved by the transport of lactate coupled with a proton, through the cell plasma membrane. It is widely known that acidification of the extracellular microenvironment has important implications in tumour progression and, therefore, MCTs have been suggested as potential targets for cancer therapy. We have recently described the up-regulation of monocarboxylate transporter 1 (MCT1) in breast carcinomas and its association with poor prognostic variables, such as basal-like subtype and high grade tumours. Therefore, we aimed to evaluate the effect of MCT1 inhibition in different breast cancer cell lines.

The human breast cancer cell lines MCF-7/AZ, SkBr3, MDA-MB-468, BT-20, MDA-MB-231 and Hs578T were used for this study. MCT1, MCT4 and CD147 expressions, as well as CAIX and GLUT1, were evaluated by immunocytochemistry in paraffin cell blocks. Also, the effect of the MCT1 inhibition in cancer cell viability and metabolic profile was evaluated using the MCT1 classical inhibitor α-cyano-4-hydroxycinnamate (CHC).

MCT1, MCT4 and CD147 were differently expressed among the breast cancer cell lines, being MCF-7/AZ, SkBr3 and MDA-MB-231 negative and MDA-MB-468, BT-20 and Hs578T positive for MCT1 plasma membrane expression. In MDA-MB-468, the CHC-induced inhibition (IC_{50} value of 9.3 mM) was accompanied by a decrease in both glucose consumption and lactate production. Although negative for MCT1 expression, MDA-MB-231 showed the lowest IC_{50} value after inhibition with CHC (5.3 mM); however, this inhibition in cell proliferation/viability was not accompanied by a decrease in glucose consumption and lactate production. BT20 showed the highest IC_{50} value (23.5 mM), with no effect on glucose or lactate extracellular concentrations.

In this study, we evaluated, for the first time, the sensitivity of different breast cancer cell lines to the classical MCT1 inhibitor CHC. The results obtained here led us to hypothesise that MCT1 inhibition may be a good therapeutic strategy to treat breast cancer, with special emphasis on basal-like subtype, which so far does not have a specific molecular therapy.

Keywords: Monocarboxylate transporter, breast carcinoma, lactate, CHC, metabolism.
Abbreviations: AE (anion exchanger); CA (carbonic anhydrase); CHC (α-cyano-4-hydroxycinnamate); DMSO (dimethyl sulfoxide); FBS (fetal bovine serum); FdG-PET (^{18}F-fluorodeoxyglucose positron emission tomography); GLUT (glucose transporter); HIF-1α (hypoxia inducible-factor 1 alpha); MCT (monocarboxylate transporter); siRNA (small-interfering RNA).

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INTRODUCTION

The increased uptake of glucose by cancer cells is a widely described phenomenon, being the rationale behind the whole-body noninvasive ^{18}F-fluorodeoxyglucose positron emission tomography (FdG-PET). This imaging technique is considered to be the most reliable for the diagnosis and prognosis of breast cancer, especially regarding the detection of distant metastases, recurrent disease as well as monitoring therapy response [1]. This increased uptake of glucose, especially through glucose transporter 1 (GLUT1), is a consequence of an increased glycolytic metabolism that generates acids inside the cell, inducing the up-regulation of some pH regulators, like carbonic anhydrases IX (CAIX) [2], to maintain physiological pH inside the cell, causing extracellular acidosis. The increased glycolytic metabolism ultimately leads to an increase in lactate release by cancer cells, contributing also to microenvironmental acidosis, as well as increased invasion capacity [3] and suppression of anticancer immune response [4]. More recently, a metabolic symbiosis between glycolytic and oxidative cancer cells was described, in which the peripheral and oxygenated oxidative cells consume the lactate produced by the central and less oxygenated glycolytic cells [5]. In this context, lactate has a central role and lactate transporters are currently seen as potential therapeutic targets in cancer treatment, with promising results using *in vitro* and *in vivo* models [5-11]. Presently, monocarboxylate transporters (MCTs) are a family of 14 members, with isoforms 1 to 4 being lactate proton symporters that exhibit different affinities for lactate [12]. MCT1 isoform has been described as the gatekeeper of cancer metabolic symbiosis, by up-taking lactate in the oxidative cancer cells [5]. Therefore, MCT1 would probably be the most promising isoform in this context and reports on MCT1 upregulation in a variety of tumours are becoming more frequent [8,10,13-17]. We also described the association of MCTs, especially MCT1, with poor prognostic variables [15,17,18], reinforcing the potential of MCT1 as cancer therapeutic target. Recently, our group described an increase of MCT1 expression in breast carcinoma, when comparing with normal tissue. Importantly, this enhanced MCT1 expression, as well as the expression of CD147 (MCT1 chaperone), were associated with basal-like subtype tumours and other poor prognostic parameters [17].

Over the last years, different approaches have been used to inhibit lactate efflux from cancer cells, including MCT small-molecule inhibitors like α-cyano-4-hydroxycinnamic acid (CHC) [5,6,9] and MCT small-interfering RNA (siRNA) [5,8,10]. Continued inhibition of lactate efflux inhibits pyruvate reduction to lactate and, therefore, prevents NAD^+ recycling, causing metabolic crisis in tumour cells. MCT inhibition studies are providing evidence for the effectiveness of targeting the end stage of glycolysis, with data showing a reduction of tumour malignancy, enhancement of radio-sensitivity and induction of cell-death in MCT targeted tumour tissue (for review, see [11]).

Despite the promising results obtained by MCT inhibition, we are still far from definitely pointing at MCTs as effective targets for cancer therapy. Without doubt, more efforts are needed to increase evidence supporting that inhibition of metabolism, more specifically lactate transport and pH regulation, may be an alternative therapeutic strategy to use in cancer treatment, in a near future. Therefore, the present work is an attempt to provide new data supporting in the exploitation of MCTs as targets in breast cancer therapy.

MATERIALS AND METHODS

Cell lines

Human breast cancer cell lines MCF-7/AZ,

SkBr3, MDA-MB-468, BT-20, MDA-MB-231 and Hs578T were obtained from ATCC or from collections developed in the laboratories of Drs Elena Moisseva (Cancer Biomarkers and Prevention Group, Departments of Biochemistry and Cancer Studies, University of Leicester, UK), Marc Mareel (Laboratory of Experimental Cancerology, Ghent University Hospital, Belgium) and Eric Lam (Imperial College School of Medicine, Hammersmith Hospital, London, UK).

Cell culture

All cell lines were grown in commercially available culture medium (DMEM, Invitrogen), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Invitrogen) and 1% antibiotic solution (penicillin–streptomycin, Invitrogen). All cell lines were routinely cultured at 37ºC, in a humidified atmosphere with 5% CO_2.

Protein expression assessment

Paraffin cytoblock

Concentrated cell suspensions were made by centrifuging fresh cell suspensions at 1200 rpm for 5 minutes. After discarding the supernatant, cell pellets were incubated with formaldehyde 3.7% overnight and re-centrifuged. Cell pellets were then processed in an automatic tissue processor (TP1020, Leica), before inclusion into paraffin (block-forming unit, EG1140H, Leica).

Immunocytochemistry

Immunocytochemistry was performed for MCT1, MCT4, CD147 (MCT1 and MCT4 chaperone [19-21]), GLUT1 and CAIX as previously described [15,22]. Briefly, deparaffinised and rehydrated 4 μm cytoblock sections were submitted to the adequate antigen retrieval, followed by inactivation of endogenous peroxidase activity. Slides were then incubated with the primary antibody and immunoreactivity was visualised with 3,3'-diamino-benzidine (DAB+ Substrate System, Dako). Please see Table 1 for detailed aspects of each antibody used. Negative controls were performed by using adequate serum control for the primary antibodies (N1699, Dako). Cytoblock sections were counterstained with haematoxylin and permanently mounted. Cells were evaluated for positive expression, distinguishing cytoplasmic from membrane expression.

Table 1. Detailed aspects of the immunocytochemical procedure used to visualise the different proteins.

Protein	Positive control	Antigen retrieval	Detection System	Antibody	Antibody dilution and incubation time
MCT1	Colon carcinoma	Citrate buffer (0.01 M, pH=6), 98ºC, 20'	R.T.U. VECTASTAIN Elite ABC Kit (Universal) Vector Laboratories	AB3538P Chemicon International	1:200, overnight
MCT4	Colon carcinoma	Citrate buffer (0.01 M, pH=6), 98ºC, 20'	R.T.U. VECTASTAIN Elite ABC Kit (Universal) Vector Laboratories	AB3316P Chemicon International	1:200, overnight
CD147	Colon carcinoma	EDTA (1 mM, pH=8), 98ºC, 20'	Ultravision Detection System Anti-polyvalent, HRP Lab Vision Corporation	18-7344 Zymed Laboratories Inc.	1:500, 2 hours
GLUT1	Skin	Citrate buffer (0.01 M, pH=6), 98ºC, 10'	Ultravision Detection System Anti-polyvalent, HRP Lab Vision Corporation	ab15309 AbCam	1:500, 2 hours
CAIX	Gastric carcinoma	Citrate buffer (0.01 M, pH=6), 98ºC, 20'	Ultravision Detection System Anti-polyvalent, HRP Lab Vision Corporation	ab15086 AbCam	1:2000, 2 hours

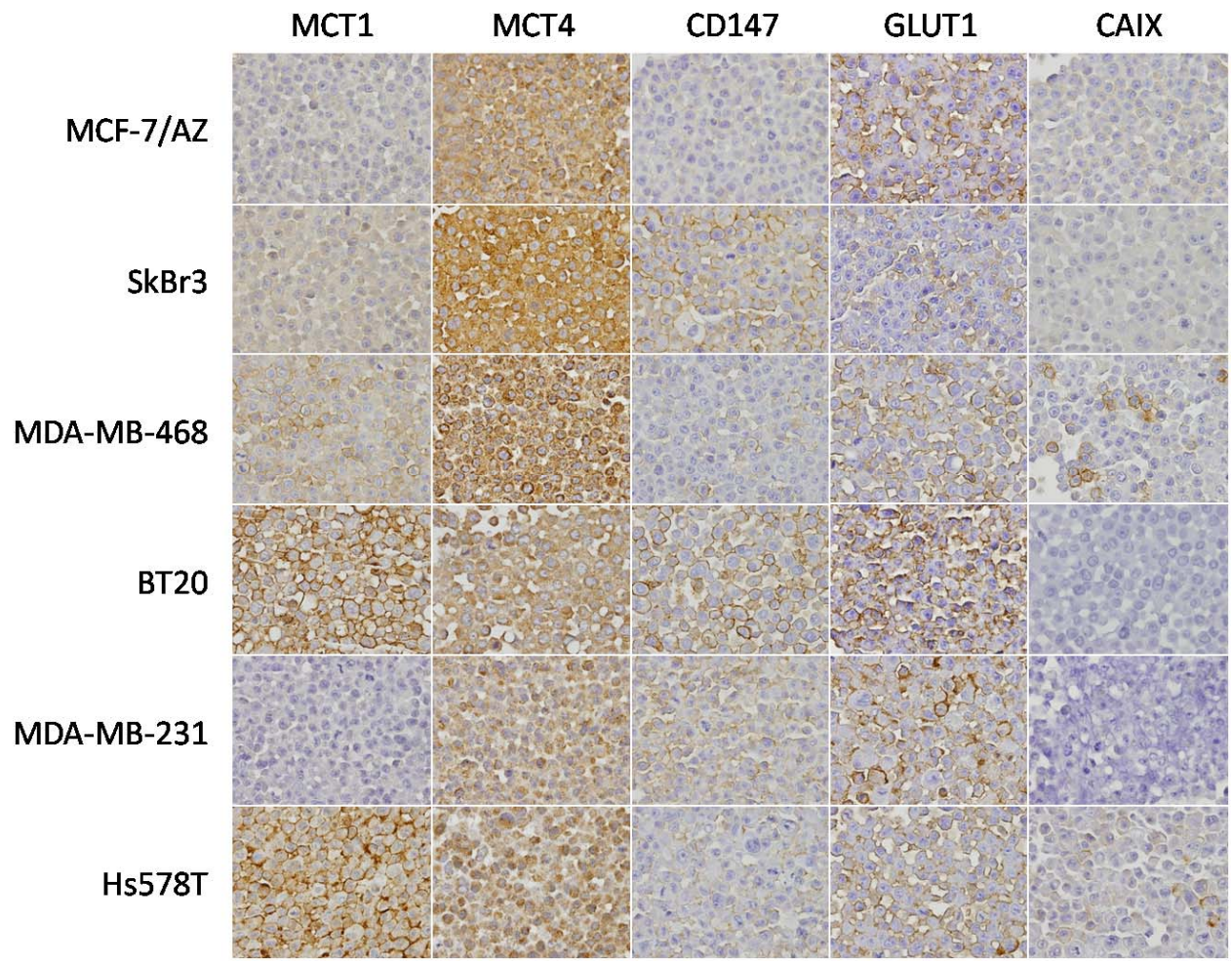

Figure 1. Immunocytochemical expression of different proteins in human breast carcinoma cell lines (400x magnification). MCT1 was mainly found in the plasma membrane of MDA-MB-468, BT20 and Hs578T cells, while MCT4 was only found in the cytoplasm of all cell lines. CD147, GLUT1 and CAIX were observed at the plasma membrane of some tumour cell lines, at varying levels.

Evaluation of the metabolism/Metabolic behaviour of human breast cancer cell lines

Glucose and lactate quantification

The metabolic behaviour of the different cell lines was determined by analysing basic metabolic parameters, i. e., extracellular concentrations of glucose and lactate. For metabolic studies, to minimise variations due to cell growth and size, as well as obtain measurable variations of glucose and lactate in a short period of time, assays were performed with confluent cells, in 24-well plates. After reaching confluence, spent medium was replaced with fresh complete culture medium (supplemented with FBS and antibiotics) and aliquots were retrieved, after 24 and 48 hours. Glucose and lactate were quantified using commercial kits (Roche and SpinReact, respectively), according to the manufacturer's protocol but scaled down to microplate volumes. Results are expressed as total µg/500 µl medium.

MCT1 *in vitro* inhibition using the classical inhibitor CHC

IC_{50} estimation

To determine the CHC IC_{50} value for the different breast cancer cell lines, cells were plated in 96-well plates, at a density ranging from 10000 to 20000 cells per well, depending on cell growth rates. Cells were allowed to grow overnight prior to incubation with culture medium (without serum) containing 3-15 mM of CHC (Sigma-Aldrich, stocks at 0.3-1.5 M in dimethyl sulfoxide (DMSO)). Controls

were performed by adding DMSO to cell culture medium at the same dilution (1:100). The effect of CHC on cell number (total biomass) was evaluated by the Sulforhodamine B assay (SRB, TOX-6, Sigma-Aldrich), following the manufacturer's recommendations. IC_{50} values were estimated from three independent experiments, each one in triplicate, using the GraphPad Prism 5 software, applying a sigmoidal dose-response (variable slope) nonlinear regression, after logarithmic transformation.

Glucose and lactate quantification

For metabolic studies, as previously described, assays were performed with confluent cells, in 24-well plates. After reaching confluence, cells were incubated with 2.5, 5 and/or 10 mM of CHC, as indicated in the results section. Cell culture medium (30 µl) was retrieved at 4, 8 and 12 hours, assuring confluences similar to the ones observed in the control. Glucose and lactate were quantified as mentioned above. Data from two independent experiments, each one in triplicate, was stored in GraphPad Prism 5 software. All conditions were examined for statistical significance using two-tailed Student's t-test for mean comparison, being threshold for significance *p* values <0.05.

RESULTS

Protein expression

Human breast cancer cell lines MCF-7/AZ, SkBr3, MDA-MB-468, BT-20, MDA-MB-231 and Hs578T were evaluated for MCT1, MCT4, CD147, GLUT1 and CAIX immunocytochemical expression, as depicted in Figure 1. Membrane expression of MCT1 was only observed in MDA-MB-468, BT20 and Hs578T cell lines, while MCT4, although differently expressed in the cytoplasm of all cell lines analysed, was not found in the plasma membrane of any of the cell lines. CD147 was observed in the plasma membrane of the 6 cell lines studied; however, MCF-7/AZ, MDA-MB-468 and Hs578T expressed this protein at very low levels. GLUT1 was expressed at the plasma membrane, at different levels in all cell lines, and CAIX was only detected in a low percentage of cells in MDA-MB-468 and Hs578T and, at even lower levels in MCF-7/AZ.

Metabolic behaviour of human breast cancer cell lines

Glucose and lactate quantification in the culture medium showed different metabolic behaviours among the breast cancer cell lines studied (Figure 2A and 2B).

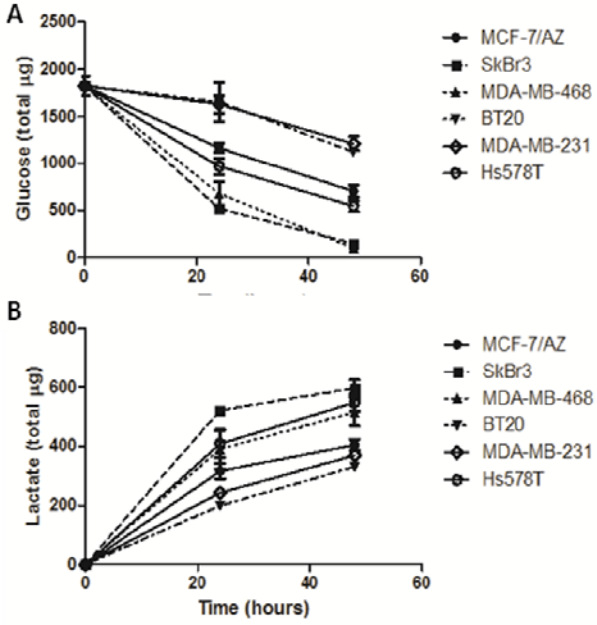

Figure 2. Extracellular amounts of glucose (A) and lactate (B) in the different human breast carcinoma cell lines, along time. Results are expressed as mean±SEM.

In general, cells consuming more glucose along time also produced more lactate. SkBr3 and MDA-MB-468 showed the highest glucose consumption rates, followed by Hs578T and MCF-7/AZ, while BT20 and MDA-MB-231 presented the lowest consumption of glucose. On the other hand, SkBr3, Hs578T and MDA-MB-468 produced more lactate

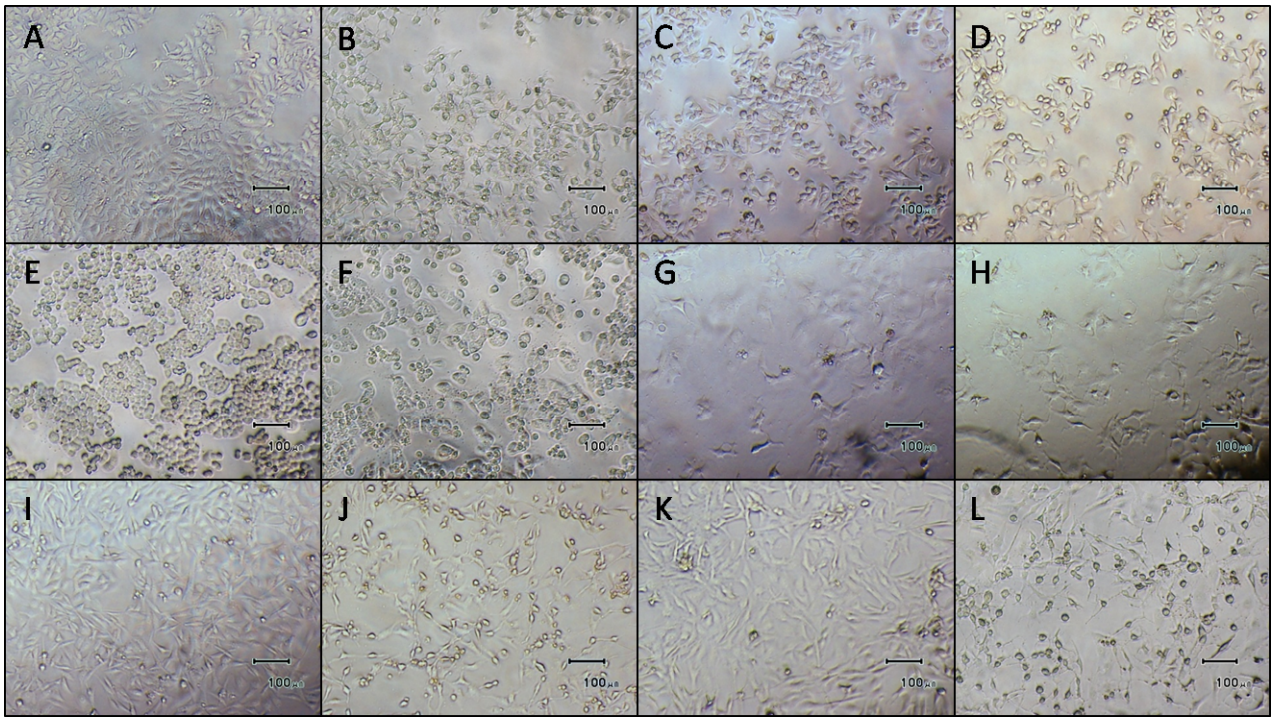

Figure 3. CHC-induced phenotypic alterations in the human breast carcinoma cell lines MCF-7/AZ (A- control, B- CHC treated), SkBr3 (C- control, D- CHC treated), MDA-MB-468 (E- control, F- CHC treated), BT20 (G- control, H- CHC treated), MDA-MB-231 (I- control, J- CHC treated) and Hs578T (K- control, L- CHC treated).

than MCF-7/AZ, MDA-MB-231 and BT20, by this order.

MCT1 inhibition using CHC

Human breast cancer cell lines were treated once with CHC at increasing concentrations (3-15 mM) and, 24 hours later, total cell biomass was determined with SRB assay (estimation of IC_{50} values). Microscopically, morphological alterations were detected after 24 hours of treatment with CHC. In general, cell volume decreased and cells became round (Figure 3), possibly due to loss of cell viability. For MDA-MB-468, which in normal growth conditions has a very round shape (Figure 3E), it was possible to observe a more elongated form in part of the population (Figure 3F), after CHC treatment. In the case of BT20, the morphological alterations were very subtle (Figure 3G *vs*. Figure 3H), anticipating a lower sensibility to CHC.

IC_{50} values show that MDA-MB-231 is the cell line most sensitive to CHC-induced decrease in cell biomass (Figure 4E), MCF-7/AZ and MDA-MB-468 have an intermediate sensitivity to CHC (Figure 4A and 4C, respectively), while SkBr3 and Hs578T have the highest IC_{50} value (Figure 4B and 4F, respectively) being slightly less sensitive than the formers. For BT20, the IC_{50} was not within the range of concentrations used (Figure 4D), thus this cell line was considered resistant to CHC, for 24 hours of treatment.

To understand if the inhibitory effect induced by CHC in the different cell lines was due to metabolic disturbance, glucose consumption and lactate production were analysed, using a CHC concentration around the IC_{50} value (5 mM for MDA-MB-231 and 10 mM for the remaining cell lines), as well as a lower concentration (2.5 mM for MDA-MB-231 and 5 mM for the other cell lines) to observe if cancer cell metabolism is affected, at concentrations inducing a reduced effect in total biomass. Quantification of extracellular glucose and lactate along time showed that MDA-MB-468 is the only cell line where the CHC-induced inhibition leads to a significant decrease in glucose consumption and lactate production, the

latter in a dose dependent manner (Figure 5A and 5D, respectively). MCF-7/AZ, SkBr3 and Hs578T produced a significantly lower amount of lactate (soon after 4 hours for MCF-7/AZ and Hs578T) for both CHC concentrations used, and in a dose dependent manner for Hs578T (Figure 5B, 5C and 5G, respectively), however, this alteration in lactate production was not accompanied by a decrease in glucose consumption (data not shown). BT20 also showed a decrease in lactate production, but only when treated with 10 mM of CHC (Figure 5E) and also with no decrease in glucose consumption (data not shown), while MDA-MB-231 showed no alteration in both CHC concentrations at the different time points (Figure 5F).

DISCUSSION

We have previously described the up-regulation of MCT1 in breast cancer and, importantly, we observed that MCT1 was more frequently expressed in high-grade tumours, as well as in the basal-like subtype [17]. Basal-like tumours have a more aggressive clinical behaviour when compared to

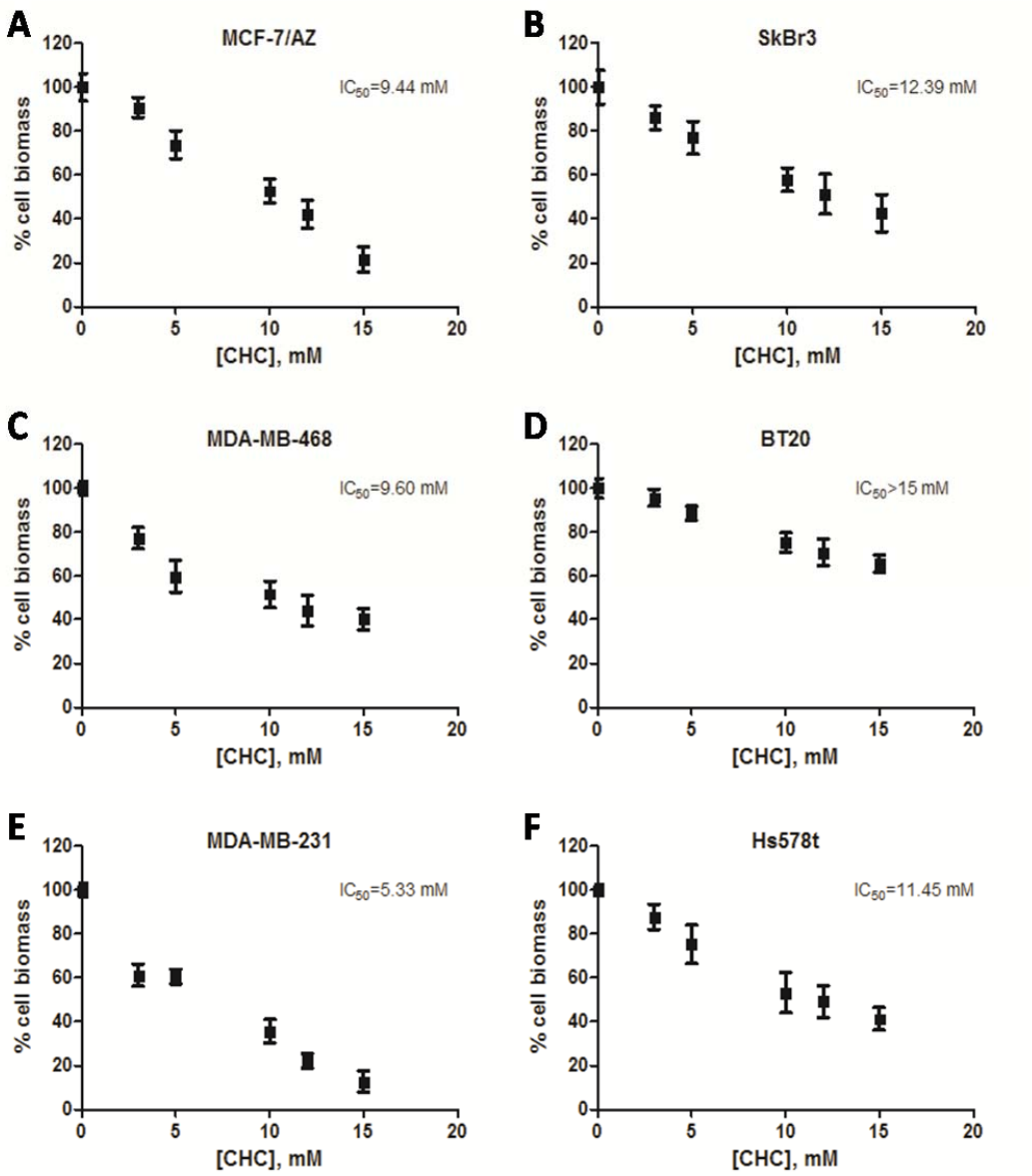

Figure 4. Effect of CHC in total cell biomass, evaluated by SRB assay. Results are represented as mean±SD.

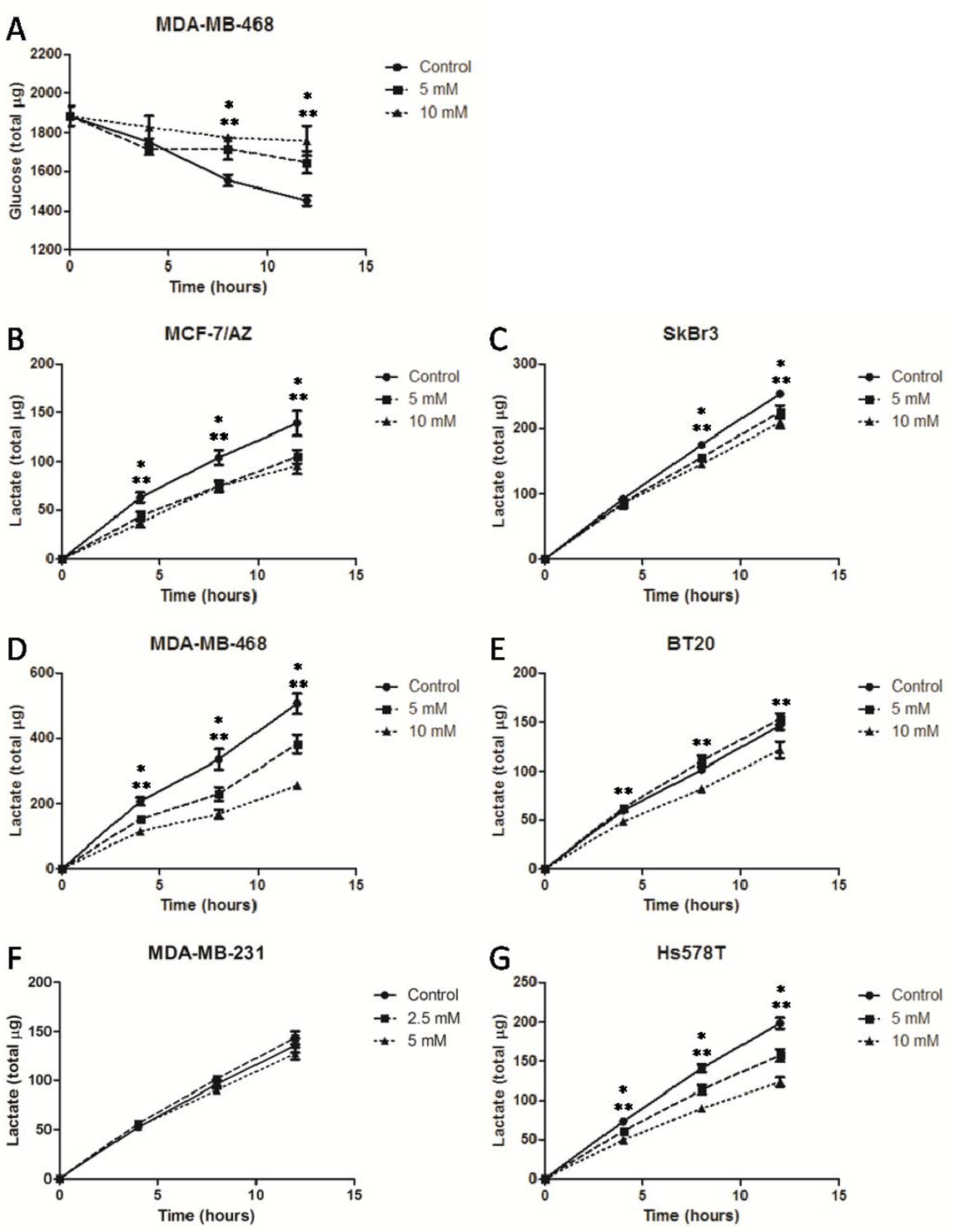

Figure 5. Effect of CHC in MDA-MB-468 glucose uptake (A) and in lactate production in the human breast carcinoma cell lines. Results are represented as mean±SEM.

luminal and normal-like breast carcinomas [23-27] and, in contrast to the other groups, do not have a specific molecular therapy [28,29]. This entails the search for new molecular targets in this aggressive group of tumours, and, considering the increased expression of MCT1 in basal-like tumours as well as recent evidence for using MCTs as effective anti-cancer targets [5,6,8-10], MCT1 emerges as a promising therapeutic target, that needs to be further explored in breast cancer.

In the present work, we analysed MCT1 expression, as well as the expression of other relevant proteins in cancer metabolism, in a variety of human breast cancer cell lines. Plasma membrane expression of MCT1 was found in MDA-MB-468, BT20 and Hs578T, which are basal-like subtype cell lines, being in accordance with our findings in human breast carcinoma samples [17]. However, in MDA-MB-231, another basal-like subtype cell line, MCT1 was not detected, as described by others [19,30,31], and the same was observed for the luminal subtype cell line (MCF-7/AZ) and the HER2 positive subtype cell line (SkBr3). The other MCT isoform involved in the glycolytic phenotype, MCT4, was strongly expressed in some cell lines, namely MCF-7/AZ, SkBr3 and MDA-MB-468, however, the immunocytochemical staining did not reveal any plasma membrane expression. This strong expression of MCT4 may reflect the involvement of MCT4 in other cell functions, such as in lactate/pyruvate transport through the mitochondrial/peroxisomal membranes. Actually, MCT4, as well as MCT1, has been described to be present in the mitochondrial membrane [32,33]. To infer on MCT4 but especially on MCT1 activity, CD147 expression was analysed, since proper plasma membrane expression and activity of these MCT isoforms requires this chaperone [19-21]. However, MDA-MB-468 and Hs578T, having a strong plasma membrane expression of MCT1, presented low and absent CD147 expression, respectively. This indicates that CD147 may not be the only chaperone required for MCT trafficking to the plasma membrane, hypothesis already raised by us and others in previous studies [17,34,35]. As expected, since all cell lines consume glucose, GLUT1, one of the major glucose transporters, was expressed in all cell lines analysed. However, GLUT1 staining intensity was not associated with the glucose consumption rates observed for each cell line, which may be explained by the presence of other GLUT isoforms, such as GLUT12 [36]. CAIX, which is highly induced by hypoxia inducible-factor 1 alpha (HIF-1α), was analysed as a surrogate marker of HIF-1α stabilisation. Although HIF-1α also induces MCT1, MCT4 and GLUT1 expressions [37-39], no associations were observed between these proteins and CAIX. Actually, focal CAIX expression was only found in MDA-MB-468, Hs578T and with a very faint staining in MCF-7/AZ. Probably, if cell lines were cultured up to a higher confluence, CAIX would be more extensively expressed, as described in confluent but not in sub-confluent MDA-MB-231 cells [40].

Inhibition of lactate transport can be achieved by different ways but, considering the outstanding results obtained by Sonveaux and colleagues [5] with CHC, we decided to use this MCT1 inhibitor in our studies in human breast cancer cells. Importantly, we applied a not so conventional assay to determine the inhibitory effect of CHC, the SRB assay, since it has been described by others that conventional assays like MTS or MTT are not adequate when inhibiting MCTs' activity [8].

Our results show that the human breast cancer cell lines studied have a different response to CHC and the underlying mechanisms seem to vary among these cell lines. In fact, the most CHC sensitive cell line, MDA-MB-231, besides being negative for MCT1 and producing less lactate then the other lines, showed no alterations in glucose consumption or lactate production after CHC treatment. Additionally, CHC has been described as an effective MCT1 inhibitor *in vitro* and *in vivo* at concentrations ≥10 mM [9]; however, we determined the lowest IC_{50} for MDA-MB-231, which is negative for MCT1. These puzzling results led us to hypothesise that CHC, in this particular cell line, should have other means of action, not related to lactate efflux or the glycolytic metabolism. CHC has also been described as a potent inhibitor of the mitochondrial pyruvate transporter [41], however, permeability studies carried out by

others show that CHC is not internalised in U-87MG glioma cells [9]. Additionally, CHC has also been pointed out as an inhibitor of the anion exchanger 1 (AE1) [42], an important pH regulator that is responsible for Cl^-/HCO_3^- membrane exchange [43]. Therefore, further studies to unveil the alterative target for CHC inhibitory activity are warranted. In contrast, MDA-MB-468 and Hs578T, both basal-like, MCT1 positive cell lines, which consume high amounts of glucose and produce high amounts of lactate, suffered a significant decrease in glucose consumption (only for MDA-MB-468) and lactate production in a dose dependent manner, simultaneously with a decrease in total biomass. This was the expected result in a highly glycolytic cell line after inhibition of MCT1. The blockage of lactate efflux likely led to accumulation of lactate in the cytoplasm, arrest of glycolysis and subsequent decrease in glucose uptake. Inhibition of cell proliferation and/or induction of cell death will be caused not only by arrest of the metabolic pathway of energy production but also due to a decrease in intracellular pH, as MCT1 also functions as a pH regulator [7]. Additionally, BT20 basal-like subtype cell line, although positive for MCT1, was insensitive to CHC treatment. This may be due to the low glycolytic metabolism of this cell line and dependence on other metabolic pathways for energy production. The reason why BT20 expresses high levels of MCT1 in the plasma membrane, regardless of the low amounts of lactate produced, remains unclear, but has probably to do with the uptake of an alternative source of energy from the culture medium, that is also a substrate for MCT1, such as pyruvate. Unexpectedly, MCF-7/AZ and SkBr3 were sensitive to CHC-induced decrease in total biomass in a metabolic dependent manner (extracellular lactate was significantly decreased, although independent from CHC dose and at a lower magnitude than that observed for MDA-MB-468 and Hs578T), albeit being negative for MCT1 expression at the plasma membrane. MCT4 could be pointed as another target of CHC, however, K_i values for MCT4 should be 5-10 times higher than for MCT1, ranging the 50 to 100 mM, which is not the case observed in this cell lines.

The results herein presented are very promising and need to be completed with other functional analysis such as apoptosis, invasion and migration assays to further characterise the alterations induced by CHC as well as anticipate the practical effect of MCT1 inhibition. However, one should always be careful when using CHC as a MCT1 inhibitor, always confirming the inhibition of the metabolism, avoiding looking only to viability and/or proliferating rates. In this perspective, other more particular approaches such as siRNA or specific MCT1 inhibitors like the ones developed by AstraZeneca [44-46] should be used. We are currently optimising the use of MCT1 and MCT4 siRNA to evaluate the effect of MCT silencing in cancer cell viability, proliferation, apoptosis, invasion and migration.

The importance of metabolism as a disease progression driving force and a strategic therapeutic target in cancer has becoming more explored over the last years. Evidence provided by experimental data, as well as mathematical models, show that the hyperglycolytic, acid-resistant phenotype of cancer cells, besides providing a growth advantage over normal cells, is also critical for the ability of cancer cells to invade the surrounding tissue [47-52]. Therefore, the targeting of this phenotype will probably retard or prevent transition from *in situ* to invasive cancer. Here, by targeting lactate efflux through CHC-induced inhibition of MCT1 activity, we took a little step forward in the direction to accept metabolism targeting as an effective way to control tumour and, in particular, to consider the development of therapeutic approaches targeting MCT1 as a promising strategy to treat basal-like breast tumours. However, other *in vitro* as well as *in vivo* approaches are necessary to confirm these conjectures.

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Chapter 6. FURTHER INSIGHTS ON MCT REGULATION

6.1. CHAPTER OVERVIEW

The regulatory mechanisms of MCT expression are far from being completely understood, however, regulation by the chaperone CD147 is a well characterised mechanism, supported by many *in vitro* studies [1-8]. Other regulatory mechanisms have also been identified but further evidence is needed. One of those alternative mechanisms is MCT regulation by CD44, as only one study describes the association between this protein and MCT1, MCT4, as well as CD147 [9].

Therefore, a tumour screening was performed aiming to confirm CD44 as a putative chaperone of MCTs. For that, CD44 and CD147 expressions, as well as MCT1, MCT2 and MCT4 were evaluated, in four different types of tumours: breast carcinoma, colon adenocarcinoma, non-small cell lung cancer and ovarian adenocarcinoma. In this work, published in an international periodical with referees, evidence is presented for the possible association of CD44 and MCT1 in lung cancer. Further, MCT1 was associated with CD147 in ovarian cancer, while MCT4 was associated with CD147 in breast and lung cancer. Notably, a relatively important number of cases expressed MCT1 at the plasma membrane of cancer cells but neither CD147 nor CD44 were present, suggesting that other mechanisms of regulation for correct trafficking of MCTs to the plasma membrane might exist. Also, MCT4 plasma membrane expression was decreased in lung cancer, as compared to normal tissue, while MCT1 was expressed in a considerable number of ovary tumours, warranting further studies in larger series of these types of tumours.

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6.2. PUBLISHED RESULTS

This chapter comprises the following paper, **published** in an international scientific periodical with referees:

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6.2.1. EXPRESSION OF MONOCARBOXYLATE TRANSPORTERS 1, 2 AND 4 IN HUMAN TUMOURS AND THEIR ASSOCIATION WITH CD147 AND CD44

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Research Article

Expression of Monocarboxylate Transporters 1, 2, and 4 in Human Tumours and Their Association with CD147 and CD44

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Monocarboxylate transporters (MCTs) are important cellular pH regulators in cancer cells; however, the value of MCT expression in cancer is still poorly understood. In the present study, we analysed MCT1, MCT2, and MCT4 protein expression in breast, colon, lung, and ovary neoplasms, as well as CD147 and CD44. MCT expression frequency was high and heterogeneous among the different tumours. Comparing with normal tissues, there was an increase in MCT1 and MCT4 expressions in breast carcinoma and a decrease in MCT4 plasma membrane expression in lung cancer. There were associations between CD147 and MCT1 expressions in ovarian cancer as well as between CD147 and MCT4 in both breast and lung cancers. CD44 was only associated with MCT1 plasma membrane expression in lung cancer. An important number of MCT1 positive cases are negative for both chaperones, suggesting that MCT plasma membrane expression in tumours may depend on a yet nonidentified regulatory protein.

1. Introduction

Uncontrolled tumour cell proliferation is a pivot mechanism in tumourigenesis, which consequently leads to significant metabolic changes. In tumour cells, the preference by anaerobic glycolysis, even in the presence of oxygen, phenomenon known as "the Warburg effect", stimulates the conversion of pyruvate to lactic acid [1, 2]. To allow proliferation through continuous glycolysis and avoid acid-induced apoptosis, cells must develop mechanisms to oppose the increased generation of lactic acid. Thus, several plasma membrane transporters and exchangers have been implicated in the maintenance of the intracellular pH of cancer cells, by exporting the accumulating acid, leading to acidification of the extracellular milieu [3]. Currently, it is acknowledged that this acidic tumour microenvironment is associated with tumour aggressiveness features, such as growth advantage, increased survival, migration, invasion, and angiogenesis [1, 4].

Monocarboxylate transporters (MCTs) are among the most important cellular pH regulators likely involved in cancer pH homeostasis [3, 5]. The MCT family comprises fourteen members, being the isoforms 1, 2, 3, and 4 responsible for the H^+-linked transport of monocarboxylates such as lactic acid across the plasma membrane [6]. The underlying molecular events involved in MCT regulation are poorly understood; however, it was recently demonstrated that proper plasma membrane expression and activity of MCTs, particularly MCT1 and MCT4, require the presence of a chaperone, CD147 [7–9], also known as EMMPRIN and basigin. Interestingly, CD147 expression seems to be also dependent on MCT1 and MCT4 expressions [10, 11]. Most recently, it was suggested that constitutive interactions between hyaluronan and CD44 also contribute to regulation of MCT localization and function [12]. In the past few years, some studies reported abnormal expression of MCTs, particularly MCT1, 2, 3, and 4 in distinct solid tumours, however, with contradictory conclusions [13–24]. Besides

acting as MCT chaperone, CD147 plays many other roles, including production of matrix metalloproteinases and vascular endothelial growth factor, being upregulated in a variety of human cancers [25–28]. Additionally, activation of CD44 has been described as important in various aspects of cancer progression including cell growth control, adhesion, migration, invasion, and chemoresistance [29, 30].

The aim of the present study was to perform a comprehensive analysis of MCT1, MCT2, and MCT4 protein expression in a variety of tumours, namely, breast, colon, lung, and ovary neoplasms in order to elucidate their pattern of expression and their role in the development of these tumours. In addition, CD147 and CD44 expressions were analysed to infer the contribution of these chaperones to MCT expression in these different tumours.

2. Materials and Methods

2.1. Cases. A commercial human multitumour tissue microarray (TARP) (NCI Tumour Repository MTA, MD, USA), containing 200 tumour samples, was used to perform the immunohistochemical reactions, corresponding to 50 breast carcinomas (42 ductal, 5 lobular, and 3 not classified), 50 colon adenocarcinomas, 50 nonsmall cell lung cancers, and 50 ovarian adenocarcinomas (32 serous, 8 clear cell, 4 mucinous, 4 endometrioid, and 2 not classified). Additionally, to allow comparison between nonneoplastic and malignant tissues, 15 normal breast samples and 11 normal lung samples were included in the analysis. Since ovarian normal tissues are not readily available, they were not included in this study. MCT expression in nonneoplastic colon epithelia was already described by our group [19].

2.2. Immunohistochemistry

2.2.1. MCT Detection. Immunohistochemistry was performed according to the avidin-biotin-peroxidase complex principle (R.T.U. VECTASTAIN Elite ABC Kit (Universal), Vector Laboratories, Burlingame, CA, USA), with the primary antibodies for MCT1 (AB3538P, Chemicon International, Temecula, CA, USA), MCT2 (sc-14926, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and MCT4 (AB3316P, Chemicon International, Temecula, CA, USA), diluted 1 : 200 for both MCT1 and MCT2 and 1 : 100 for MCT4, as previously described by our group [18, 19].

2.2.2. CD147 Detection. Immunohistochemistry was performed according to the avidin-biotin-peroxidase complex principle (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA, USA), using a primary antibody raised against CD147 (18-7344, ZYMED Laboratories Inc., South San Francisco, CA, USA) diluted 1 : 750, as previously described by our group [31].

2.2.3. CD44 Detection. Immunohistochemistry was performed according to the avidin-biotin-peroxidase complex principle (Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA, USA), using a primary antibody raised against total CD44 (clone 156-3C11, Cell Signalling Technology, Beverly, MA, USA), diluted 1 : 100. Briefly, deparaffinised and rehydrated sections were immersed in 0.01 M citrate-buffered solution (pH 6.0), heated up to 98°C in a water bath for 15 minutes, and washed in PBS. Endogenous peroxidases were inactivated with 3% hydrogen peroxide in methanol for 10 minutes, followed by washing in PBS. Tissue sections were incubated with blocking solution for 10 minutes and incubated at room temperature with the primary antibody for 30 minutes. Sections were then sequentially washed in PBS, incubated with biotinylated goat anti-polyvalent antibody for 10 minutes, streptavidin peroxidase for 10 minutes, and developed with 3,3′-diamino-benzidine (DAB+ Substrate System, Dako, Carpinteria, CA, USA) for 10 minutes. Negative controls were performed by using the adequate serum control (N1698, Dako, Carpinteria, CA, USA) and tonsil was used as positive control. Tissue sections were counterstained with haematoxylin and permanently mounted.

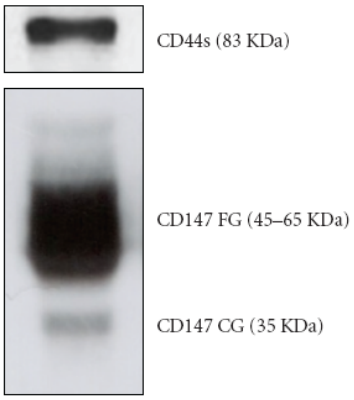

Figure 1: Western-blot for CD44 (breast cancer cell line MDA MB 231) and CD147 (human colon tissue). The protein molecular weights observed are in accordance with the predicted for these proteins. FG: fully glycosylated, CG: core glycosylated.

The specificity of CD147 and CD44 antibodies was further demonstrated by Western-blot, as shown in Figure 1. Antibodies for the MCT isoforms have been previously validated by our group by Western-blot [19].

2.2.4. Immunohistochemical Evaluation. MCTs, CD147, and CD44 immunoreactions were evaluated semiquantitatively using the criteria previously described [19, 31]. Immunoreaction extent was scored semiquantitatively as follows: 0: 0% of immunoreactive cells, 1: <5% of immunoreactive cell, 2: 5%–50% of immunoreactive cells, and 3: >50% of immunoreactive cells. Also, intensity of staining was scored semi-qualitatively as 0: negative, 1: weak, 2: intermediate, and 3: strong. Immunoreaction final score was defined as the sum of both parameters (extent and intensity), and grouped as negative (scores 0 and 2) and positive (3–6). Finally, since plasma membrane location is essential for MCT1 and MCT4 membrane localization and activity, we also analysed the plasma membrane positive cases separately. Evaluation

Table 1: Frequency of MCTs, CD147, and CD44 expressions in tumour samples.

Tumour type	Expression												
	MCT1 Positive (%)			MCT2 Positive (%)			MCT4 Positive (%)			CD147 Positive (%)		CD44 Positive (%)	
	n	Cyt[a]	PM	n	Cyt[a]	PM	n	Cyt[a]	PM	n	Cyt[a]/PM	n	Cyt[a]/PM
Breast carcinoma	22	21 (95.5)	5 (22.7)	24	34 (100.0)	0 (0.0)	24	19 (79.2)	1 (4.2)	28	13 (46.4)	27	8 (29.6)
Colon adenocarcinoma	32	23 (71.9)	19 (59.4)	29	29 (100.0)	0 (0.0)	26	19 (73.1)	0 (0.0)	34	13 (38.2)	31	4 (12.9)
Nonsmall cell lung cancer	23	14 (60.9)	5 (21.7)	21	21 (100.0)	0 (0.0)	23	16 (69.6)	0 (0.0)	28	13 (46.4)	27	12 (44.4)
Ovarian adenocarcinoma	24	18 (75.0)	10 (41.7)	30	18 (93.3)	0 (0.0)	28	13 (46.4)	1 (3.6)	26	11 (42.3)	28	2 (7.1)

Cyt: cytoplasm; PM: plasma membrane; [a]with or without plasma membrane staining.
Note: for CD147 and CD44, frequency results are the same for cytoplasm and plasma membrane expression.

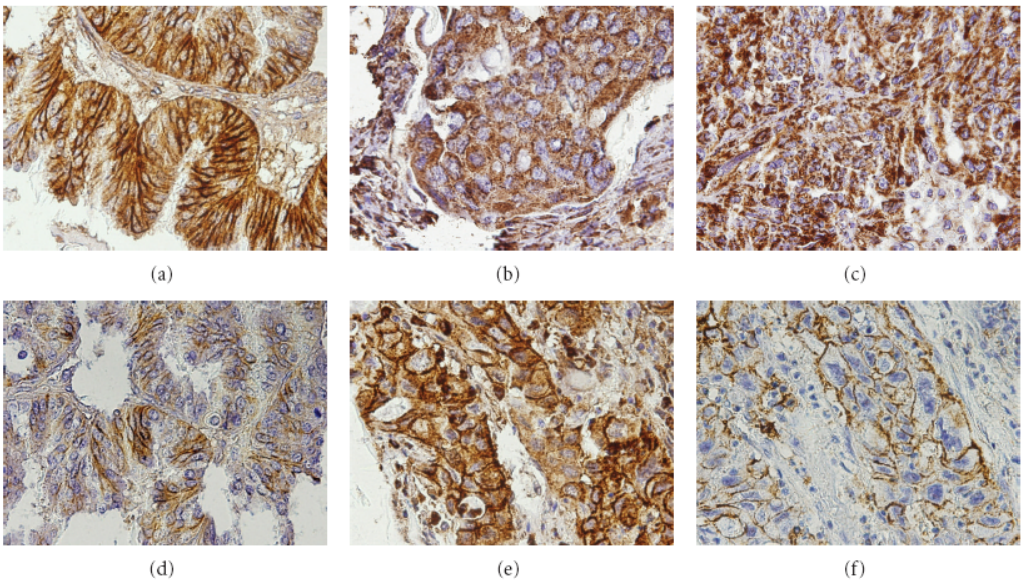

Figure 2: Representative immunohistochemical expression of MCT1 in ovarian carcinoma (a), MCT2 in breast carcinoma (b), MCT4 in ovarian carcinoma (c), CD147 in ovarian carcinoma (d), MCT1 in lung cancer (e), and CD44 in lung cancer (f). Plasma membrane staining for both MCT1 (a) and CD147 (d) is shown in the same tumour area of an ovary cancer case and for both MCT1 (e) and CD44 (f) in the same area of lung cancer case.

was performed blindly by two independent observers (AL, FS). Discordant results were discussed in a double-head microscope and a final score was agreed.

2.3. Statistical Analysis. Data were stored and analysed using the SPSS statistical software (version 16.0, SPSS Inc., Chicago, IL, USA). All comparisons were examined for statistical significance using Pearson's Chi-square (χ^2) test and Fisher's exact test (when $n < 5$), being threshold for significance P values <.05.

3. Results

From the 200 tumour samples, only approximately 60% were suitable for analysis. The remaining tumour spots were missing, dropped out during immunohistochemistry protocol, or did not contain representative tissue, with some heterogeneity between TARP slides. Tumour samples were assessed for MCT1, MCT2, MCT4, CD147, and CD44 immunohistochemical expressions and the results are summarised in Table 1. Positive MCT1 expression was observed in both plasma membrane and cytoplasm (Figures 2(a) and 2(e)), while MCT2 expression was only observed in the cytoplasm (Figure 2(b)) and MCT4 was commonly found in the cytoplasm (Figure 2(c)) and rarely in the plasma membrane. Regarding CD147 and CD44, expression was always present in the plasma membrane (Figures 2(d) and 2(f), resp.), with some cytoplasmic staining. There was a strong tendency for differences in MCT1 and MCT4 expression frequencies among the different tumours ($P = .056$ and $P = .061$, resp.); however, differences were only significant when considering MCT1 plasma membrane staining ($P = .012$), being highest for colon, followed by ovary. MCT2 and CD147 expression frequencies were not significantly different among the tumour types studied. CD44 expression

Table 2: Frequency of MCTs expression in nonneoplastic and malignant breast and lung tissues.

	Breast tissues					Lung tissues				
		Cyt[a]		PM			Cyt[a]		PM	
	n	Positive (%)	*P*	Positive (%)	*P*	*n*	Positive (%)	*P*	Positive (%)	*P*
MCT1			.001		.067			1		.612
Normal	15	7 (46.7)		0 (0.0)		6	4 (66.7)		2 (33.3)	
Tumour	22	21 (95.5)		5 (22.7)		23	14 (60.9)		5 (21.7)	
MCT2			.142					.300		
Normal	15	13 (86.7)				9	8 (88.9)			
Tumour	24	24 (100.0)				21	21 (100.0)			
MCT4			.004		1			.289		.001
Normal	15	5 (33.3)		0 (0.0)		6	6 (100.0)		4 (66.7)	
Tumour	24	19 (79.2)		1 (4.2)		23	16 (69.6)		0 (0.0)	

Cyt: cytoplasm; PM: plasma membrane; [a]with or without plasma membrane staining.

was also significantly different among tumours ($P = .004$), being more frequent in lung, followed by breast. Overall, and looking at each tumour entity, we observed that MCT2 was the most frequently expressed MCT isoform, followed by MCT1 and 4. MCT1 exhibited the highest frequency of staining at the plasma membrane, while MCT4 was barely present and MCT2 was absent.

Comparison of MCT expression frequencies in breast and lung malignant tissues with the corresponding normal tissues is depicted in Table 2. In breast carcinomas, there was a significant increase in MCT1 and MCT4 staining ($P = .001$ and $P = .004$, resp.), while in lung cancer, there was a significant decrease in MCT4 plasma membrane expression ($P = .001$).

In order to assess the association of CD147 and CD44 to MCT expression in tumours, we searched for associations among expressions of these proteins (Tables 3 and 4). Overall, considering all tumour entities, both MCT1 and MCT4 immunoreactions correlated with CD147 ($P = .001$ and $P < .001$, resp.), while, for plasma membrane localisation, this association was only observed for MCT1 ($P = .025$), as illustrated in Figures 2(a) and 2(d). No association was observed between CD147 and MCT2. Considering the different tumours individually, associations between CD147 and MCT4 immunoreaction were only observed in breast and lung cancers ($P = .024$ and $P = .023$, resp.); however, other values showed a tendency for significance but the low number of cases limited statistical power. Regarding CD44, the only significant association was with MCT1 plasma membrane expression in lung cancer ($P = .039$), as illustrated in Figures 2(e) and 2(f). Importantly, among the 36 cases positive for MCT1 plasma membrane expression, 12 were negative for both CD147 and CD44 (data not shown).

4. Discussion

Upregulation of glycolysis and adaptation to acidosis are key events in the transition from *in situ* to invasive cancer [1] and MCTs may play an important role through their involvement in exporting lactate [6]. MCTs have been described to be up-regulated in several cancers [13–19]; however, there are still some controversies [22, 23]. Moreover, their regulation in cancer is starting to be unravelled.

In the present study, we observed a high and heterogeneous frequency of MCT expression among the different tumour entities. Importantly, only MCT1 presented a relevant expression at the plasma membrane, a fact which is essential for lactate transporter activity. It appears that the pair MCT1/CD147 is the most relevant in the tumours studied, likely by promoting lactate efflux from cancer cells. Lactate efflux allows continuous proliferation, avoiding apoptosis by intracellular acidification, thus conferring a proliferative advantage to cancer cells [1, 4]. The high frequencies of MCT2 and MCT4 in the cytoplasm might mean that they are involved in other functions in the cell, such as in lactate/pyruvate transport through the mitochondrial/peroxisomal membrane. Actually, MCT2 and MCT4, as well as MCT1, have been described to be present in the mitochondrial membrane [32–34].

In the present study, we showed that MCT1 is upregulated in breast carcinomas, which does not corroborate a previous report in breast cancer, pointing to a possible silencing of MCT1 expression by gene promoter hypermethylation [10, 22]. However, this study only presented the results of MCT1 promoter methylation and was not supported by MCT1 protein expression. Taking into consideration that MCT2 is not present in the plasma membrane and MCT4 is expressed at low levels, it is reasonable to hypothesise that MCT1 is the main isoform responsible for lactate plasma membrane transport in breast carcinoma. To confirm this hypothesis, we are currently evaluating MCT expression in a larger series of breast carcinomas. The important role of MCTs in substrate transport in colonic epithelium has been vastly studied [23, 24, 35–38]. However, data on MCT expression in colorectal carcinoma are still contradictory [17, 19, 23, 24]. Our findings on MCT expression are in agreement with our previous report [19] in which we described MCT upregulation, especially MCT1 and MCT4. Comparative studies on lung cancer showed an absent MCT expression in normal lung but a high MCT plasma

Table 3: Correlations between MCTs and CD147 expressions in tumours.

		CD147 expression										
		Breast			Colon			Lung			Ovary	
		n	Positive (%)	*P*	*n*	Positive (%)	*P*	*n*	Positive (%)	*P*	*n*	Positive (%)	*P*
MCT1	Cytoplasm[a]			.381			.184			.074			.245
	Negative	1	0 (0.0)		7	1 (14.3)		8	2 (25.0)		3	0 (0.0)	
	Positive	20	13 (65.0)		21	11 (52.4)		14	10 (71.4)		15	7 (46.7)	
	Plasma membrane			.131			.114			1			.050
	Negative	17	9 (52.9)		10	2 (20.0)		17	9 (52.9)		9	1 (11.1)	
	Positive	4	4 (100.0)		18	10 (55.6)		5	3 (60.0)		9	6 (66.7)	
MCT4	Cytoplasm[a]			.024			.118			.023			.214
	Negative	4	0 (0.0)		5	0 (0.0)		6	1 (16.7)		12	3 (25.0)	
	Positive	19	13 (68.4)		19	9 (47.4)		16	12 (75.0)		12	7 (58.3)	
	Plasma membrane			1			—			—			1
	Negative	22	12 (54.5)		24	9 (37.5)		22	13 (59.1)		23	10 (43.5)	
	Positive	1	1 (100.0)		0	—		0	—		1	0 (0.0)	

[a] with or without plasma membrane staining.

Table 4: Correlations between MCTs and CD44 expressions in tumours.

		CD44 expression										
		Breast			Colon			Lung			Ovary	
		n	Positive (%)	*P*	*n*	Positive (%)	*P*	*n*	Positive (%)	*P*	*n*	Positive (%)	*P*
MCT1	Cytoplasm[a]			.364			.218			1			.250
	Negative	1	1 (100.0)		6	2 (33.3)		9	3 (33.3)		5	1 (20.0)	
	Positive	21	7 (33.3)		20	2 (10.0)		13	5 (38.5)		15	0 (0.0)	
	Plasma membrane			.309			.591			.039			1
	Negative	17	5 (29.4)		9	2 (22.2)		17	4 (23.5)		10	1 (10.0)	
	Positive	5	3 (60.0)		17	2 (11.8)		5	4 (80.0)		10	0 (0.0)	
MCT4	Cytoplasm[a]			1			.539			.613			.482
	Negative	4	1 (25.0)		5	1 (20.0)		6	1 (16.7)		13	2 (15.4)	
	Positive	18	6 (33.3)		18	2 (11.1)		15	6 (40.0)		11	0 (0.0)	
	Plasma membrane			.318			—			—			1
	Negative	21	6 (28.6)		23	3 (13.0)		21	7 (33.3)		23	2 (8.7)	
	Positive	1	1 (100.0)		0	—		0	—		1	0 (0.0)	

[a] with or without plasma membrane staining.

membrane expression in cancer cells, especially MCT1 [16]. Although at a significantly lower level (20% versus 100%) we also detected MCT1 in the membrane of cancer cells, the same was not observed for MCT2 and MCT4. Therefore, it appears that MCT1 is the most important MCT isoform likely involved in lactate efflux from lung cancer cells. To the best of our knowledge, there are no reports on MCT expression in ovarian cancer. Thus, the results here presented are novel and may shed some light onto the metabolic alterations occurring in this type of cancer. Indeed, the upregulation of glycolytic enzymes in ovarian cancer [39–41] points to an increased production of lactate and, consequently, to the need for upregulation of lactate transport. In harmony with this line of evidence, we found a relevant MCT1 expression in ovarian carcinoma, which warrants further studies on MCT expression in this type of carcinoma.

Importantly, we assessed the association between MCTs and the glycoproteins CD147 and CD44 in human tumour samples. We observed a close association between CD147 expression and MCT1 and MCT4 isoforms, which corroborates the data from the literature stating that CD147 is essential for MCT1 and MCT4 regulation [7, 9, 10] and that CD147 maturation and cell surface expression is dependent on MCT1 and MCT4 [10, 11]. It is important to note that these associations were not homogeneous among tumour entities, which might indicate that regulation of these proteins diverges from tumour to tumour. Thus, additional studies are necessary to unveil the possible synergism between MCT and CD147 in malignant progression of tumours from different origins. Since it was recently described that CD44 was also involved in MCT regulation in breast cancer [12], we analysed MCT and CD44 coexpression. However, we only found an association

between CD44 and MCT1 plasma membrane expression in lung cancer. Notably, we observed that there are still an important number of cases positive for MCT1, which are negative for both CD147 and CD44, suggesting that MCT1 plasma membrane expression in tumours may depend on a yet nonidentified regulatory protein.

In summary, we analysed the expression of MCT1, MCT2, MCT4, CD147, and CD44 in different primary tumours. Importantly, we evaluated for the first time MCT expression in ovarian carcinoma and the association between MCT and both CD147 and CD44 expressions in four different types of tumours. The results herein presented can contribute to understanding of MCTs' regulation and role in human tumours. Nevertheless, additional studies, including larger and well-characterized tumour series, especially of breast and ovary, will definitely complement this work and pave the way to the possible exploitation of MCTs as targets for cancer therapy.

Competing Interests

These authors declare no competing interests.

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Chapter 7. GENERAL DISCUSSION

7.1. OVERVIEW ON THE CONTRIBUTION TO THE STATE OF THE ART

Carcinogenesis has been viewed as an evolutionary process described as "somatic evolution" as it requires a sequence of genetic changes; however, recent models of carcinogenesis integrate the neo-Darwinian evolution, stating that phenotypic properties are retained or lost based on their contribution to fitness for survival, with cell-environment interactions. This new concept of carcinogenesis was applied to explain the Warburg phenomenon, i.e., the preference for the glycolytic phenotype, even in the presence of oxygen. Thus, as cancer progression proceeds, mutations in tumour cells increase and traits that are found in invasive cancers, like the hyper-glycolytic and acid-resistant phenotypes, arise as adaptive mechanisms to environmental proliferative constraints, such as hypoxia. Many players have been associated with these cellular adaptations; however, although an important role of lactate transporters could be anticipated in the context of the Warburg effect, the underlying role of MCTs in solid tumours are far from being characterised. Although pointed out some times as potential therapeutic targets, information on MCTs' expression in human solid tumours is still very scarce. To be used as therapeutic targets in cancer, MCTs must be differently expressed, either in quantity or in isoform. Thus, there is an urgent need to determine MCTs' expression during the carcinogenesis process. Some information is provided for human tumours from the nervous system, colon and lung [1-8], but similar studies are lacking for other types of tumours. Importantly, only one of these studies analysed the clinico-pathological value of MCT1 [7]. Also, additional functional studies are needed to comprehend the overall contribution of MCTs to tumour malignancy.

7.1.1. MCT EXPRESSION IN SOLID TUMOURS

In this work, an important step forward was taken towards the possibility of exploiting MCTs as targets for cancer therapy. In fact, colorectal, cervical and breast carcinomas were found to express MCTs, especially MCT1, more frequently than the corresponding normal tissues (Table 1).

Importantly, MCT1 was associated with poorer patient prognosis, especially in gastric and breast carcinomas. Although MCT1 was not up-regulated in gastric carcinoma, MCT1/CD147 co-expression was associated with parameters of poor prognosis, such as tumour invasion and metastasis, implying a role of MCT1 in tumour aggressiveness.

Table 1. Overview on MCT1, MCT2 and MCT4 expression and prognosis in different tumour types.

Tumour type	MCT1	MCT2	MCT4
Brain	Strongest in high grade glial neoplasms, compared to low grade glial neoplasms [2] (+) in glioblastoma and (-) in normal tissue [5]	↑ in glioblastoma, compared to normal tissue [5]	(-) in glioblastoma [5]
Breast	↓ due to gene hypermethylation [9] **↑ in tumour cells, compared to normal epithelium / associated with basal-like subtype, high histological grade, estrogen and progesterone receptors, cytokeratins 5 and 14 and vimentin (alone or co-expressed with CD147)**	**(+) in tumour cells cytoplasm, but not in plasma membrane (+) normal epithelium**	**Tendency to be ↑ in tumour cells, compared to normal epithelium**
Cervical	**↑ from preinvasive to invasive / associated with metastases in AC (when co-expressed with CD147)**	**No progressive change from preinvasive to invasive / ↑ ASC**	**↑ from preinvasive to invasive / ↑ AC**
Colorectal	↓ from normality to malignancy [1,3] (+) in tumour cells but (-) normal epithelium [6] **↑ in tumour cells, compared to normal epithelium / associated with vascular invasion**	Not detected in either normal or tumour tissues [3] + in tumour cells cytoplasm, but not in plasma membrane [6] **↑ in cytoplasm expression but ↓ in tumour cells plasma membrane compared to normal epithelium**	Not detected in either normal or tumour tissues [3] Weak in tumour microenvironment [6] **↑ in tumour cells, compared to normal epithelium**
Gastric	**No change along progression / associated with advanced gastric cancer, Lauren's intestinal type, stage III+IV and lymph-node metastases when (co-expressed with CD147)**		**↓ from normal tissue, to primary tumour, to lymph-node metastases / associated with early gastric cancer and Lauren's intestinal type**
Lung	Cytoplasmic accumulation in alveolar soft-part sarcoma [4] (+) in tumour cells but (-) normal epithelium [8] **(+) in tumour cells and normal epithelium**	(+) in tumour cells but (-) normal epithelium [8] **(+) in tumour cells cytoplasm, but not in plasma membrane (+) normal epithelium**	(+) in tumour cells but (-) normal epithelium [8] **↓ in tumour cells, compared to normal epithelium**
Ovarian	**(+) in tumour cells[10]**	**(+) in tumour cells cytoplasm, but not in plasma membrane[10]**	**(+) in tumour cells**
Neuroendocrine	(+) in neuroblastoma / associated with age >1 year at diagnosis, stage 4 disease, unfavourable Shimada histopathology, DNA diploid index, *n-myc* amplification and high-risk clinical group (COG criteria) [7]		

Results from this thesis are shown in bold. ↑, up-regulated;↓, down-regulated; (+) positive, (-), negative. Abbreviations: AC, adenocarcinoma; ASC, adenosquamous carcinoma; COG, Children's Oncology Group.

Additionally, it is important to highlight that both MCT4 and CD147 alone, or MCT1 in association with CD147, were more frequently observed in gastric tumours of intestinal-type

(Lauren's classification). This may indicate that the carcinogenic process leading to intestinal-type gastric carcinoma, which includes evolution of normal gastric mucosa through chronic gastritis, atrophic gastritis (which are common to the diffuse-type gastric carcinogenesis), as well as intestinal metaplasia and adenoma/dysplasia (exclusive of the intestinal-type carcinogenesis) [11], may require metabolic adaptations involving MCT up-regulation, which are not required during carcinogenesis towards diffuse-type gastric carcinomas. Also, in breast carcinoma, an important association between MCT1 and basal-like subtype was found. This finding is of great importance as, although being a less frequent group of breast tumours, accounting for around 15% of cases [12], basal-like subtype tumours have a more aggressive clinical behaviour and, until now, does not have a specific molecular therapy. In this scenario, MCT1 arises as a promising therapeutic target for this particular group of breast tumours. Considering these findings in the light of the microenvironmental model of carcinogenesis, where MCTs shall have a vital role in the emergence of both the hyper-glycolytic and acid-resistant phenotypes, one can easily justify the up-regulation of MCTs, as well as their contribution to a more aggressive tumour behaviour. By enabling lactate efflux from cancer cells, as well as regulate the intracellular pH and, consequently, acidifying the extracellular microenvironment, MCT activity will promote the aggressive behaviour by enhancing migration, invasion and angiogenesis, among others [13-33].

Furthermore, in view of the recent model of metabolic symbiosis between glycolytic and oxidative cancer cells, where lactate plays a key role as the metabolic intermediate, MCTs are essential players in this process. It is proposed in this model that lactate release from glycolytic/hypoxic cancer cells occurs through the low-affinity lactate transporter MCT4 and lactate uptake by the oxidative/oxygenated cancer cells occurs through MCT1. Moreover, MCT1 inhibition will lead to a switch from lactate oxidation to glycolysis in oxidative tumour cells, thereby preventing the necessary glucose delivery to glycolytic cells, which will die from glucose starvation [34]. However, it is important to note that, in the breast carcinoma series herein analysed, in contrast to the previous model, MCT1 was expressed in the peri-necrotic/hypoxic regions and was expressed in the same cells than the hypoxia markers CAIX and GLUT1, important in glycolytic metabolism. This indicates that MCT1, besides being important in oxidative cancer cells, may also be important in glycolytic cancer cells. In fact, the kinetic parameters of MCT1 make this isoform suitable for both the uptake and efflux of substrates, therefore, MCT1 is also appropriate for lactate efflux from glycolytic cancer cells. The role of MCT1 in both types of metabolic cancer cells makes it an even more interesting therapeutic target.

Characterisation of MCT expression in series of human solid tumours provided the first evidence of this thesis, suggesting MCT1 as promising therapeutic target for cancer therapy, in

sub-groups of colorectal, cervical and breast carcinomas. However, in this regard, gastric carcinoma was an exception as, besides not having MCT up-regulation, MCT4 expression was, actually, decreased along progression to malignancy. Therefore, MCT1 will probably not be a good therapeutic target in gastric cancer, since is not increased in comparison to the normal tissue, but may be an additional marker for prognosis, as it is associated, when co-expressed with CD147, to patient's poor outcome.

Due to the lack of relevant alterations in the expression of MCT2 in colorectal and cervical carcinomas, as well as the lack of associations with clinic-pathological variables, data from this MCT isoform expression was not included in the subsequent series. Indeed, MCT2 was also evaluated in gastric and breast carcinomas, however with no relevant results (data not shown). Importantly, one should never exclude a possible role of MCT2, especially in tumours with non-glycolytic metabolism. Actually, preliminary results in prostate carcinoma suggest an up-regulation of MCT2 in tumour cells, as compared to normal epithelium. Importantly, the similar specificity and sensitivity of MCT2 staining, as compared with the prostate tumour marker AMACR, led to the hypothesis that this MCT isoform may be explored as a new marker of prostate carcinoma.

7.1.2. MCT REGULATION

With this thesis, some contribution was also made to understand the possible mechanisms underlying MCT regulation.

Firstly, the regulation of MCT1 and MCT4, but not MCT2, by CD147, was supported by evidence on human tissues, complementing the *in vitro* and some *in vivo* studies previously described by others [35-42]. Indeed, the prognostic value of CD147 appears to be associated with its co-expression with MCT1, as observed in breast and gastric carcinomas. Therefore, targeting CD147, which will also impair MCT activity, appears to be a rational therapeutic approach against human cancer, as already described both *in vitro* and *in vivo* [41-43]. Besides the role of CD147 as chaperone for MCT1 and MCT4 plasma membrane trafficking and activity, these MCT isoforms also have been implicated in CD147 proper membrane expression [38,40]. Thus, the contribution of MCTs to the malignant phenotype is not limited to their own function as lactate transporters and pH regulators, but may also be further enhanced by their role in regulating CD147 expression. If so, MCTs may also have major roles in tumour growth and angiogenesis, as well as cancer cell migration and invasion, as described for CD147 [44-46].

Additionally, CD44 was analysed as a putative chaperone of MCT1 and MCT4 [47]. Although CD44 was only associated with MCT1 in lung cancer, the number of cases was small and the results obtained demand further analysis, with a higher number of cases. Indeed, as for CD147, which was not associated with MCT1 in the breast carcinoma screening but the tendency observed was confirmed in the study with the larger series (with a significant association between MCT1 and CD147), CD44 association with MCTs will most likely be also confirmed in larger series. As a result, MCT expression may also have a role in cell growth control, adhesion, migration, invasion, and chemoresistance [48-50], through interaction with CD44. Importantly, although CD147 was associated with both MCT1 and MCT4 in all series analysed, in cervical cancer, association was only at the overall expression level and not at the plasma membrane level. Additionally, in the other tumour types (gastric and breast carcinoma), there was a relevant number of cases with MCT plasma membrane expression that lacked CD147 plasma membrane co-expression. Further, in the tumour screening, this absence of CD147 was not overcome by CD44. These findings suggest that a not yet identified chaperone may be involved in MCT trafficking to the plasma membrane.

HPV is present in virtually all cervical cancer cases, being a necessary cause for this type of cancer [51]. In this thesis, an association between HPV and both MCT1 and MCT4 expression was observed in pre-malignant cervical lesions, presenting the first evidence for a possible HPV-dependent MCT regulation. High-risk HPVs exert their malignant action by constitutively expressing two major oncoproteins E6 and E7 [52]. The well-known functions of E6 and E7 are that E6 targets p53, resulting in cell resistance to apoptosis, and E7 associates with retinoblastoma protein (pRB), promoting cell cycle progression [53]. However, other mechanisms of HPV-induced carcinogenesis take place, and include activation of transcription factors such as HIF-1α and c-myc [54,55]. As these transcription factors are regulators of MCT expression [56-60], one can hypothesise that the molecular alterations induced by HPV oncoproteins, which are required for cervical carcinogenesis, include up-regulation of MCTs.

Study on MCT regulation by hypoxia has provided puzzling results, especially in what concerns regulation of MCT1 isoform [34,57,58,60-63]. While MCT4 expression has been associated with hypoxic conditions and promoter regulation by HIF-1α [58,60-63], MCT1 regulation by hypoxia points to both directions: induction and repression [34,57,58,60,61,63]. Here, additional evidence on MCT regulation by hypoxia is provided, by evaluating the co-expression of MCTs with the hypoxia-inducible proteins GLUT1 and CAIX, recognised as tumour hypoxia markers [64], in breast carcinomas. MCT4, unexpectedly, was not increased in GLUT1 or CAIX positive tumours; however, MCT1 was more frequently expressed in both GLUT1 and

CAIX positive tumours, pointing to a hypoxia dependent up-regulation of this MCT isoform that is accompanied by co-expression of its chaperone CD147. Therefore, it seems that, in breast carcinoma, the MCT isoform induced by the hypoxic microenvironment is MCT1, since this isoform is present in tumours presenting a hypoxia induced profile, which, in turn, is also associated with the basal-like subtype. Importantly, these findings are relevant for the metabolic characterisation of basal-like breast tumours, and may have important therapeutic implications as new potential targets may arise for this aggressive tumour type.

7.1.3. EFFECT OF MCT INHIBITION IN *IN VITRO* MODELS OF SOLID TUMOURS

As MCTs play a key role in tumour cell viability and, probably, aggressiveness, some groups have performed *in vitro* inhibition studies targeting MCT expression and activity [5,7,34,65,66]. Although with very promising results, these studies are still very scarce and further efforts are needed to support the exploitation of MCTs as therapeutic targets.

Importantly, in this thesis, some *in vitro* studies were performed for the first time in breast cancer cell lines. CHC induced a decrease in total cancer cell biomass, in accordance to results described by others in different *in vitro* models. In the majority of the cell lines, this inhibition was accompanied by a decrease in lactate efflux. MDA-MB-468 cell line, presented the most notable results, with a CHC-induced inhibition of proliferation, accompanied by a dose dependent inhibition of glucose uptake and lactate production. Interestingly, this is a basal-like subtype breast cancer cell line, supporting the important role of MCT1 expression demonstrated in the results obtained with the breast cancer series. However, this was not observed in all cell lines studied and, surprisingly, the most sensitive breast cancer cell line, MDA-MB-231, was negative for MCT1 and maintained the same level of lactate production and glucose consumption. Therefore, a CHC-induced inhibition of proliferation that does not have an impact on cancer cell metabolism suggests an off-target effect of CHC. In fact, CHC has been described as a potent inhibitor of other transporters, such as AE1 [67], but, if those off-target effects of CHC are, in fact, harmful for cancer cells as observed in MDA-MB-231, this CHC lack of specificity could actually be an advantage, by inducing a more effective cancer cell death. However, to correctly infer on the role of MCTs in cancer survival and aggressiveness, more specific inhibitions are needed. One of those more directed strategies is the use of siRNAs, which have already shown promising results in other models [5,7,34], and some preliminary results in the cervical cancer cell line HeLa point in the same direction (data not shown). Also, AstraZeneca MCT1 specific inhibitors currently studied

in immunosupression for allotransplantation also anticipate promising results in the cancer research field.

Additionally, it is important to draw attention to the fact that some compounds described as MCT inhibitors have been pointed out as chemopreventive agents, such as quercetin, NSAIDs and cholesterol synthesis inhibitors (statins) [68-75], as well as effective anti-cancer agents in clinical trials, like lonidamine [76]. In fact, NSAIDs and statins were shown to prevent ER-negative breast cancer [72,74], which are described in this thesis as having an increased MCT1 expression. Hence, MCT1 inhibition may contribute to the chemopreventive and anti-cancer effects of these compounds, and further studies on the additional mechanisms underlying their beneficial properties are needed.

When considering MCTs as targets for therapy, it is imperative to bear in mind and evaluate toxicity to normal tissue. Systemic delivery of MCT inhibitors, more specifically MCT1, will probably affect almost every organ of the body. In skeletal muscle, as MCT1 participates in the lactate shuttle, possible side effects will include muscle fatigue and inability to tolerate moderate- to high-intensity training due to accumulation of lactate and H+ in the extracellular milieu, as oxidative muscle fibers will no longer perform lactate uptake. In the colon, as MCT1 is responsible for butyrate transport in colonic epithelium, MCT1 inhibition may inhibit cell proliferation and proper differentiation [77]. Importantly, as MCT1 is fundamental for T cell activation and, as MCT1 specific inhibition is being studied for immunosupression in transplantation, MCT1 inhibition in the cancer context will likely cause immunosupression and further inhibition of the immune response against cancer cells. However, in practice, MCT1 inhibition may have manageable side-effects like myalgia, asthenia, testicular pain, and gastrointestinal discomfort, with no serious organ toxicity or myelosupression, as observed for lonidamine. Nevertheless, these adverse effects may be decreased or eliminated in association with other therapies [76]. Although MCT1 inhibition is prone to cause some important side-effects, one should never forget that some drawbacks are acceptable if the benefit is justified, as it happens with virtually all drugs.

7.2. CONCLUSIONS AND FUTURE PERSPECTIVES

In general, the results here presented support the hypothesis of a major role of MCTs in the emergence of the hyper-glycolytic and acid-resistant phenotypes, as adaptations to the hypoxic microenvironment. The up-regulation of MCTs in the plasma membrane of colorectal, cervical

and breast cancer cells is most likely an adaptive mechanism to allow continuous high glycolytic rates, by exporting the accumulating end-product, lactate, as well as to counteract acid-induced apoptosis or necrosis. This may not be the case for all tumour types as gastric carcinoma revealed no up-regulation of MCTs. As "aerobic glycolysis" is considered a new hallmark of cancer [78,79], and MCTs are key players in the adaptations to hypoxia and acidosis, further efforts should be made on the exploitation of these proteins, especially MCT1, as therapeutic targets in cancer.

Although much was achieved with this work, many other doors are now open that should be explored. Importantly, characterisation of MCT expression in other tumour series would be of great value, as additional promising results are anticipated. Ovarian carcinoma, which presented high amounts of MCT1 in the tumour screening study, as well as lung cancer that, similarly to gastric carcinoma, presented, in opposition to results from other groups [8], a loss of MCT4 expression, would be two interesting tumour series to proceed with. Additionally, characterisation of MCT expression in tumours that are known to have a different metabolic behaviour, such as prostate tumours, which rely mainly on fatty acid oxidation rather than glycolysis [80], will also be of great interest. Many other tumours, where the contribution of MCTs is still unknown, could benefit from additional metabolic studies with evaluation of MCT expression.

Additional *in vitro* studies with MCT specific inhibition, evaluating parameters of aggressive behaviour, such as migration, invasion and colony formation capacity will shed some light on the true value of MCTs. Indeed, MCT4 has already been associated with migration capacity [40,81]. Also, by studying the possible contribution of MCT inhibition to the chemopreventive and/or anti-cancer effects of compounds such as quercetin, NSAIDs, statins and lonidamine, MCTs may stand out as important molecular players in cancer and promising therapeutic targets. However, one should always bear in mind that the main goal of MCT1 inhibition is the possible clinical application, so, MCT inhibitors should be subjected to experimental an clinical studies.

As an alternative not yet identified chaperone is suggested to be involved in MCT trafficking to the plasma membrane, additional studies assessing protein interactions with MCTs may bring some new knowledge on MCT regulation. For that, databases of protein interactions would be an important tool to explore.

Besides the more obvious lines that can be further explored, other directions on the assessment of the role of MCTs can be taken, such as the study of other metabolic pathways important in cancer, like glutaminolysis (where lactate is also produced), microenvironmental conditions like acidity and hypoxia, and other players in MCT regulation such as, among others,

HIF-1α, AKT, c-myc and, possibly, HPV. Also, other mechanisms by which MCT expression may be controlled, including gene mutations or methylation, could also provide relevant information regarding MCT tumour alterations.

Importantly, since cell culture does not mimic all real tumour conditions, including O_2 and nutrient limitation, key factors in metabolism, it is fundamental to assess the effects of MCT inhibition in animal models, in what concerns tumour survival and aggressiveness. Therefore, *in vivo* studies, evaluating the effect of MCT inhibition in, among others, tumour growth, angiogenesis and metastisation will be essential. In parallel, as MCTs are also important in physiological homeostasis, toxicological studies to determine MCT inhibition side effects will determine the real potential of MCTs as therapeutic targets in cancer.

In conclusion, the results herein presented encourage the exploitation of MCTs, especially MCT1, as potential targets for cancer therapy, and pave the way for further efforts to understand the role of MCTs in solid tumours. Although major advances have been made with the present work, it elicits for many other studies to complement the present knowledge on MCTs' role in tumour survival and aggressiveness.

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APPENDIX I

Atlas of Genetics and Cytogenetics in Oncology and Haematology

SLC16A1 (solute carrier family 16, member 1 (monocarboxylic acid transporter 1))

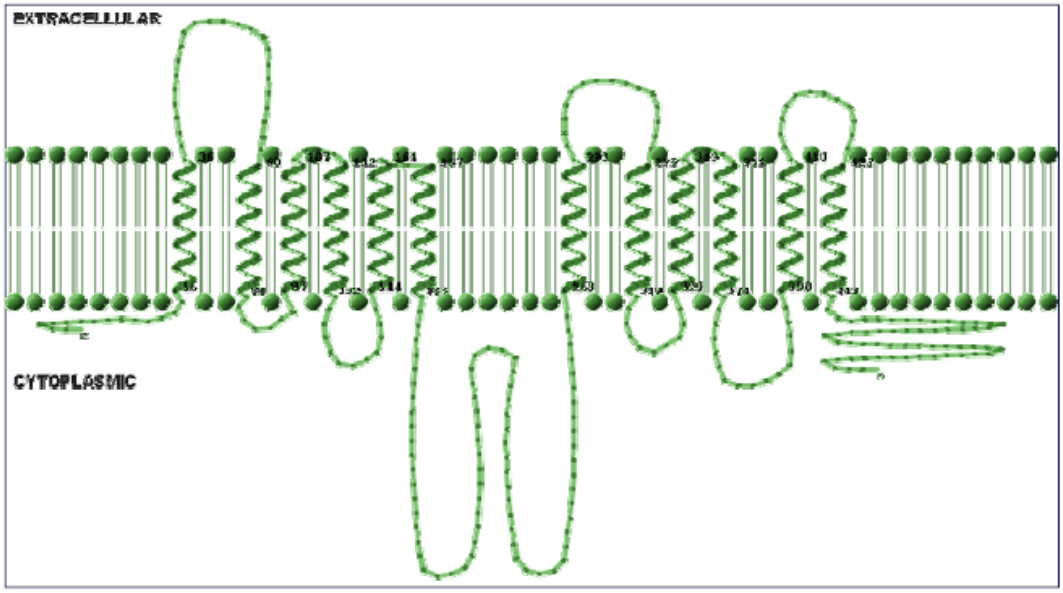
Identity

Other names	FLJ36745 HHF7 MCT MCT1 MGC44475
HGNC (Hugo)	SLC16A1
Location	1p13.2
Location_base_pair	Starts at 113454472 and ends at 113498685 bp from pter (according to hg19-Feb_2009) [Mapping]

DNA/RNA

Note	Human SLC16A1 was firstly cloned in 1994, by Garcia and colleagues. Structural gene organization as well as isolation and characterization of SLC16A1 promoter was achieved in 2002, by Cuff and Shirazi-Beechey.
Description	44507 bp lenght, containing 5 exons. Various SNPs have been described in SLC16A1 gene.
Transcription	6 transcripts have been described for this gene (4 with protein product, 2 with no protein product): SLC16A1-001 (5 exons; 3910 bps transcript length; 500 residues translation length); SLC16A1-002 (5 exons; 2101 bps transcript length; 456 residues translation length); SLC16A1-003 (4 exons; 865 bps transcript length; 215 residues translation length); SLC16A1-004 (2 exons; 452 bps transcript length; no translation product); SLC16A1-005 (4 exons; 1099 bps transcript length; 296 residues translation length); SLC16A1-006 (2 exons; 430 bps transcript length; no translation product).
Pseudogene	1 related pseudogene identified - AKR7 family pseudogene (AFARP1), non-coding RNA.

Protein



Protein diagram drawn following UniProtKB/Swiss-Prot database prediction, using TMRPres2D software.

Description	500 amino acids; 53958 Da; 12 transmembrane domains, intracellular N- and C-terminal and a large intracellular loop between transmembrane domains 6 and 7.
Expression	Ubiquitous.
Localisation	Plasma membrane; also described in rat mitochondrial and peroxisomal membranes.

Function	Catalyses the proton-linked transport of metabolically important monocarboxylates such as lactate, pyruvate, branched-chain oxo acids derived from leucine, valine and isoleucine, and ketone bodies (acetoacetate, beta-hydroxybutyrate and acetate).
Homology	Belongs to the major facilitator superfamily (MFS). Monocarboxylate porter (TC 2.A.1.13) family. SLC16A1 gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, and zebrafish.

Implicated in

Entity	Various cancers
Note	MCT1/SLC16A1 has been described to be upregulated in a variety of tumours.
Disease	High grade glial neoplasms (Mathupala et al., 2004; Fang et al., 2006), colorectal (Koukourakis et al., 2006; Pinheiro et al., 2008), lung (Koukourakis et al., 2007), cervical (Pinheiro et al., 2008), and breast carcinomas (Pinheiro et al., in Press).

Entity	Breast cancer
Prognosis	In breast cancer, MCT1/SLC16A1 was found to be associated with poor prognostic variables such as basal-like subtype and high grade tumours (Pinheiro et al., in Press).
Oncogenesis	SLC16A1 is expressed in normal breast tissue, but is silenced in breast cancer due to gene methylation (Asada et al., 2003).

Entity	Gastric cancer
Note	The prognostic value of CD147 (a MCT1/SLC16A1 and MCT4/SLC16A3 chaperone required for plasma membrane expression and activity) was associated with MCT1/SLC16A1 co-expression in gastric cancer cells (Pinheiro et al., 2009).
Prognosis	Co-expression of MCT1/SLC16A1 with CD147 was associated with advanced gastric carcinoma, Lauren's intestinal type, TNM staging and lymph-node metastasis, in gastric cancer.

Entity	Colorectal carcinoma
Note	MCT1/SLC16A1 has been described to be downregulated in colorectal carcinoma (Lamber et al., 2002).

Entity	Erythrocyte lactate transporter defect
Note	Merezhinskaya et al. (2000) identified two heterozygous transitions in the SLC16A1 gene, in patients with erythrocyte lactate transporter defect: 610A-G transition (resulting in a lys204-to-glu (K204E) substitution in a highly conserved residue) and 1414G-A transition (resulting in a gly472-to-arg (G472R) substitution halfway along the cytoplasmic C-terminal chain). These substitutions are not conserved, but were not identified in 90 healthy control individuals. Erythrocyte lactate clearance in patients with these mutations was 40 to 50% that of normal control values.

Entity	Hyperinsulinemic hypoglycemia familial 7
Note	Otonkoski et al. (2007) identified two heterozygotic alterations in the SLC16A1, in affected members of a Finnish family segregating autosomal dominant exercise-induced hyperinsulinemic hypoglycemia. First, a 163G-A transition in exon 1 located within a binding site for nuclear matrix protein-1 and predicted to disrupt the binding sites of 2 potential transcriptional repressors, and, secondly, a 25-bp insertion at nucleotide -24 introducing additional binding sites for the ubiquitous transcription factors SP1, USF and MZF1. The first variation leads to a 3-fold increase in transcription while the second variation leads to a 10-fold increase in transcription. These mutations were not found in 92 Finnish and German controls.

External links

	Nomenclature
HGNC (Hugo)	SLC16A1 10922
Entrez Gene (NCBI)	SLC16A1 6566 solute carrier family 16, member 1 (monocarboxylic acid transporter 1)
	Cards
Atlas	SLC16A1ID44046ch1p13
GeneCards (Weizmann)	SLC16A1
Ensembl (Hinxton)	ENSG00000155380 [Gene_View] SLC16A1 [Vega]
AceView (NCBI)	SLC16A1
Genatlas (Paris)	SLC16A1
euGene (Indiana)	6566
SOURCE (Stanford)	NM_001166496 NM_003051
Gene Expression (Array Express)	ENSG00000155380
	Genomic and cartography
GoldenPath (UCSC)	SLC16A1 - 1p13.2 chr1:113454472-113498685 - 1p12 [Description] (hg19-Feb_2009)
Ensembl	SLC16A1 - 1p12 [CytoView]

Mapping of homologs : NCBI	SLC16A1 [Mapview]
OMIM	245340 600682 610021
	Gene and transcription
Gene : Genbank (Entrez)	AI656300 AJ438942 AJ438943 AK000641 AK094064
Reference sequence (RefSeq transcript) : SRS	NM_001166496 NM_003051
Reference transcript : Entrez	NM_001166496 NM_003051
RefSeq genomic : SRS	AC_000044 AC_000133 NC_000001 NG_015880 NT_032977 NW_001838594 NW_922462
RefSeq genomic : Entrez	AC_000044 AC_000133 NC_000001 NG_015880 NT_032977 NW_001838594 NW_922462
Consensus coding sequences : CCDS NCBI	SLC16A1
Cluster EST : Unigene	Hs.75231 [SRS] Hs.75231 [NCBI]
Alternative Splicing : Fast-db (Paris)	17105
	Protein : pattern, domain, 3D structure
Protein : UniProt/SwissProt	P53985 (SRS) P53985 (Expasy) P53985 (Uniprot)
With graphics : InterPro	P53985
Splice isoforms : VarSplice FASTA	P53985(VarSplice FASTA)
Domaine pattern : Prosite (SRS)	MFS (PS50850)
Domain pattern : Prosite (Expaxy)	MFS (PS50850)
Domains : Interpro (SRS)	MFS_1 MFS_general_subst_transpt Monocarb_transpt
Domains : Interpro (EBI)	MFS_1 MFS_general_subst_transpt Monocarb_transpt
Related proteins : CluSTr	P53985
Domain families : Pfam SRS	MFS_1 (PF07690)
Domain families : Pfam Sanger	MFS_1 (PF07690)
Domain families : Pfam NCBI	pfam07690
Blocks (Seattle)	P53985
Crystal structure of protein : PDB SRS	
Crystal structure of protein : PDBSum	
Crystal structure of protein : IMB	
Crystal structure of protein : PDB RSDB	
Human Protein Atlas	ENSG00000155380
HPRD	02816
	Protein Interaction databases
DIP (DOE-UCLA)	P53985
IntAct (EBI)	P53985
FunCoup	ENSG00000155380
	Polymorphism : SNP, mutations, diseases
Single Nucleotide Polymorphism (SNP) : dbSNP NCBI	SLC16A1
SNP : GeneSNP Utah	SLC16A1
SNP : HGBase	SLC16A1

Domains : Interpro (EBI)	MFS_1 MFS_general_subst_transpt Monocarb_transpt
Related proteins : CluSTr	P53985
Domain families : Pfam SRS	MFS_1 (PF07690)
Domain families : Pfam Sanger	MFS_1 (PF07690)
Domain families : Pfam NCBI	pfam07690
Blocks (Seattle)	P53985
Crystal structure of protein : PDB SRS	
Crystal structure of protein : PDBSum	
Crystal structure of protein : IMB	
Crystal structure of protein : PDB RSDB	
Human Protein Atlas	ENSG00000155380
HPRD	02816
	Protein Interaction databases
DIP (DOE-UCLA)	P53985
IntAct (EBI)	P53985
FunCoup	ENSG00000155380
	Polymorphism : SNP, mutations, diseases
Single Nucleotide Polymorphism (SNP) : dbSNP NCBI	SLC16A1
SNP : GeneSNP Utah	SLC16A1
SNP : HGBase	SLC16A1
Genetic variants : HAPMAP	SLC16A1
Somatic Mutations in Cancer : COSMIC	SLC16A1
Mutations and Diseases : HGMD	SLC16A1
Hereditary diseases : OMIM	245340 600682 610021
Hereditary diseases : GENETests	245340 600682 610021
Diseases : Genetic Association	SLC16A1
	General knowledge
Homologs : HomoloGene	SLC16A1
Homology/Alignments : Family Browser UCSC	SLC16A1
Phylogenetic Trees/Animal Genes : TreeFam	SLC16A1
Chemical/Protein Interactions : CTD	6566
Keywords Ontology : AmiGO	protein binding membrane fraction plasma membrane plasma membrane pyruvate metabolic process monocarboxylic acid transmembrane transporter activity mevalonate transmembrane transporter activity symporter activity secondary active monocarboxylate transmembrane transporter activity organic anion transport monocarboxylic acid transport mevalonate transport integral to membrane transmembrane transport
Keywords Ontology : EGO-EBI	protein binding membrane fraction plasma membrane plasma membrane pyruvate metabolic process monocarboxylic acid transmembrane transporter activity mevalonate transmembrane transporter activity symporter activity secondary active monocarboxylate transmembrane transporter activity organic anion transport monocarboxylic acid transport mevalonate transport integral to membrane transmembrane transport
Pathways : BIOCARTA	
Pathways : KEGG	

Other databases

Probes

Probes : Imagenes	SLC16A1 Related clones (RZPD - Berlin)

Literature

PubMed	**52 Pubmed reference(s) in Entrez**
PubGene	SLC16A1

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Histopathology In press

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EBI NCBI

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URL : http://AtlasGeneticsOncology.org/Genes/SLC16A1ID44046ch1p13.html

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APPENDIX II

Atlas of Genetics and Cytogenetics in Oncology and Haematology

SLC16A3 (solute carrier family 16, member 3 (monocarboxylic acid transporter 4))

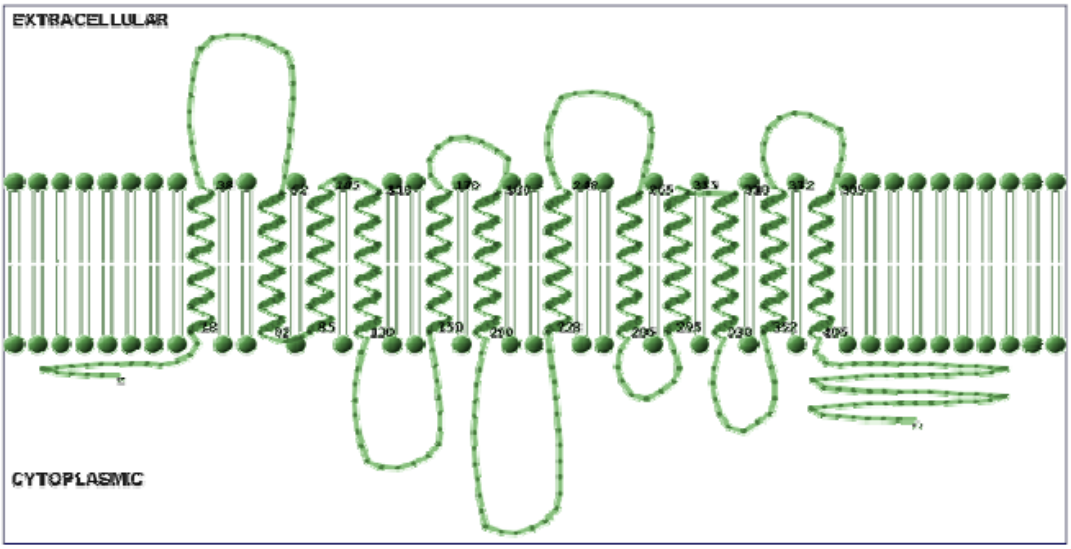
Identity

Other names	MCT3 MCT4 MGC138472 MGC138474
HGNC (Hugo)	SLC16A3
Location	17q25.3
Location_base_pair	Starts at 80186293 and ends at 80197368 bp from pter (according to hg19-Feb_2009)

DNA/RNA

Note	SLC16A3 was first cloned from human circulating blood by Price et al. (1998).
Description	11077 bp lenght, 5 exons.
Transcription	3 transcripts have been described for this gene (all with protein product): SLC16A3-201, (5 exons; 2033 bps transcript length; 465 residues translation length); SLC16A3-202 (4 exons; 4222 bps transcript length; 465 residues translation length); SLC16A3-203 (5 exons; 2054 bps transcript length; 465 residues translation length).

Protein



Protein diagram drawn following UniProtKB/Swiss-Prot database prediction, using TMRPres2D software.

Description	465 residues; 49469 Da; 12 transmembrane domains; intracellular N- and C-terminals.
Expression	SLC16A3/MCT4 is expressed in tissues such as white skeletal muscle fibres, astrocytes, white blood cells, chondrocytes, testis, lung, placenta, heart and some mammalian cell lines (Halestrap and Meredith, 2004; Meredith and Christian, 2008).
Localisation	Plasma membrane.
Function	Proton-linked monocarboxylate transporter. Catalyzes plasma membrane transport of monocarboxylates such as lactate, pyruvate, branched-chain oxo acids derived from leucine, valine and isoleucine, and the ketone bodies acetoacetate, beta-hydroxybutyrate and acetate.
Homology	Belongs to the major facilitator superfamily (MFS). Monocarboxylate porter (TC 2.A.1.13) family. The SLC16A3 gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, and M. grisea.

Implicated in

Entity	Colorectal carcinoma
Note	SLC16A3/MCT4 protein is overexpressed in colorectal cancer (Pinheiro et al., 2008a).
Entity	Cervical cancer
Note	SLC16A3/MCT4 protein is overexpressed in cervical cancer (Pinheiro et al., 2008b). SLC16A3/MCT4 protein overexpression in cervical cancer correlated with positivity for high-risk HPV (Pinheiro et al., 2008b).
Entity	Bladder cancer
Note	SLC16A3 gene expression was upregulated in some bladder tumours and induced by hypoxia in bladder cancer cell lines, but not in cultures of normal urothelium (Ord et al., 2005).
Entity	Breast cancer
Note	Induction was also seen in two breast cancer cell lines. Expression of SLC16A3 gene is higher in breast cancer distant metastasis as compared to primary tumours or regional metastasis. SLC16A3 gene was then included in the 'VEGF profile' of breast cancer, associated with promotion of vessel formation, survival under anaerobic conditions and loss of dependence upon fibroblasts (Hu et al., 2009).
Entity	Ovarian cancer
Note	SLC16A3 gene expression was described to be downregulated in malignant ovarian tumours as compared to normal ovarian surface epithelial cells. Additionally, the non-tumorigenic cell line TOV-81D presented higher expression that tumorigenic cell lines (Wojnarowicz et al., 2008). SLC16A3 gene, among other transporter genes, was differentially expressed in a chemotherapy resistant ovarian cancer cell line and tumour tissue as compared to a chemosensitive cell line and tumour tissue. It was suggested that these transporters might be involved in drug influx/efflux, modulating chemotherapy response (Cheng et al., 2010).
Entity	Mitochondrial myopathy
Note	SLC16A3/MCT4 overexpression was described in a patient with a mitochondrial myopathy (Baker et al., 2001).
Entity	Chronic obstructive pulmonary disease
Note	SLC16A3/MCT4 downregulation was described in the vastus lateralis muscle of patients with chronic obstructive pulmonary disease as compared with healthy controls (Green et al., 2008).

External links

	Nomenclature
HGNC (Hugo)	SLC16A3 10924
Entrez Gene (NCBI)	SLC16A3 9123 solute carrier family 16, member 3 (monocarboxylic acid transporter 4)
	Cards
Atlas	SLC16A3ID44573ch17q25
GeneCards (Weizmann)	SLC16A3
Ensembl (Hinxton)	ENSG00000141526 [Gene_View] SLC16A3 [Vega]
AceView (NCBI)	SLC16A3
Genatlas (Paris)	SLC16A3
euGene (Indiana)	9123
SOURCE (Stanford)	NM_001042422 NM_001042423 NM_004207
Gene Expression (Array Express)	ENSG00000141526
	Genomic and cartography
GoldenPath (UCSC)	SLC16A3 - 17q25.3 chr17:80186293-80197368 + 17q25 [Description] (hg19-Feb_2009)
Ensembl	SLC16A3 - 17q25 [CytoView]
Mapping of homologs : NCBI	SLC16A3 [Mapview]
OMIM	603877
	Gene and transcription
Gene : Genbank (Entrez)	AK127319 AK223040 BC112267 BC112269 CR604145
Reference sequence (RefSeq transcript) : SRS	NM_001042422 NM_001042423 NM_004207

Reference transcript : Entrez	NM_001042422 NM_001042423 NM_004207
RefSeq genomic : SRS	AC_000060 AC_000149 NC_000017 NT_010663 NW_001838459 NW_926918
RefSeq genomic : Entrez	AC_000060 AC_000149 NC_000017 NT_010663 NW_001838459 NW_926918
Consensus coding sequences : CCDS NCBI	SLC16A3
Cluster EST : Unigene	Hs.500761 [SRS] Hs.500761 [NCBI]
	Protein : pattern, domain, 3D structure
Protein : UniProt/SwissProt	O15427 (SRS) O15427 (Expasy) O15427 (Uniprot)
With graphics : InterPro	O15427
Splice isoforms : VarSplice FASTA	O15427(VarSplice FASTA)
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Domain pattern : Prosite (Expaxy)	MFS (PS50850)
Domains : Interpro (SRS)	MFS_1 MFS_general_subst_transpt Monocarb_transpt
Domains : Interpro (EBI)	MFS_1 MFS_general_subst_transpt Monocarb_transpt
Related proteins : CluSTr	O15427
Domain families : Pfam SRS	MFS_1 (PF07690)
Domain families : Pfam Sanger	MFS_1 (PF07690)
Domain families : Pfam NCBI	pfam07690
Blocks (Seattle)	O15427
Crystal structure of protein : PDB SRS	
Crystal structure of protein : PDBSum	
Crystal structure of protein : IMB	
Crystal structure of protein : PDB RSDB	
Human Protein Atlas	ENSG00000141526
HPRD	04854
	Protein Interaction databases
DIP (DOE-UCLA)	O15427
IntAct (EBI)	O15427
FunCoup	ENSG00000141526
	Polymorphism : SNP, mutations, diseases
Single Nucleotide Polymorphism (SNP) : dbSNP NCBI	SLC16A3
SNP : GeneSNP Utah	SLC16A3
SNP : HGBase	SLC16A3
Genetic variants : HAPMAP	SLC16A3
Somatic Mutations in Cancer : COSMIC	SLC16A3
Mutations and Diseases : HGMD	SLC16A3
Hereditary diseases : OMIM	603877
Hereditary diseases : GENETests	603877

Diseases : Genetic Association	SLC16A3
	General knowledge
Homologs : HomoloGene	SLC16A3
Homology/Alignments : Family Browser UCSC	SLC16A3
Phylogenetic Trees/Animal Genes : TreeFam	SLC16A3
Chemical/Protein Interactions : CTD	9123
Keywords Ontology : AmiGO	membrane fraction plasma membrane integral to plasma membrane pyruvate metabolic process monocarboxylic acid transmembrane transporter activity symporter activity secondary active monocarboxylate transmembrane transporter activity organic anion transport monocarboxylic acid transport transmembrane transport
Keywords Ontology : EGO-EBI	membrane fraction plasma membrane integral to plasma membrane pyruvate metabolic process monocarboxylic acid transmembrane transporter activity symporter activity secondary active monocarboxylate transmembrane transporter activity organic anion transport monocarboxylic acid transport transmembrane transport
Pathways : BIOCARTA	
Pathways : KEGG	
	Other databases
	Probes
Probes : Imagenes	SLC16A3 Related clones (RZPD - Berlin)
	Literature
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