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Isabel Cristina Pinto Valença

O papel da dinâmica peroxissomal no desenvolvimento de cancro

Unraveling the role of peroxisome dynamics in cancer development

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Thesis submitted at University of Aveiro to fulfil the requirements to obtain the Doctor degree in Biomedicine, held under the scientific guidance of Dr. Daniela Ribeiro, Assistant Researcher at the Department of Medical Sciences from University of Aveiro, Dr. Valdemar Máximo, Assistant Professor at the Faculty of Medicine from University of Porto and Dr. Georg Luers, Assistant Researcher at the Department of Experimental Medicine from University Hospital Hamburg Eppendorf

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resumo

Os peroxissomas são organelos intracelulares multifuncionais, dinâmicos e essenciais para a saúde e desenvolvimento humano.

Ao longo dos anos, vários estudos têm mostrado uma associação direta entre os peroxissomas e diferentes tipos de cancro. O cancro da próstata (PCa) apresenta um perfil metabólico exclusivo. Em contraste com a maioria das células cancerígenas, que usam glucose com a principal fonte de energia, o PCa, em estadios iniciais, consome pequenas taxas de glucose e os lípidos são a principal fonte de energia, sendo a β-oxidação indicada como a via bioenergética dominante nas células do PCa.

O transportador de monocarboxilatos 2 (MCT2), um transportador de membrana tipicamente associado ao metabolismo da glucose, foi mostrado estar sobre-expresso e deslocalizado em tecidos do PCa. O objetivo deste trabalho foi compreender o papel do MCT2 no PCa. Os nossos resultados demonstram que o MCT2 está localizado nas membranas peroxissomais das células do PCa, sugerindo um possível papel nos mecanismos relacionados com o peroxissoma na transformação maligna da próstata, provavelmente associado a um aumento das taxas da β-oxidação. Os nossos resultados também demonstram que o PCa se apodera da maquinaria de transporte membranar peroxissomal para direcionar o MCT2 para os peroxissomas. Além disso, o papel importante deste organelo, nos estadios iniciais do PCa, é suportado pela observação do aumento do importe de proteínas peroxissomais da matriz e da membrana, para potenciar as suas capacidades metabólicas, assim como pela observação do aumento do transporte de ácidos gordos ramificados e sua degradação para a produção de energia. Os nossos dados claramente mostram que o MCT2 está diretamente associado com as alterações da morfologia e número dos peroxissomas no PCa.

Para além disso, os nossos resultados demonstram que o MCT2 promove a migração e proliferação do PCa e que, notavelmente, a localização peroxissomal do MCT2 é essencial para a proliferação do PCa.

Os nossos resultados também indicam que o MCT2 está localizado nos peroxissomas do cancro do fígado e do colo do útero, sugerindo um possível papel nestes cancros.

De um modo geral, os nossos resultados realçam a importância da interação entre os peroxissomas e o MCT2 no PCa e abrem um leque de possíveis alvos para a sua terapia.

keywords Peroxisomes, MCT2, prostate cancer

abstract

Peroxisomes are multifunctional and highly dynamic intracellular organelles, essential for human health and development.

Over the years, several reports showed a direct association between peroxisomes and different types of cancer. Prostate cancer (PCa) displays an exclusive metabolic profile. In contrast with most cancer cells, that use glucose as main energy source, PCa in early stages consumes low rates of glucose and the lipids are the main energy source, being β-oxidation pointed as the dominant bioenergetic pathway in PCa cells.

The monocarboxylate transporter 2 (MCT2), a membrane transporter typically associated with glucose metabolism, was shown to be overexpressed and mislocalized in PCa tissues. The aim of this work was to understand the role of MCT2 in PCa. Our results demonstrate that MCT2 localizes at the peroxisomal membranes in PCa cells and suggest a possible role for peroxisome-related mechanisms in prostate malignant transformation, likely associated with increased β-oxidation rates. We have also shown that PCa takes advantage of the peroxisomal membrane transport machinery to target MCT2 to peroxisomes. Furthermore, the important role of this organelle in the early stages of PCa is supported by the observations of increased import of peroxisomal matrix and membrane proteins to potentiate their metabolic capacity, as well as the increased transport of branched fatty acids and their degradation for energy production. Our data clearly show that MCT2 is directly associated with changes in peroxisomal morphology and number in PCa.

Furthermore, our results showed that MCT2 promotes PCa migration and proliferation and, remarkably, that MCT2's peroxisomal localization is essential for PCa proliferation.

Moreover, our results indicate that MCT2 is localized at peroxisomes in liver and cervix cancer, suggesting a putative role in these cancers.

Altogether, our results highlight the importance of the interplay between peroxisomes and MCT2 in PCa, exposing a range of possible targets for its therapy.

List of Abbreviations

ACAA1	3-ketoacyl-CoA thiolase
ACBD5	acyl-CoA-binding domain-containing protein 5
Acetyl-CoA	acetyl coenzyme A
ACOT	acyl-coenzyme A thioesterase
ACOXs	acyl-CoA oxidases
Acyl-CoA	long-chain fatty acid-Acyl-CoA
ALDP	adrenoleukodystrophy protein
ALDR	Adrenoleukodystrophy-related protein
ALOX15	15-lipoxygenase-1
AMACR	alpha-methylacyl-CoA racemase
ATP	adenosine triphosphate
ATP5A	ATP synthase subunit alpha
ATP5B	ATP synthase subunit beta
BCFA	branched-chain fatty acids
BrdU	5-bromo-2´-deoxyuridine
BT	benign tissue
CACT	carnitine/acylcarnitine translocase
CAFS	cancer-associated fibroblasts
CAT	catalase
cDNA	complementary DNA
CF	cytosolic and microsomal subcellular fraction
CoA	coenzyme A
COXIV	cytochrome c oxidase subunit 4
CPT	carnitine palmitoyl transferase
CRAT	carnitine acetyltransferase
CROT	carnitine octanoyltransferase
DAB	3'-diaminobenzidine
DBP	D-bifunctional protein
DCA	dicarboxylic acids
DHCA	dihydroxycholestanoic acid
DLP1	dynamin-like protein
DNA	deoxynucleic acids
DNA	deoxyribonucleic Acid
EDTA	ethylenediamintetraacetic acid
ER	endoplasmic reticulum
$FADH_2$	reduced form of flavin adenine dinucleotide
FIS1	mitochondrial fission factor 1

h	hour
H_2O_2	hydrogen peroxide
HER2	human epidermal growth factor receptor 2
HRP	horse radish peroxidase
HSDL2	hydroxysteroid dehydrogenase-like 2
IHC	immunohistochemistry
LBP	L-bifunctional protein
LCFAS	long-chain acyl-CoA synthetase
LONP2	lon protease 2
LOOH	alkyl peroxides
MCTs	monocarboxylate transporters
MFF	mitochondrial fission factor
min	minutes
MIRO1	mitochondrial Rho GTPase 1
MOPS	3-(N-morpholino)propanesulfonic acid
mPTS	membrane targeting signal
MT	metastatic tissue
NADH	nicotine adenine dinucleotide
NNT	non-neoplastic tissue
NO	nitric oxide
OH	hydroxyl radical
OXPHOS	oxidative phosphorylation
PBD	peroxisome biogenesis disorders
PBS	phosphate buffered saline
PCa	prostate cancer
PEDs	peroxisomal enzyme deficiencies
PF	peroxisomal-enriched subcellular fraction
PIN	prostatic intraepithelial neoplasia
PMPs	peroxisomal membrane proteins
PPARs	proliferator-activated receptors
PTSs	peroxisome targeting signal
RCDP	rhizomelic chondrodysplasia puntacta
RNA	ribonucleic acid
ROS	reactive oxygen species
SCPx	sterol carrier protein X
SEM	standard error of mean
siRNA	small interference RNA
TCA	tricarboxylic acid
TH	total homogenate
THCA	trihydroxycholestanoic acid

TMAs	tissue microarray blocks
TOM20	mitochondrial import receptor subunit
TT	prostate tumour tissue
TUB	tubulin
VDAC1	voltage-dependent anion-selective channel protein 1
VLCFA	very long-chain-fatty acids
VLCFAS	very long-chain acyl-CoA synthetase
ZIP	zinc transporters
µm	micrometer

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Legal considerations

The author declares that part of the results presented in this thesis were published under the name of Valença I., and that she has participated in the planning and execution, as well as in the preparation and interpretation of the data.

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1. General Introduction

1.1. Peroxisomes

Peroxisomes are cytoplasmic organelles, with 0.1–1 μm of diameter enclosing a dense proteinaceous matrix bounded by a single membrane. Peroxisomes were discovered in mouse kidney by Rodin J. in 1949 [1]. Although initially called microbodies, further biochemical studies revealed the presence of several oxidases involved in hydrogen peroxide (H_2O_2) production and degradation, leading to the actual name “peroxisome” [2]. Peroxisomes are found in almost all eukaryotic cells, with exception of erythrocytes and sperm cells [3].

1.1.1. Peroxisome biogenesis

After decades of intense research on peroxisome biogenesis, it is now accepted that peroxisomes arise both from growth and division of existing peroxisomes and *de novo* formation [4, 5]. For over 20 years peroxisomes were considered autonomous organelles that arose from pre-existing peroxisomes through growth and division, importing newly synthesized matrix and membrane proteins directly from the cytosol [6, 7]. According to this model, a certain stimulus is followed by an elongation, constriction, and final peroxisomal fission. PEX11β promotes the deformation and elongation of the peroxisomal membrane [8], recruiting mitochondrial fission protein 1 (FIS1) and mitochondrial fission factor (MFF) to the membrane-constricted regions, which in turn recruit dynamin-like protein 1 (DLP1). Together these proteins promote the fission during peroxisomal division [7, 9, 10]. This model was supported by the evidence of dumbbell-shaped interconnected structures that were more abundant when peroxisome proliferation was induced by pharmacological means or by partial hepatectomy [11, 12] . However, in yeast and mammals’ mutant cells lacking some specific proteins involved in peroxisome biogenesis, only non-functional remnant peroxisomes designated ghosts can be observed [13, 14]. Remarkably, re-expression of these proteins is enough to generate new peroxisomes, showing that peroxisome biogenesis could occur *de novo* [13]. According to the *de novo* generation theory, peroxisomes are semiautonomous organelles, whose membrane is derived from the endoplasmic reticulum (ER) and matrix proteins are post-translationally imported into peroxisomes after being synthesized on free ribosomes, not associated with ER [15, 16]. Some studies have shown numerous contacts between peroxisomes and smooth ER, suggesting that peroxisomes arise from dilated regions of this organelle [17, 18]. Van der Zand *et al* suggested the existence of pre-peroxisomal vesicles, precursors of peroxisomes, which bud from the ER [18]. Moreover, it was shown that

peroxisomes can form *de novo*, even in cells lacking pre-existing peroxisomes upon re-introduction of the PEX16 [19]. Recently, an essential role for mitochondria was found in the *de novo* generation of peroxisomes in mammalian cells [20] . In the absence of peroxisomes, mitochondria were able to emerge PEX3 and PEX14-containing vesicles that import some peroxisomal proteins forming pre-peroxisomal vesicles that fuse with ER PEX16-containing vesicles to form mature peroxisomes [20].

1.1.2. Peroxisomal protein import

Peroxisomal proteins are targeted to peroxisomes by distinct machineries, depending on their localization. The transport of matrix proteins to peroxisomes is highly selective and mediated by specific import sequences, the peroxisomal targeting sequences (PTSs). This well characterized pathway involves four consecutive steps: recognition via PTSs by receptors, protein targeting to peroxisomal membrane, translocation of protein across peroxisomal membrane and receptor recycling [21]. Peroxisomal proteins are recognized by their receptors via two different PTSs. Most proteins that are targeted to the peroxisomal matrix contain a C-terminal peroxisomal targeting sequence PTS1, a tripeptide SKL, and are recognized by PEX5 [22, 23] at the cytoplasm. However, some other matrix proteins contain an internal peroxisomal targeting sequence PTS2, a nonapeptide RLx5HL, near the N-terminal of the protein and are recognized by PEX7 [24, 25]. After recognition, the complexes formed between the protein and the respective receptor travel to the peroxisomal membrane (with support of PEX5 in case of PEX7), docking onto the peroxisomal docking complex, composed by PEX13 and PEX14 (in mammals) [21, 26, 27]. Once docked, the matrix protein is translocated across the peroxisomal membrane into the peroxisomal matrix and the PEX5 is released to the cytosol to be recycled for the next targeting cycle [21, 28].

Proteins lacking the PTS signals can also be imported into peroxisomes. They can bind directly to the N-terminal of PEX5 or by piggyback import, through association with a protein containing the PTS signal [29–31].

The transport of peroxisomal membrane proteins (PMPs) is not as well characterized as the transport of peroxisomal matrix proteins. The class I PMPs contain a peroxisomal membrane targeting signal (mPTS), a positive charged and hydrophobic region, that is recognized by the import receptor, PEX19 [32–34].

PEX19 acts as a bifunctional chaperone/import receptor that binds and stabilizes newly synthesized PMPs in the cytosol, being essential for PMPs targeting and import to peroxisome [32]. After the formation of PEX19/PMP complex in the cytosol, PEX19 docks on PEX3 at the peroxisomal membrane, forming a trimeric complex that promotes the release of the PMP [35,

36]. The PMPs without a mPTS recognition motif belong to the class II PMPs and their targeting is independent of PEX19 [32]. According to several studies, class II PMPs are inserted into the ER membrane prior to their transit to peroxisomes [14, 37]. Although the mechanisms that mediate their insertion into the ER bilayer are not yet clear, it was shown, in mammals and yeast, that proteins such as PEX16, PEX22 and PEX3 are exported from the ER in a vesicle-mediated transport [14, 37, 38]. However, it was reported PEX19 is required for PEX3 release from the ER, and PEX16 is needed for its docking to the peroxisomal membrane [39–41].

1.1.3. Peroxisomal metabolism

Peroxisomes are involved in a wide range of metabolic pathways, being essential for human health and development. Although peroxisomes are present in almost all eukaryotic cells, their functions may vary depending on protein content, which diverges across species. However, oxidation of fatty acids and H_2O_2 degradation by catalase (CAT) are common functions to all organisms [42–46]. In general, peroxisomes are involved in processes of biosynthesis, degradation and signaling. Focusing on animals, it is known that peroxisomes are involved in several metabolic pathways including α-oxidation of fatty acids, glyoxylate detoxification, synthesis of ether phospholipids and bile acids, antiviral defense and signaling and, most notably, β-oxidation of fatty acids and metabolism of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [47–51].

1.1.3.1. Degradation of fatty acids

Fatty acids can be acquired by dietary uptake and/or through biosynthesis. Cells use fatty acids and their coenzyme A (CoA) esters in multiple processes, as components of cell membranes, carbon source (triacylglycerols), enzyme and membrane channels regulators, ligands of nuclear receptors, precursor molecules for hormones, signaling molecules and sources for energy production [52]. Depending on their composition, fatty acids can undergo different degradation pathways (α-, β- or ω-oxidation) in three distinct organelles (peroxisomes, mitochondria and ER) [52, 53].

In yeasts and plants, fatty acid β-oxidation occurs exclusively in peroxisomes. However, in higher eukaryotes, β-oxidation occurs in both peroxisomes and mitochondria [45, 54]. Peroxisomes and mitochondria share the catalytic mechanism of chain shortening of fatty acids between carbons 2 and 3, through a sequential cycle of oxidation, hydration, dehydrogenation and thiolytic cleavage [45, 54, 55]. However, the enzymes involved in these steps have distinct specificities and are encoded by different genes, with the exceptions of 3-

hydroxy-3-methylglutaryl-CoA lyase and alpha- methylacyl-CoA racemase (AMACR). The initial step of dehydrogenation is also different between these organelles: in peroxisomes, the reduced form of flavin adenine dinucleotide ($FADH_2$) is reoxidized, producing H_2O_2, while in mitochondria, its reoxidation is coupled to the electron transport chain to produce ATP [56].

It is known that at least three types of fatty acids rely fully on peroxisomal β-oxidation: the fatty acids composed by 22 carbons or more, known as very long chain fatty acids (VLCFAs), the 2-methyl branched-chain fatty acids (BCFA), such pristanic acid, bile acid synthesis intermediates dihydroxycholestanoic acid (DHCA) and trihydroxycholestanoic acid (THCA) and long-chain dicarboxylic acids (DCA) [57]. In contrast, long chain, medium chain, and short chain fatty acids with 18 carbons or less are degraded in mitochondria [57].

To be degraded by peroxisomes, fatty acids undergo several processes, including activation, import to peroxisomes, α-oxidation/racemization (in case of phytanic acid), β-oxidation and export from peroxisomes, which will be discussed in the following sub-chapters and are depicted in Fig.1.

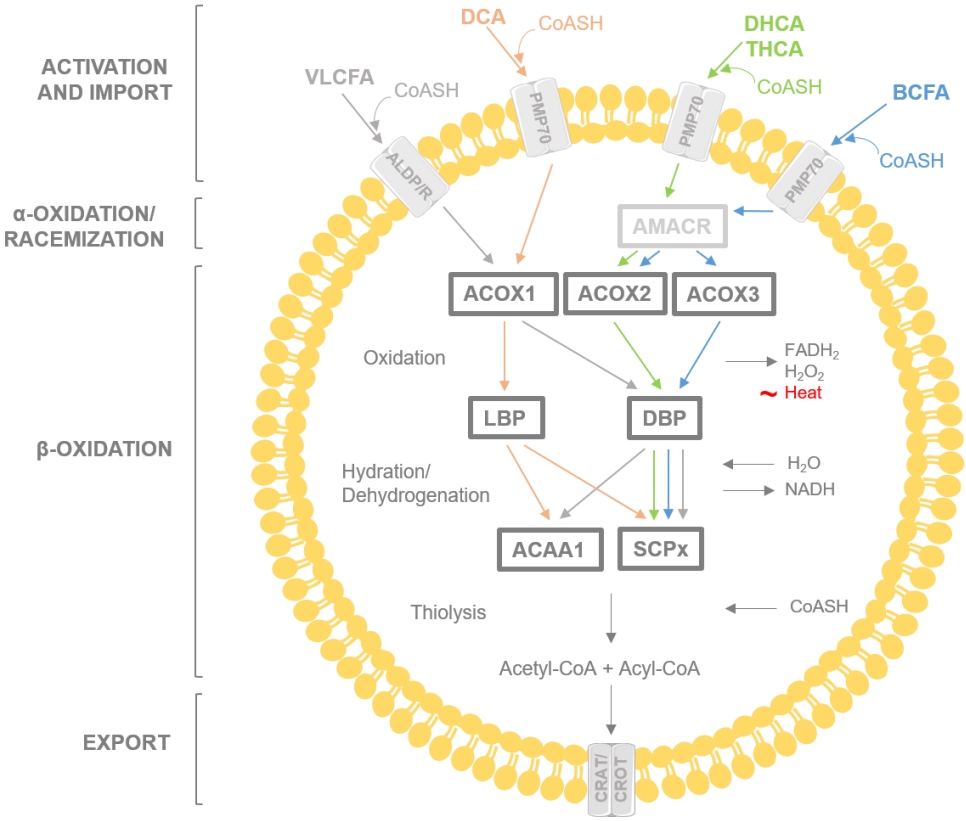

Figure 1. Peroxisomal degradation of fatty acids in mammals.

1.1.3.1.1. Peroxisomal fatty acids activation and import

Before degradation at peroxisomes, fatty acids must be activated to its CoA derivative by one of the two acyl-CoA synthetases that are present at the organelle's membrane: a long-chain acyl-CoA synthetase (LCAS) for long-chain fatty acids activation and a very long-chain acyl-CoA synthetase (VLCAS) for very long-chain fatty acids activation [58–60]. Due to the variety of fatty acids, peroxisomes display three different proteins that able to transport fatty acids across their membrane: adrenoleukodystrophy protein (ALDP), adrenoleukodystrophy-related protein (ALDPR) and PMP70. ALDP and ALDPR are associated with the to the transport VLCFA-CoA, being ALDP more linked with saturated VLCFA and ALDPR with unsaturated VLCFA [61–63]. The transport of BCFAs, DCAs and D/THCAs seem to be operated by PMP70 [63, 64] (Fig.1).

1.1.3.1.2. Peroxisomal fatty acids β-oxidation

After fatty acids activation and import, β-oxidation begins with an oxidation reaction, with exception of the 3-methyl branched fatty acids. This group of fatty acids requires an initial α-oxidation, in which the terminal carboxyl group is removed, leading to the production of a 2-methyl branched-chain fatty acid, which can be degraded by β-oxidation. (e.g., phytanic acid undergoes α-oxidation and is converted into pristanic acid, which can then undergo β-oxidation [65, 66]). Racemization is also required, since the first enzymes in the β-oxidation cycle are stereospecific for S-isoforms of their substrates. As the 2-methyl branched fatty acyl-CoAs resulting from α-oxidation, display both R- and S-isoforms and all bile acid intermediates are in the R-orientation, AMACR is required to convert all substrates into their S-orientation [66, 67].

Depending on fatty acid composition, two distinct pathways can be activated: the classical peroxisome proliferator-inducible pathway, for the degradation of straight-chain substrates, and the non-inducible pathway for branched-chain substrates [68]. In both pathways acyl-CoA is desaturated to a 2-trans-enoyl-CoA, in a FAD-dependent manner, leading to H_2O_2 production. Since peroxisomes are not associated to a respiratory chain, the electrons from $FADH_2$ are transferred directly to O_2, producing H_2O_2 and energy in form of heat (not ATP, as in mitochondria) [6, 56]. In the oxidation step, three acyl-CoA oxidases (ACOXs) have been associated, ACOX1, ACOX2 and ACOX3. These enzymes are flavoproteins that differ in substrate range, properties, and tissue distribution [52, 65, 68, 69]. ACOX1, initially known as palmitoyl-CoA oxidase, can only desaturate straight-chain substrates and is expressed in most tissues. ACOX2, also known as branched-chain acyl-CoA oxidase, is ubiquitously expressed and desaturates 2-methylacyl-CoAs, being cholestanoyl-CoA and pristanoyl-CoA its main

substrates. ACOX3, originally named pristanoyl-CoA oxidase, also desaturates 2-methylacyl-CoAs and it is highly expressed in rat liver, although some studies have shown that apparently this is not the case for humans and mice [70, 71]. Recently, ACOX3 has been associated to prostate cancer (PCa), displaying high levels of expression comparatively to non-tumour cells [69]. Although both ACOX2 and ACOX3 degrade 2-methylacyl-CoAs, the substrate recognition is stereospecific, where ACOX2 only recognizes 2-S-methylacyl-CoAs [72, 73]. The energy produced in the oxidation step is conserved in the form of the high energy level electrons of the reduced form of nicotinamide adenine dinucleotide (NADH). Posteriorly, hydration and dehydrogenation reactions occur, where the unsaturated intermediate is firstly converted to L-3-hydroxyacyl-CoA or D-3-hydroxyacyl-CoA and then is dehydrogenated to a 3-ketoacyl-CoA, through L- or D- bifunctional proteins (LBP or DBP), respectively. Finally, a thiolytic cleavage, where two thiolases, 3-ketoacyl-CoA thiolase (ACAA1) and sterol carrier protein X (SCPx), convert 3-ketoacyl-Coa in acetyl-CoA and acyl-CoA two carbon atoms shorter than the original molecule (Fig.1). Depending on the substrate structure, this process occurs as many times as required for the complete fatty acid degradation [45, 52].

1.1.3.1.3. Peroxisomal fatty acids export

The final products of β-oxidation can have different destinations. After β-oxidation, substrates can be targeted to mitochondria for ATP production, fatty acids biosynthesis in peroxisomes, phospholipid biosynthesis in the ER, bile acids biosynthesis or excreted via blood/urine [71]. Focusing on the targeting to mitochondria, acyl-CoA and acetyl-CoA esters can be converted in carnitines and exported from peroxisomes through carnitine acetyltransferase (CRAT), with specificity for short-chain acetyl-CoA esters, and through carnitine octanoyltransferase (CROT) in case of medium-/long-chain acyl-CoA esters [74]. These acylcarnitines can be incorporated into mitochondria, for ATP production, by the action of several proteins, including carnitine palmitoyl transferase 1 (CPT1), the carnitine/acylcarnitine translocase (CACT) and carnitine palmitoyl transferase 2 (CPT2) [75]. Also, acyl-CoA and acetyl-coA esters can be cleaved by the acyl-CoA thioesterases 4 and 8 (ACOT4/8), releasing free fatty acids that after peroxisomal export can be incorporated into mitochondria [76].

1.1.3.2. Peroxisomal ROS/RNS metabolism

Peroxisomes, as multifunctional organelles, play a role in several metabolic pathways. However, their contribution in various cellular processes has its costs, since many of the enzymes involved in these pathways produce ROS, each time a catalytic cycle in completed.

The degradation of fatty acids, glyoxylate detoxification, amino acid catabolism, and polyamine oxidation involve the action of different peroxisomal oxidases, leading to H_2O_2 or superoxide ($O_2^{\bullet-}$) production. H_2O_2 at low concentrations can act as secondary messenger in several cellular processes, including cell division, differentiation, and migration [77]. However, when it is decomposed into the highly reactive hydroxyl radical ($^{\bullet}OH$) or alkyl peroxides (LOOH), it is extremely destructive for the cell. Other peroxisomal oxidases also lead to production of $O_2^{\bullet-}$ and nitric oxide ($^{\bullet}NO$), that can be converted in peroxynitrite ($ONOO^-$). To avoid the destructive effects of these molecules, peroxisomes also contain various antioxidant enzymes, glutathione peroxidase, peroxiredoxin 5, glutathione S-transferase kappa 1, epoxide hydrolase 2, superoxide dismutase 1 and CAT [78–80]. The main function of CAT is H_2O_2 degradation, being targeted to peroxisomes via a modified PTS1 (KANL) to degrade H_2O_2 and may act in cytosol to degrade extra-peroxisomal ROS [81, 82].

1.1.4. Peroxisome dynamics

Peroxisomes are versatile and highly dynamic organelles that, in the presence of a cellular and/or environmental stimulus, respond with rapid modifications of their size, number, morphology and function [83–86]. In response to intra- or extracellular stimuli, there is a regulation of the expression of several genes coding for peroxisomal proteins, allowing an adaptation of peroxisome proliferation and degradation rates.

1.1.4.1. Peroxisome proliferation

Peroxisome proliferation is induced when certain stimuli initiate signaling cascades that culminate with the expression of peroxisomal genes. In mammals, peroxisome proliferation is highly associated with the activation of nuclear peroxisome proliferator activated receptor (PPAR) α, by peroxisome proliferators [45, 87]. Despite this mechanism being highly controlled by PPAR, several other compounds, environmental factors or stimuli, which appear to be independent of this nuclear receptor can also induce peroxisome proliferation, including extracellular signals such as ROS, growth factors, arachidonic acid and ultraviolet light [80, 87].

1.1.4.2. Peroxisome motility and heritance

Studies concerning peroxisome motility in yeast and plants revealed a mechanism based on actin and myosin filaments [88]. However, evidences showed that mammalian peroxisomes move bidirectionally along microtubules, with the support of both kinesin and dynein motors

[88, 89]. Firstly it was assumed that peroxisomes could be shared between daughter cells by simple portioning of the mother cell, in a stochastic event [90, 91]. Currently, it is known that peroxisome motility, distribution and heritance are well-organized events, where peroxisomes occupy specific intracellular localizations during cell division using microtubules to align them at the mitotic spindle [92]. Recently, it was found that the mitochondrial Rho GTPase 1 (MIRO1) is also involved in peroxisome distribution and motility. It was reported that MIRO1 supports membrane dynamics by providing directionality [89].

1.1.4.3. Peroxisome degradation

The half-life of mammalian peroxisomes is approximately 1.5–2 days, suggesting that biogenesis and degradation of peroxisomes are dynamic and concerted processes, whose balance is essential for peroxisome homeostasis. In mammals, three mechanisms for peroxisome degradation have been described: pexophagy, proteolysis by peroxisomal Lon protease 2 (LONP2), and 15-lipoxygenase-1 (ALOX15)-mediated autolysis [93]. Pexophagy is a selective autophagy process and seems to be the main mechanism for peroxisome degradation, being the responsible for 70-80% of liver peroxisomes degradation [94]. This mechanism involves six sequential steps, including initiation, membrane nucleation and phagophore formation, phagophore elongation and cargo sequestration, formation of autophagosome (phagophore closure), formation of autolysosome (fusion of the autophagosome with the lysosome) and degradation in the autolysosome [95]. The contribution of pexophagy to homeostasis is crucial, since it prevents the accumulation of functionally compromised peroxisomes and maintains redox balance by removing excess or damaged peroxisomes [48, 96].

1.1.5. Peroxisomes in disease

Since peroxisomes play an essential role in several cellular catabolic and anabolic pathways, important for human health and development, their malfunctioning results in severe abnormalities which are sometimes lethal. Mutations in the genes encoding for peroxisomal proteins leads to several peroxisomal disorders, where severity is dependent of the affected genes. Peroxisomal disorders are divided in two distinct groups; peroxisome biogenesis disorders (PBDs) and single peroxisomal enzyme deficiencies (PEDs). PBDs are characterized by the absence of peroxisomes or by ghost peroxisomes (empty membrane compartments), including Zellweger spectrum disorders, rhizomelic chondrodysplasia puntacta type 1(RCDP1) and type 5 (RCDP5) and the peroxisomal fission defects [97]. Zellweger spectrum disorders result from mutations in PEX genes involved in peroxisomal

biogenesis, being mutations in PEX3, PEX16 and PEX19 that cause the most severe phenotype [97]. Mutations in PEX7 and PEX5 are associated with RCDP1 and RCDP5 respectively. A number of patients have been reported with mutations in the peroxisomal fission machinery genes, including DLP1, MFF and PEX11β. Regarding PEDs, numerous severe clinical aberrations were described, being associated with mutations in peroxisomal matrix enzymes as well as peroxisomal membrane proteins involved in metabolite transport, including ACOX1, DBP, AMACR, CAT (acatalasemia), SCPx, ALDP (X-linked adrenoleukodystrophy), PMP70, among others [71, 97].

Besides peroxisomal disorders, several studies have associated peroxisomes with other pathologies, including vitiligo [98], amyotrophic lateral sclerosis [99], schizophrenia [99], viral infections [100–102] and age-related disorders [103], including obesity, hypertension, type 2-diabetes, Alzheimer's, Parkinson's disease and cancer [99, 103–105].

1.2. Peroxisomes and cancer

Over the years, several studies showed evidences of a direct association between peroxisomes and several types of cancer, including breast, colon, thyroid, colorectal, liver, brain, bladder, kidney, ovarian and prostate cancer [105, 106]. Since metabolic reprogramming is one of the major hallmarks of cancer, it would be expected that peroxisomes, as metabolic organelles, would be involved in cancer [107].

The specific role of peroxisomes in cancer is still far from being understood. Some tumours seem to be favoured by the absence of peroxisomes, however, evidences have grown showing that many cancers can take advantage of several peroxisomal processes, including peroxisome biogenesis and degradation, ROS metabolism, transport machinery, crosstalk between peroxisomes and other organelles, fatty acids oxidation and biosynthesis of ether phospholipids [105, 106].

The first evidences reported the absence of peroxisomes associated with an imbalance in ROS metabolism, leading to malignant transformation. In colon carcinoma, it was observed a reduced number of peroxisomes as well as reduced peroxisomal protein abundance (CAT, PMP70 and PMP22) and enzymes activities (CAT, ACOX1), comparatively to benign tissues [108–111]. The same pattern was observed in breast, renal cell and hepatocellular carcinomas [111–114]. Intriguingly, the mRNA levels of these enzymes were stable in colon carcinomas, suggesting that this discrepancy is due to an incompetence in peroxisomes biogenesis or an increase of protein degradation [109]. According to Walter *et al,* the absence of peroxisomes in some cancers might be explained by their degradation in hypoxic conditions [115].

Intriguingly, Cai *et a*l, suggested that peroxisomes are indispensable for the viability of liver cancer in xenografts, since the loss of peroxisome function leads to a mislocalization of CAT to the cytosol, leading to an increase of ROS levels and consequently cell death [116]. Contradictory results were reported showing that cytosolic CAT, resulting from reduced import, provides protection for the redox balance of the cell, comparatively with peroxisomal CAT [81, 82]. The fact that most studies showing the absence of peroxisomes in several tumours rely on CAT detection creates controversy since, in specific conditions such oxidative stress, CAT can localize in the cytosol, hindering peroxisome detection [81].

The import of peroxisomal matrix proteins seems to be also associated with many cancers [117]. The reduced PEX5 levels seen in mulibrey nanism, that are associated with defects in PEX5 ubiquitination that prevents its proteasomal degradation, decrease the peroxisomal matrix protein import, creating an imbalance in the peroxisomal ROS quenching machinery that might increase the risk of cancer [117, 118].

In contrast, recent findings reported an active role of peroxisomes in malignant transformation and progression of some cancers. The increased overexpression of enzymes involved in peroxisomal fatty acids oxidation has been associated with several cancers. Contrarily, to what it was observed in colon carcinomas, the activity of ACOX1 seems to be increased in liver cancer comparatively to normal liver tissues, despite this activity not being reflected at protein levels. The authors suggest that the increased ACOX1 activity lead to excess H_2O_2 generation in the tumour cells, promoting the malignant transformation [119]. A high-throughput analysis showed that ACOX1 mRNA levels were increased in the majority of luminal and triple negative breast cancers tissues, comparatively to breast normal tissues (lack of the estrogen receptor, progesterone receptor, and HER2) [120].

PPARs, members of the nuclear hormone receptor superfamily, control complex gene expression involved in lipid metabolism and adipogenesis, as well as inflammation, and metabolic homeostasis, playing a significant role in cancer. However, the three PPAR isotypes, PPARα, PPARβ/δ and PPARγ, have generated a lot of controversy, since some studies implicated PPARs in the promotion and development of cancer while others show their protective role against this disease [121]. In rodents, abnormal upregulation of ACOX1 by PPARα activation stimulates hepatic fatty acid oxidation, leading to H_2O_2 accumulation and consequently contribute to the development of liver cancer [122, 123]. On the other hand, it was reports that ACOX1-null mice develop progressive liver diseases, due to increased intrahepatic H_2O_2 levels derived from mitochondrial and microsomal fatty acid oxidation and other sources [124, 125]. In humans, PPARα activation does not seem to induce hepatocellular carcinoma [126].

Brain tumours also take advantage of peroxisome dynamics and fatty acids oxidation: peroxisomes increase in number (increased expression of peroxisomal membrane proteins

PEX14 and PMP70) and β-oxidation levels (increased expression of peroxisomal proteins, ACOX1 and ACAA1) with malignancy [127]. It was suggested that the PPARα activation, due to the oxidative stress conditions, leads to peroxisomal proliferation and increases peroxisomal β-oxidation [127].

Peroxisomes seem to be associated with resistance to chemotherapy. The treatment of lymphoma with Vorinostat is affected by peroxisome proliferation. With the increased peroxisome number, lymphoma cells acquire more antioxidant capacity, counteracting ROS-mediated apoptosis by Vorinostat [128].

Also, the auxiliary enzyme of fatty acids oxidation, AMACR has been associated with several cancers, including breast, colon, kidney, liver and prostate, suggesting that peroxisomal branched-chain metabolism might be associated with a broader variety of tumours [129].

Other peroxisomal function, the biosynthesis of ether phospholipids, mainly plasmalogens, seems to be widely used by many cancers. The fact that plasmalogens are integral components of cell and organelle membranes and are involved in membrane dynamics, cell differentiation, cell signalling and oxidative stress, renders them essential for cancer proliferation and progression [130]. Elevated plasmalogens and mRNA levels of key proteins involved in their biosynthesis were observed in lymph, skin, liver, colon, colorectal, gastric and breast cancer [130]. However, it was observed a decrease in plasmalogens levels in some colon, esophageal, pancreatic and colorectal cancer tissues [130]. Also, it was reported that some plasmalogens have anti-tumorigenic role in MDA-MB-231 breast cancer cells [131, 132].

The peroxisomal protein hydroxysteroid dehydrogenase-like 2 (HSDL2), involved in lipid metabolism, is also upregulated in gliomas and ovarian cancer, promoting tumorigenesis and tumour progression [133, 134].

Recently, peroxisomes were associated as controllers of the balance between cell growth and differentiation in skin epithelial cells [135]. During mitosis, the correct peroxisome positioning at the spindle poles is required for the correct asymmetric cell division. Perturbations associated with peroxisome mislocalization, resulting in a mitotic delay and in basal daughters displaying differentiation markers but still proliferating features, typically associates with cancer [135].

1.2.1. Peroxisomes in prostate cancer

PCa is the second most frequent cancer and the fifth leading cause of cancer death worldwide in men [136] . Currently, it is known that age, family history, ethnicity, and internal steroid hormones levels are risk factors. Furthermore, recent studies point diet lifestyle as a carcinogenic factor [137, 138].

PCa initiation has been attributed to several events, including inflammation, oxidative stress, and cellular senescence. In a brief way, in >95% of PCas, upon an inflammation, ROS accumulation and certain drive mutations normal prostate epithelial cells undergo to several processes, becoming prostatic intraepithelial neoplasia (PIN), which lead to adenocarcinoma and later to metastasis [139].

The adaptation of cancer cells to a new status requires a profound reprograming of metabolism and redox homeostasis. PCa has an exclusive metabolic profile, which is remodelled accordingly to the tumour stage.

1.2.1.1. Prostate cancer metabolism

PCa displays a unique metabolic profile, contrasting with other types of cancer. The different cancer stages of PCa, from initiation to progression require metabolic remodelling, two different metabolisms: localized tumour metabolism (early stage) and metastasis metabolism (late stage) (Fig.2).

1.2.1.1.1. Normal prostate metabolism

Prostate glandular epithelial cells exhibit a peculiar metabolism, differing from any other kind of cell. Although, usually, acetyl-CoA enters in the tricarboxylic acid (TCA) cycle for ATP production, in normal prostate epithelium the TCA cycle is not active, leading to a massive citrate production. Consequently, citrate is accumulated and excreted into the prostatic fluid to fuel sperm cells [140]. As ATP production through oxidative phosphorylation (OXPHOS) is impaired, prostate epithelial cells rely on aerobic glycolysis to survive and sustain citrate production [141] (Fig.2).

1.2.1.1.2. Localized tumour metabolism

In malignant transformation, prostate cells adjust their metabolism to support the high proliferative rates that the new status imposes. The produced citrate in non-tumour state is now oxidized, functioning as an intermediate in the TCA cycle as well as a substrate for *de novo* fatty acid synthesis [142]. To relieve TCA cycle, PCa cells downregulate zinc transporters, mainly ZIP1, avoiding zinc accumulation and consequently the inhibition of m-aconitase [143]. Despite being an attractive substrate for ATP production, citrate is not enough for a cancer cell energetic demand.

Most tumour cells consume high rates of glucose to produce energy, via glycolysis, even when oxygen is not limiting – Warburg effect [144]. Glucose is converted to pyruvate which is then almost totally converted to lactate. The accumulation of lactate forces cancer cells to

overexpress monocarboxylate transporters (MCTs) in order to externalize it, avoiding intracellular acidification and consequently apoptosis [145] (Fig.3). MCTs belong to the SLC16 gene family that is composed by 14 members and are proteins responsible for the proton-linked transport of important monocarboxylate metabolites such as pyruvate, lactate and ketone bodies [146]. MCT1-4 are the most studied, differing in the substrate and inhibitor affinities, its regulation, tissue distribution and intracellular localization [146].

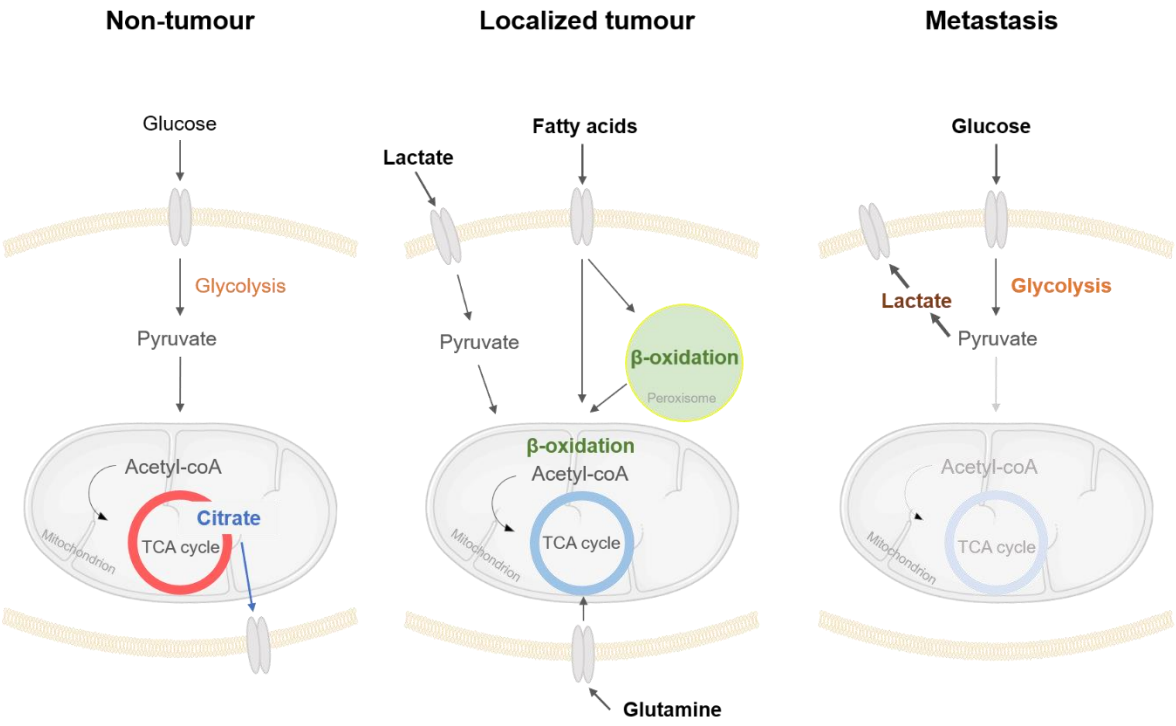

Figure 2. Prostate cells metabolism at different stages of carcinogenesis; non-tumour, early (localized tumour) and late (metastasis or advanced tumour) stages.

Aerobic glycolysis yields a much smaller amount of ATP, 2ATPs per molecule of glucose comparatively to oxidative phosphorylation (36ATP/molecule of glucose), however, this process confers some advantages to cancer cells, by leading to a faster ATP production (glucose to pyruvate and pyruvate to lactate), that results in a higher proliferative capacity [147]. Also, it allows the synthesis of glucose-derived macromolecules required for cell division [148, 149]. Moreover, the final product of aerobic glycolysis, lactate, promotes the survival, growth, invasion of tumour cells [150–152] and suppresses anticancer T cell immune responses [153]. Despite, most cancer cells use glucose as main energy source, PCa displays an exclusive metabolic profile. PCa cells consume low rates of glucose, and the lipids become the main energy source [154]. Lipids seem to have a central role in malignancy of PCa. The increased uptake of circulating lipids, the transfer of fatty acids from stromal adipocytes to PCa

cells, the *de novo* fatty acids synthesis and the fatty acids oxidation support the central role of lipids in PCa [155–157].

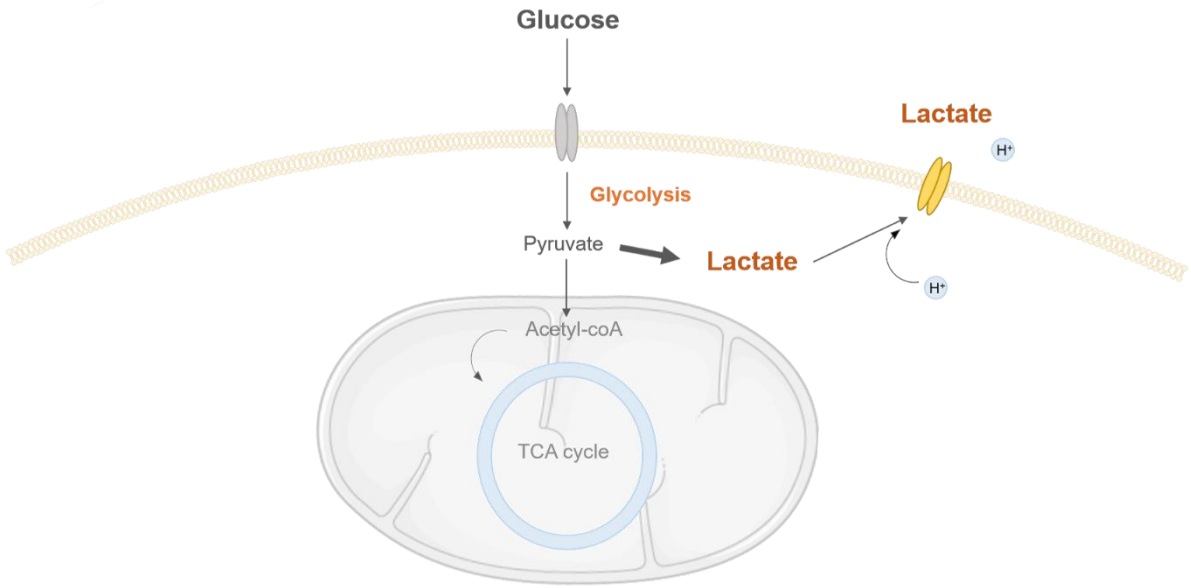

Figure 3. The Warburg effect in cancer cells. Glucose is converted to pyruvate and the majority of pyruvate is converted in lactate, even in the presence of oxygen. To avoid apoptosis and to support progression, cancer cells externalize lactate, through MCTs.

In humans, lipids can be acquired from circulating lipids in blood or from *de novo* synthesis of fatty acids, that is mainly restricted to liver and adipose tissues [158]. In normal conditions, the *de novo* fatty acids synthesis is inhibited by dietary fatty acids, promoting their degradation. However, some tumours, including PCa are able to increase lipogenesis for ATP production, synthesis of new phospholipids to build new cell membranes, production of signalling molecules and to escape to drugs and oxidative stress [159, 160] . Recent studies have shown the role of increased fatty acids oxidation in PCa, pointing it as the dominant bioenergetic pathway in PCa [69, 161].

Aberrant lipid metabolism of PCa targeted the attention to peroxisomes. Data showed that PCa induces peroxisomal branched chain fatty acid β-oxidation, by increasing the overexpression and activity of enzymes involved in this pathway [69, 162, 163]. The AMACR was the first enzyme associated with PCa, being overexpressed in PCa tissues comparatively to normal prostate tissues [129, 162, 163]. The strong association of AMACR with PCa led AMACR to be considered a new biomarker of PCa [163]. Posteriorly it was shown that not only AMACR was overexpressed in PCa, but also key enzymes involved in peroxisomal β-oxidation, including DBP and ACOX3 [69].

As peroxisomal β-oxidation is not directly associated with ATP production, it would be expected that mitochondrial β-oxidation would also be increased in PCa. However, Zha *et al.*,

did not observe an increase of mRNA levels of proteins involved in mitochondrial β-oxidation in PCa. Interestingly, it was shown that the inhibition of CPT1 by etomoxir induces cell death in PCa cell lines [164].

Furthermore, to ensure the energetic demands, PCa cells also take advantage from cells of the tumour microenvironment, forcing them to provide metabolic substrates, including lactate and glutamine. A *reverse Warburg effect* is observed in this type of metabolism, where cancer-associated fibroblasts (CAFs) can be corrupted to produce lactate, feeding cancer cells. The produced lactate is exported by MCT4 present in cell membrane of CAFs and, through MCT1, is internalized in cancer cells, where is converted to pyruvate for energy production through OXPHOS [165–167] (Fig.2). Glutamine is also internalized by PCa cells to be used as intermediate of TCA cycle and as nitrogen source [168].

1.2.1.1.3. Metastasis metabolism

For PCa progression, cancer cells need to remodel the metabolism once more to adapt to the new status. In advanced and metastatic PCa, some cancer cells start to gain the capacity to leave the primary tumour and spread to the body, through lymph and bloodstream. This process involves time expenditure and, above all, efforts to detach from primary tumour, digesting the matrix, invading the circulation and adapting to the new microenvironment.

Most cancer cells use glucose as main energy source from tumour initiation to metastasis, however, PCa only switches to the Warburg effect in the late stages of the disease [141].

2. Aims

Prostate cancer is the second most frequent cancer and the fifth leading cause of cancer death worldwide in men [136]. The late detection dramatically decreases survival rates, being imperative to develop methodologies for early detection and treatment.

PCa exhibits a unique metabolic profile, being remodelled according to tumour stage. Contrasting with the other types of cancer, at early stages, PCa relies on lipids to support the energy demands and only switches to the Warburg effect at late stages.

Interestingly, MCT2, a membrane transporter normally associated with glucose metabolism, was found upregulated and mislocalized in early stages of PCa.

Our main aim was to further unravel the role of MCT2 in PCa. To that end, we proposed the following aims:

1. Unravelling MCT2's intracellular localization and expression across prostate malignant transformation (section 3.1.)

2. Study the effect of peroxisomal MCT2 in peroxisome dynamics, cell motility and cell proliferation of PCa (section 3.2.)

3. Results

3.1. Localization of MCT2 at peroxisomes is associated with malignant transformation in prostate cancer

The results from this section were published as:

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Abstract

Previous studies on monocarboxylate transporters expression in PCa have shown that MCT2 was clearly overexpressed in prostate malignant glands, pointing it out as a putative biomarker for PCa. However, its localization and possible role in PCa cells remained unclear. In this study, we demonstrate that MCT2 localizes mainly at peroxisomes in PCa cells and is able to take advantage of the peroxisomal transport machinery by interacting with Pex19. We have also shown an increase in MCT2 expression from non-malignant to malignant cells that was directly correlated with its peroxisomal localization. Upon analysis of the expression of several peroxisomal β-oxidation proteins in PIN lesions and PCa cells from a large variety of human prostate samples, we suggest that MCT2 presence at peroxisomes is related to an increase in β -oxidation levels which may be crucial for malignant transformation. Our results present novel evidence that may not only contribute to the study of PCa development mechanisms but also pinpoint novel targets for cancer therapy.

Introduction

Metabolic adaptation is now considered a new hallmark of cancer, in which cancer cells exhibit high rates of glucose consumption and consequent lactate production [169]. The crucial role of lactate exchange within the tumour microenvironment drew attention to MCTS. In glycolytic tumours, they promote the efflux of lactic acid, being important players in the maintenance of the tumour intracellular pH, avoiding the routing to apoptosis and providing the favourable microenvironment conditions for invasion [170–172]. MCTs have been described in a large variety of tumours and their use as targets for cancer therapy have been widely suggested. However, the importance of MCTs expression in PCa is still not well understood [173].

PCa is the second most common malignancy in men, involving challenging diagnostics [174]. Several proteins, among which MCT2, have been identified as possible PCa biomarkers [175]. Previous studies point out to a consistent overexpression of MCT2 in PCa cells and its possible relevance as a putative biomarker in PCa because of its high sensitivity and specificity to detect malignant glands. MCT2 protein levels were significantly up-regulated (80–100%) in PCa and PIN lesions in human samples, in sharp contrast to the near complete lack of expression in both benign hyperplastic and normal prostate glands. These data suggest a basic mechanistic role for this protein throughout the early stages of PCa formation and prompted us to investigate this transporter in more detail [175]. Similarly to MCT1, MCT2 was also found to localize in mitochondria and peroxisomes in non-tumour liver fractions and in rat skeletal muscle [176, 177]. The presence of MCTs in mitochondria is justified by the need

of a pyruvate carrier that plays a central role in carbohydrate and fat metabolism. In contrast, the presence of MCT1 and MCT2 at peroxisomes was explained as being involved in a lactate–pyruvate shuttle system present in the membrane of this organelle. This shuttle was suggested to play an important role in the oxidation of NADH generated by β-oxidation, being crucial for the maintenance of peroxisomal viability and, consequently, β-oxidation rates. Although the role of MCT2 in cancer is not yet elucidated, a recent study showed that MCT2 knockdown suppressed KRAS mutant (the mutation in KRAS gene occurs in a big percentage of colorectal cancers and has been suggested to be associated with proliferation and decreased apoptosis in cancer cells) colorectal tumour growth in vivo, supporting the use of MCT2 as a promising target for inhibition of colorectal cancer [178]. However, so far, the precise localization and role of MCT2 in PCa is still unknown.

Importantly, MCT2 staining in PCa was comparable to AMACR, an already established biomarker in PCa. Under normal physiological conditions, AMACR is expressed at appreciable levels and is transported to both the peroxisomal and mitochondrial compartments in a variety of tissues, including liver, kidney, skeletal muscle, gall bladder and brain [55, 67, 179, 180]. AMACR is responsible for the interconversion of R-configured β-methyl groups found within various small fatty acid molecules containing branched chains (such as phytols and bile acids) to the S form, a pre-requisite for metabolism via the β-oxidation pathway [65, 181]. A similar staining pattern between MCT2 and this crucial component of the oxidative metabolism raised the hypothesis that this MCT isoform might also be involved in these peroxisomal and mitochondrial-dependent mechanisms. In this work, we aimed at unravelling MCT2's intracellular localization and expression across prostate malignant transformation using different models of disease progression.

Our results demonstrate for the first time that MCT2 is present at the peroxisomes of PCa cells and that its expression increases from non-malignant to malignant cells, directly correlating with its localization at peroxisomes. Using a large series of human prostate samples, we have also shown an increase in the expression of peroxisomal β-oxidation proteins in PIN lesions and PCa cells. Our data provide novel evidence for the importance of MCT2- and peroxisomal-dependent mechanisms in PCa initiation in humans.

Results

MCT2 localizes at peroxisomes in prostate cancer cells

To access the exact intracellular localization of MCT2 in PCa cells, we have performed immunolocalization analyses of MCT2 in different cellular models of PCa disease progression:

PNT1A (non-tumour), 22Rv1 and PC3. As McClelland et al., have shown a peroxisomal localization of MCT2 in non-tumour liver fractions [182], we firstly tested whether this protein would as well be present in this organelle in PCa cells. To that end, the localization of MCT2 was analysed in parallel with the peroxisomal marker CAT.

Our results have interestingly demonstrated that, although no co-localization was observed between MCT2 and the peroxisomal marker in PNT1A, this protein is localized at peroxisomes in all the malignant cell lines (Fig.4). We have, however, observed that the localization level varied across the different models. In 22Rv1 cells, MCT2 was mainly found at peroxisomes with a minor portion spread throughout the cytoplasm as small aggregates (Fig.4A d-f). Quantification analyses show that 60.35% of the MCT2 co-localizes with the peroxisomal marker. However, the ratio of peroxisomal MCT2/cytoplasmic MCT2 decreased with disease progression, culminating with 34% of MCT2 at peroxisomes in PC3 cells (Fig.4A g-i).

To substantiate these results, we have performed differential centrifugation analyses with lysates from 22Rv1 and PC3 cells and obtained a fraction that (although presenting some degree of contamination with light mitochondria and small vesicles such as lysosomes and endosomes) is highly enriched in peroxisomes (Fig.4B, PF). The results (Fig.4B) clearly show that MCT2 localizes at the peroxisome-enriched fractions in both cell lines. Surprisingly, the amount of MCT2 at peroxisomes appears to correlate with a change on the organelle's morphology. In fact, in cells where no MCT2 was present at peroxisomes (PNT1A), this organelle exhibits a regular phenotype (with 67.7% round and 32.3% tubular; Fig.4A a-c). Curiously, in 22Rv1 cells (where MCT2 was mainly observed at peroxisomes), this organelle appears somewhat elongated and in clusters (with only 15.6% round and 84.4% tubular and in clusters). In PC3, the highly metastatic model, peroxisomes appear similar to ones in the non-malignant cells PNT1A (with 73.1% round and 26.9% tubular; Fig.4A g-i). Further experiments will have to be performed to better analyse the correlation between MCT2 localization at the peroxisomes and the different organelle morphologies.

Strikingly, the expression level of MCT2 increases from non-tumour (PNT1A) to localized malignant cells (22Rv1) in about 784% correlating to its change in localization from cytoplasmic to peroxisomal (Fig.4C). An increase in expression level (of about 89%) was also observed for PEX14 (a peroxisomal membrane protein; Fig.4D), suggesting an increase in peroxisomal membrane surface/number accompanying the malignant transformation.

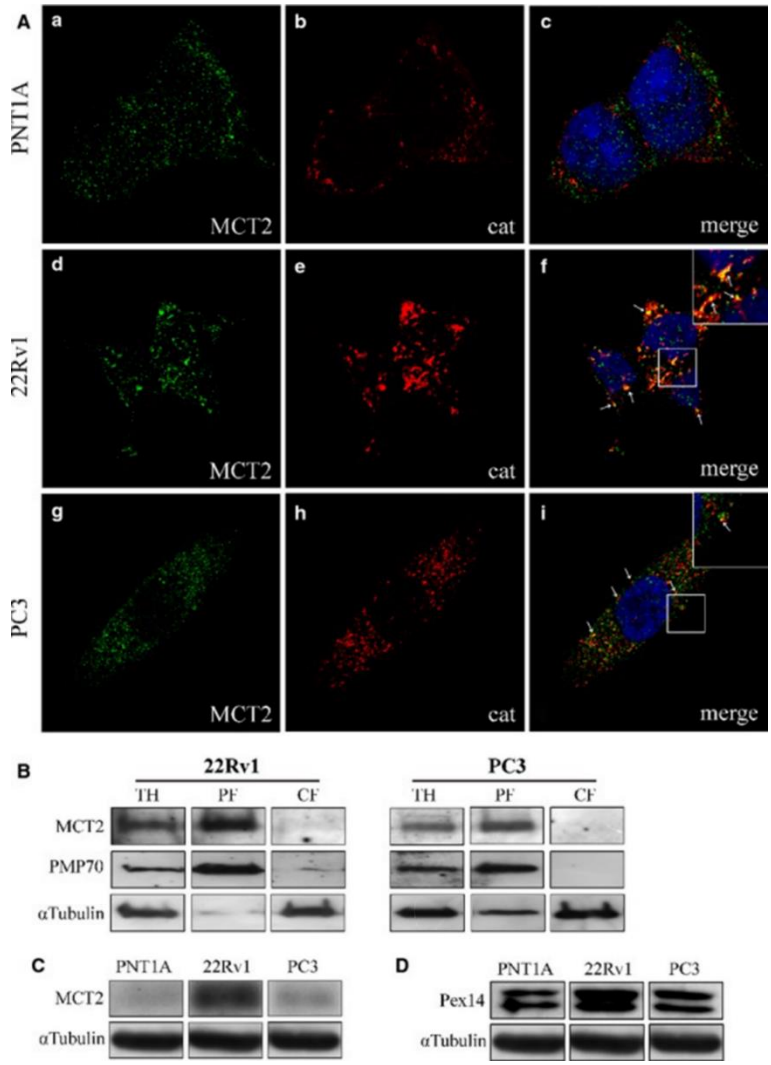

Figure 4. MCT2 localizes at peroxisomes in PCa cells. (A) a–c: MCT2 intracellular localization in PNT1A cells, (a) MCT2, (b) CAT and (c) merge image of a and b; d–f: MCT2 intracellular localization in 22RV1 cells, (d) MCT2, (e) CAT and (f) merge image of d and e; g–i: MCT2 intracellular localization in PC3 cells, (g) MCT2, (h) CAT and (i) merge image of g and h. Arrows indicate some of the co-localization sites. Nuclei are shown in blue (stained with Hoechst 33258). Bars represent 5 μm. (B) Western blot analysis of the presence of MCT2 in peroxisomal-enriched (PF) and cytosolic and microsomal (CF) subcellular fraction of 22Rv1 and PC3 cells upon differential centrifugation. TH represents total homogenate. PMP70 and α-tubulin are used as peroxisome and cytosol markers, respectively. (C) Western blot analysis showing the levels of MCT2 in the different prostate cell lines models, PNT1A, 22RV1 and PC3. (D) Western blot analysis showing the levels of PEX14 in the different prostate cell lines models, PNT1A, 22RV1 and PC3.

Other MCT isoforms are present at the peroxisomes, plasma membrane, cytoplasm and nucleus of prostate cancer cells

As McClelland *et al*., have also shown a peroxisomal localization of MCT1 in non-tumour liver fractions [182], we decided to test whether this protein would also be present in this organelle in PCa cells.

Upon immunolocalization of MCT1 together with peroxisomal markers, we have observed a small degree of co-localization with the peroxisomal marker (Fig.5A) in the tumour cell lines (5.15% of the MCT2 co-localizes with the peroxisomal marker in 22RV1 cells and 4% in PC3 cells). Differential centrifugation analyses with lysates from 22Rv1 and PC3 cells (Fig.5B) clearly confirm that MCT1 localizes at the peroxisome-enriched fractions in both cell lines.

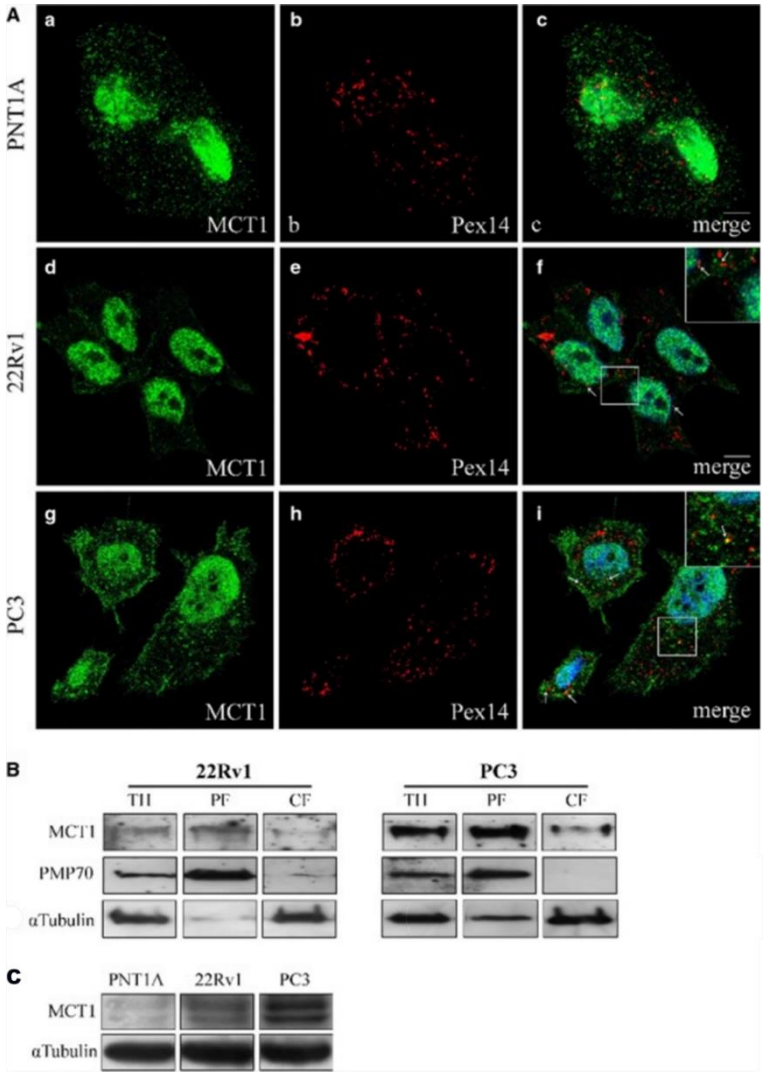

Figure 5. MCT1 localizes at peroxisomes, cytoplasm, nucleus and plasma membrane in PCa cells. (A) a–c: MCT1 intracellular localization in PNT1A cells, (a) MCT1, (b) PEX14 and (c) merge image of a and b; d–f: MCT1 intracellular localization in 22RV1 cells, (d) MCT1, (e) PEX14 and (f) merge image of d and e; g–i: MCT1 intracellular localization in PC3 cells, (g) MCT1, (h) PEX14 and (i) merge image of g and h. Arrows indicate some of the co-localization sites. Nuclei are shown in blue (stained with Hoechst 33258). Bars represent 5 μm. (B) Western blot analysis of the presence of MCT1 in peroxisomal-enriched (PF) and cytosolic and microsomal (CF) subcellular fraction of 22Rv1 and PC3 cells upon differential centrifugation. TH represents total homogenate. PMP70 and α-tubulin are used as peroxisome and cytosol markers, respectively. (C) Western blot analysis showing the levels of MCT1 in the different prostate cell lines models, PNT1A, 22RV1 and PC3.

However, MCT1 was mainly found to strongly localize at the nucleus in all cell lines (Fig.5A) and was also present at the cytoplasm and plasma membrane (Fig.5A). The expression level

of MCT1 increased from the PNT1A cells to the tumour cell lines, with a higher expression at PC3 cells then at 22RV1 cells (Fig.5C).

Interestingly, MCT1 chaperone CD147 was found to localize not only at the plasma membrane and cytoplasm but also at the nuclear envelope, mainly in 22RV1 cells (Fig.6).

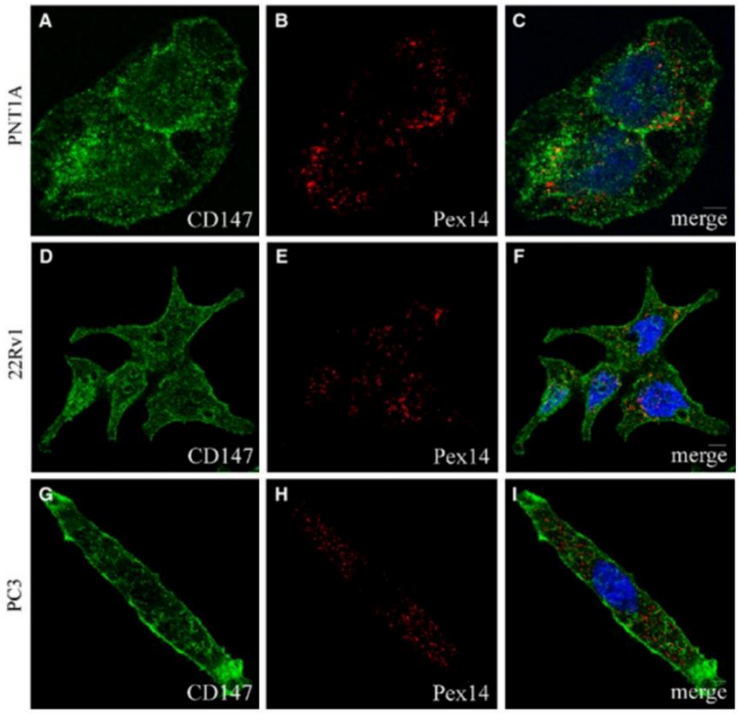

Figure 6. CD147 localizes at the cytoplasm, nuclear and plasma membranes in PCa cells. A–C: CD147 intracellular localization in PNT1A cells, (A) CD147, (B) PEX14 and (C) merge image of A and B; D–F: CD147 intracellular localization in 22RV1 cells, (D) CD147, (E) PEX14 and (f) merge image of D and E; G–I: CD147 intracellular localization in PC3 cells, (G) CD147, (H) Pex14 and (I) merge image of G and H. Nuclei are shown in blue (stained with Hoechst 33258). Bars represent 5 µm.

As the intracellular localization of MCT4 in PCa cells has never been assessed, we have also analysed it by immunolocalization with organelle markers. MCT4 was found mainly at the cytoplasm in all the three cell lines with some degree of localization at the peroxisomes (6.78% of the MCT4 co-localizes with the peroxisomal marker in 22RV1 cells and 4.77% in PC3 cells). In PC3 cells, however, a strong plasma membrane staining was also observed (Fig.7A). The peroxisomal localization in both 22Rv1 and PC3 cells was confirmed by differential centrifugation analyses (Fig.7B). The expression level of MCT4 is similar in PNT1A and PC3 cells, decreasing in 22Rv1 cells (Fig.7C).

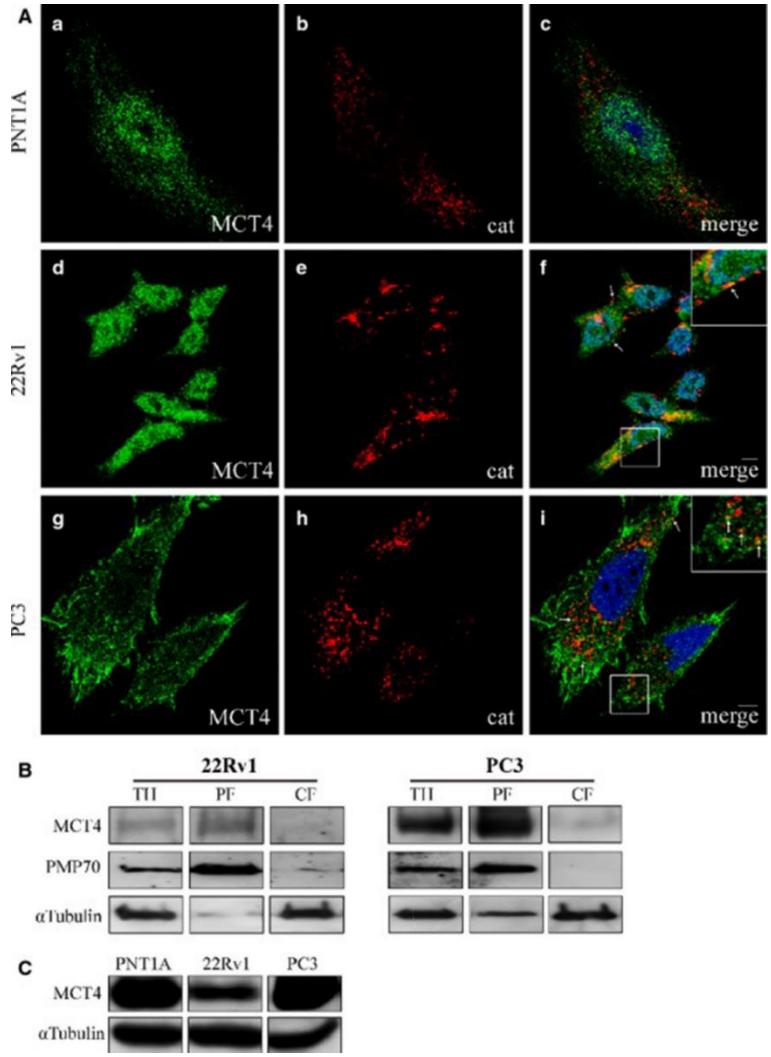

Figure 7. MCT4 localizes at peroxisomes, cytoplasm and plasma membrane in PCa cells. (A) a–c: MCT4 intracellular localization in PNT1A cells, (a) MCT4, (b) CAT and (c) merge image of a and b; d–f: MCT4 intracellular localization in 22RV1 cells, (d) MCT4, (e) CAT and (f) merge image of d and e; g–i: MCT4 intracellular localization in PC3 cells, (g) MCT4, (h) CAT and (i) merge image of a and g and h. Arrows indicate some of the co-localization sites. Nuclei are shown in blue (stained with Hoechst 33258). Bars represent 5 μm. (B) Western blot analysis of the presence of MCT4 in peroxisomal-enriched (PF) and cytosolic and microsomal (CF) subcellular fraction of 22Rv1 and PC3 cells upon differential centrifugation. TH represents total homogenate. PMP70 and α-tubulin are used as peroxisome and cytosol markers, respectively. (C) Western blot analysis showing the levels of MCT4 in the different prostate cell lines models, PNT1A, 22RV1 and PC3.

MCT2 travels to the peroxisomal membranes via interaction with PEX19

The quick movement of monocarboxylates across the membranes is imperative for cellular metabolism. These proteins are thought to require chaperones such as CD147 in the case of MCT1 and MCT4, or Gp70 in the case of MCT2, for appropriate expression and activity in the plasma membrane. As MCT2 was the main isoform found to strongly localize at peroxisomes in PCa cells, we aimed at better unravelling its targeting mechanism to this organelle. A previous study was unable to find Gp70 expression in human prostate samples that exhibited

MCT2 expression [173]. However, our studies with confocal microscopy allowed us to observe some, although scarce, Gp70 distributed in the cytoplasm without any co-localization with peroxisomal markers (results not shown). Hence, the protein that actually behaves as chaperone for MCT2's transport to peroxisomes in prostate cells remained unknown.

As PEX19 is the main responsible for the transport of peroxisomal membrane proteins to this organelle [32], we tested whether this protein could act as a chaperone for MCT2 in these cells. As in 22RV1, the majority MCT2 was present at peroxisomes, it was the chosen model to study the possible interaction between MCT2 and PEX19. 22RV1 cells transfected with Pex19-YFP were then subject to immunoprecipitation experiments. Our results showed, indeed, an interaction between MCT2 and PEX19 (Fig.8), suggesting that MCT2 is able to take advantage of peroxisomal transport machinery to reach this organelle.

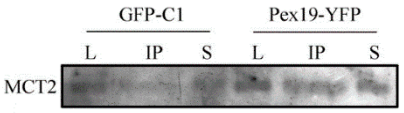

Figure 8. MCT2 interacts with PEX19. Co-immunoprecipitation analysis of the Interaction between endogenous MCT2 and overexpressed Pex19 (PEX19-YFP) in 22RV1 cells. Negative controls were performed by immunoprecipitating cells expressing GFP (GFP-C1). Western blots were performed with an antibody anti-MCT2 as well as tubulin as loading control. L represents lysate, IP represents the immunoprecipitation result and S represents the supernatant.

The expression of MCT2 and peroxisomal β-oxidation-related proteins increase in prostate malignant transformation

McClelland *et al*., observed a decrease in β-oxidation upon MCTs inhibition, suggesting that the presence of MCT2 at peroxisomes of non-malignant liver cells would be essential for the maintenance of peroxisomal viability and consequently β-oxidation rates [182] .

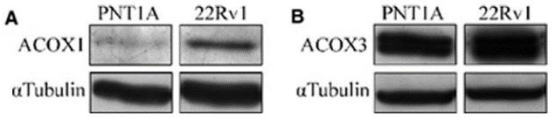

Figure 9. Peroxisomal β-oxidation proteins are overexpressed in localized prostate tumour cells. (A) Western blot analysis, showing the expression levels of ACOX1 in 22RV1 cells. (B) Western blot analysis, showing the expression levels of ACOX3 in 22 RV1 cells.

Hence, we hypothesized that the increase in MCT2 expression from non-malignant prostate cells (PNT1A) to localized prostate tumour cells (22RV1) could be related with an increase in peroxisomal β-oxidation. In fact, we observed an increase in the expression levels of ACOX1 (of about 246%; Fig.9A) and ACOX3 (about 14%; Fig.9B), two central proteins in the peroxisomal β-oxidation pathway [65]. These results interestingly suggest that, indeed, the

increase in MCT2 expression levels as well as its presence at peroxisomes, are related to an increase in β-oxidation levels which may be crucial for malignant transformation. Further experiments need to be performed to confirm this correlation.

Peroxisome-related proteins are overexpressed in human prostate cancer samples

To study the pathological relevance of the expression of proteins involved in peroxisomal β-oxidation in human samples, we characterized the expression of AMACR, ACOX3 and DBP in a large series of human prostate samples. Fig.10 shows representative immunohistochemical reactions for all proteins in BT (benign tissue), PIN lesions, primary tumour tissue (TT) and metastatic tissue (MT). We observed important changes in the expression of all the proteins studied from benign and/or adjacent non-neoplastic prostate tissue to PIN lesions and to primary tumour. AMACR, ACOX3 and DBP expressions was clearly increased from BT to TT.

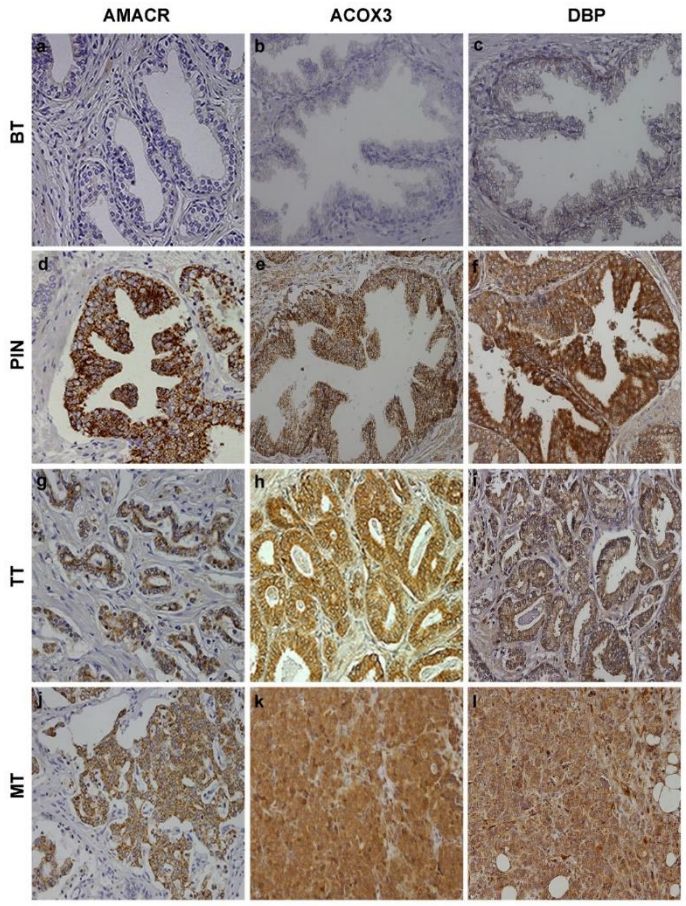

Figure 10. The expression of proteins involved in peroxisomal β-oxidation is more intense in PIN lesions and prostate tumour samples. Immunohistochemical expression of metabolic-related proteins in benign tissue (BT), PIN lesions (PIN), prostate tumour tissue (TT) and metastatic tissue (MT; 200x magnification). Images are shown with a magnification of 200x.

Fig.11A shows the specific percentage of positive cases for each protein in different tissues. Interestingly, an evident increase was verified from BT or non-neoplastic tissue (NNT) to PIN and TT. In Fig.11B and C, the distribution of the final score across different prostate tissue types for each protein is represented, showing in a general way that there is an increase in the final score in the localized tumour when compared to the benign glands.

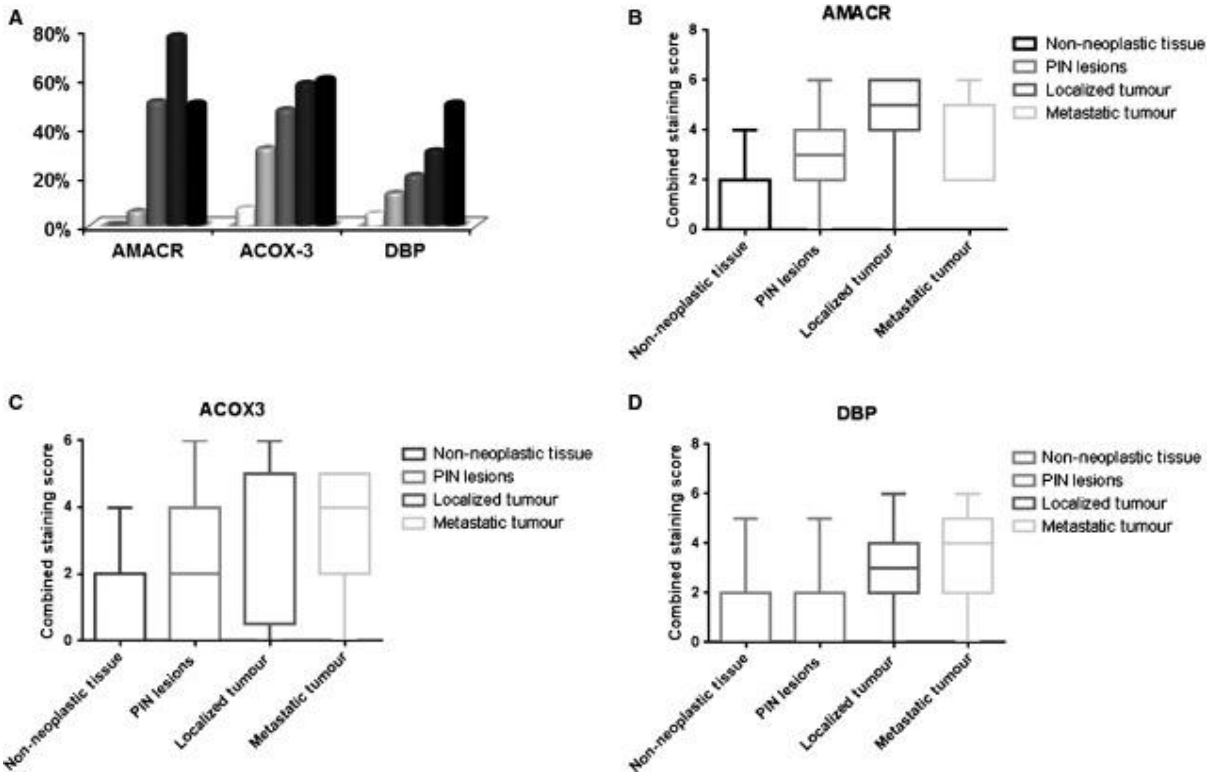

Figure 11. The percentage of cases showing increase AMACR, ACOX3 and DBP expression increases in PIN lesions and tumour samples. (A) Overall percentage of positive cases for each one of the proteins studied in the different tissue samples. (B–D) Distribution of the final staining score for AMACR (B), ACOX3 (C) and DBP (D) in different PCa tissues.

Discussion

A variety of studies pointed to the importance of MCTs in solid tumours. In contrast to MCT1 and MCT4, which were mainly described at the plasma membrane in a wide variety of malignancies, MCT2 expression in human cancers was always less evident and, when present, its expression was mainly cytoplasmic [183]. As so, and because of the major role described for MCTs as important players in the acid-resistant phenotype of tumour cells, MCT1 and MCT4 turned into the most popular isoforms explored for cancer therapy. However, a study in malignant gliomas and more recently a study in colorectal malignancies showed a significant role for MCT2 in cancer, describing that MCT2 inhibition induces senescence-

associated mitochondrial dysfunction and suppresses progression of colorectal malignancies *in vivo* [178, 184]. Similarly to what was described for colon cancer, MCT2 expression in PCa was also observed in the cytoplasm of tumour cells [175]. The observation that MCT2 was clearly expressed in PIN lesions and prostate tumour cells strongly pointed into an unexplored role of this isoform in prostate malignant transformation.

In this study, we have demonstrated for the first time that MCT2 is localized at peroxisomes in PCa cells. Importantly, its localization pattern changes across the different *in vitro* models of prostate disease progression: while no peroxisomal localization was observed in non-malignant prostate cells, the highest co-localization level was detected in the localized tumour cells, decreasing with the level of metastization. These results strongly suggest that the localization of MCT2 in PCa peroxisomes is important in disease initiation.

Peroxisomes are ubiquitous and essential subcellular organelles, versatile and highly diverse depending on the organism, cell type and developmental stage [3, 86, 185, 186]. They fulfil important functions in lipid and reactive oxygen species metabolism, influencing, among others, neuronal development and ageing [3, 86, 185, 186]. The protein composition, morphology and abundance of these dynamic organelles are tightly regulated upon external stimuli to maintain cellular homoeostasis [3, 86, 185, 186]. Peroxisome dynamics and morphology play important roles in cell pathology, and defects on these machineries lead to significant implications in health and disease [86]. Information on the role of peroxisomes in tumour development is scarce. In some studies, mainly on hepatocarcinomas, a decrease in peroxisome number in cancer cells was demonstrated by the reduction in peroxisomal CAT and the three peroxisomal β-oxidation enzymes [112].

The presence of MCT2 in peroxisomes (in non-tumour liver cells) was firstly suggested by McClelland *et al*., who proposed that this protein would be involved in a redox shuttle system at the peroxisomal membrane, consisting of a substrate cycle between lactate and pyruvate. This shuttle would stimulate the reoxidation of NADH, fuelling the organelles β-oxidation and playing a role in peroxisomal redox balance [182]. Our results seem to highlight a similar role for MCT2 at the peroxisomal membrane of PCa cells. In parallel to a clear increase on MCT2 expression from non-tumour to localized tumour cells, we have also observed a rise in the expression of specific key proteins involved in peroxisomal β-oxidation: ACOX1 and ACOX3. These results suggest that the presence of MCT2 at peroxisomes stimulates an increase in the β-oxidation rate that seems to be related with prostate tumour initiation. Importantly, these results are substantiated by the study of the expression of AMACR, ACOX3 and DBP in human prostate samples, showing a specific and consistent overexpression of proteins involved in peroxisomal fatty acid oxidation in PCa as well as in PIN lesions in contrast to benign tissue, suggesting a possible aetiological role for this pathway in malignant transformation.

The observation that the expression level of MCT2 as well as its co-localization with peroxisomes decreases from the localized tumour cells to the highly metastatic models likely demonstrates that in these cells other metabolic mechanisms play a more important role, such as hypoxia and hypoxic-related proteins involved in glycolysis, which was already suggested to be linked with disease aggressiveness [187]. To better unravel the mechanisms involved in the peroxisomal and MCT2-dependent disease initiation, we have analysed the cellular trafficking of MCT2 and demonstrated that GP70, the previously described MCT2 chaperone, is barely expressed in the PCa cells, indicating that MCT2 should rely on an alternative chaperone for its proper function in these cells. Our results show that MCT2 interacts with PEX19, the main responsible for the trafficking of intrinsic peroxisomal membrane proteins to this organelle. These data strongly suggest a highjack of the peroxisomal transport machinery to sustain malignant transformation. We have furthermore observed a clear change in peroxisome morphology across prostate malignant transformation correlated with MCT2 presence at this organelle, providing once more evidence for the involvement of these organelles in tumour initiation and progression.

In this study, we have also analysed the intracellular localization of MCT1 and MCT4 in PCa cells. Surprisingly, both proteins are also found at peroxisomes, although in a much lower extent than MCT2. It is tempting to suggest that both MCT1 and MCT4 would be present at the peroxisomal membranes as a partner for MCT2 within the substrate shuttle. In fact, McCleeland *et al*., have already shown MCT1 to be present in liver peroxisomes and to form, along with peroxisomal lactate dehydrogenase, a peroxisomal lactate shuttle. However, our results show that the amount of MCT1 at peroxisomes is much lower than the one of MCT2. Furthermore, MCT1 is also present at the plasma membrane and, surprisingly, at the nucleus. Its chaperone CD147 was found to localize at the nuclear membrane, suggesting that it was the responsible for the MCT1 transport to the nuclear membrane prior to its internalization. A nuclear localization for MCT1 has already been shown to occur in human sarcomas [188]. Also, only a small part of MCT4 was found at peroxisomes with its majority localizing in the cytoplasm and at the plasma membrane, as expected. Although the possibility of MCT1 and/or MCT4 behaving as partners of MCT2 at the peroxisomal membrane is very appealing, further experiments have to be performed to test this or other hypothesis concerning their role at this organelle. Nevertheless, our results suggest that the presence of multiple MCTs is physiologically important in cancer cells and the involvement of these isoforms in the biology of tumour cells goes beyond their classical role in the glycolytic metabolism.

Our study describes for the first time the presence of MCT2 at the peroxisomes of PCa cells and suggests a possible role for peroxisome-related mechanisms in prostate malignant transformation. These results may further be exploited for the study of peroxisomal metabolism as target for cancer therapy.

Materials and methods

Cell culture

In this study, we have used several prostate cell lines such as PNT1A (non-malignant), 22Rv1 (localized tumour) and PC3 (bone metastasis). Cells were seeded in RPMI-1640 (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% of foetal bovine serum (PAA Laboratories GmbH, Cölbe, Germany), 1% of antibiotic (penicillin/streptomycin) (PAA Laboratories GmbH) and incubated at 37°C in an atmosphere containing 5% CO2. All cell lines were cultivated under the same experimental conditions and observations were made at about 70% cell confluence.

Antibodies and plasmids

For the immunofluorescence experiments, the following antibodies were used: MCT2 (sc-50322; Santa Cruz Biotechnology, Santa Cruz, CA, USA), MCT1 (sc-365501; Santa Cruz Biotechnology), MCT4 (sc-50329; Santa Cruz Biotechnology), CD147 (sc-71038; Santa Cruz Biotechnology), gp70 (HPA017740, 1:100; Atlas Antibodies, Stockholm, Sweden), PEX14 (a gift from Dr. Dennis Crane, Griffith University, Brisbane, Australia), CAT (ab88650; Abcam, Cambridge, UK), TRITC (Jackson ImmunoResearch, West Grove, PA, USA) and Alexa 488 (Invitrogen, Life Technologies, Carlsbad, CA, USA). For the Western blot analysis, the following antibodies were used: MCT2, ACOX1 (a gift from A. Völkl, University of Heidelberg, Germany), ACOX3 (sc-135435; Santa Cruz Biotechnology), PEX14, PMP70 (SAB4200181; Sigma-Aldrich, St Louis, MO, USA) and α-Tubulin (T9026, Sigma-Aldrich). For the immunohistochemistry (IHC) staining, the following antibodies were used: AMACR (504R-16, Cell Marque, Rocklin, CA, USA), ACOX3 (sc-135435; Santa Cruz Biotechnology) and DBP (a gift from Dr. Gabriele Moller from HelmholtzZentrum München). Pex19-YFP plasmid was a gift from Dr M. Schrader, Exeter University, UK.

Immunofluorescence and microscopy techniques

Immunofluorescence analyses were performed in cells seeded on glass cover slips that were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 20 min. Afterwards, cells were permeabilized with 0.2% Triton X-100 for 10 min., blocked with 1% BSA solution for 10 min. and incubated with primary (MCT2, MCT1, MCT4, CD147, Gp70, PEX14, CAT) and secondary antibodies (TRITC or Alexa488) for 1h each. Between each step, cells were washed three times with PBS, pH 7.4. Lastly, cells were stained with Hoechst 33258 (PolySciences, Warrington, FL, USA) and mounted in slides using Mowiol 4-88 containing n-

propylgallate. Images were obtained using a Zeiss LSM 510 Meta Confocal setup (Carl Zeiss, Jena, Germany) equipped with a plan-Apochromat 100×/1.4 oil objective. Quantifications of co-localizations were performed by determining the Manders' coefficients using the JACoP (ImageJ, Bethesda, MD, USA) software.

Cell fractionation

22Rv1 and PC3 cells were collected in PBS with a rubber scraper. Upon centrifugation at 500 × g for 5 min., the pellet was homogenized in homogenization buffer (5 mM MOPS, pH 7.4, 250 mM sucrose, 1 mM EDTA, protease-inhibitor mixture) and passed gently through a 26-gauge syringe needle, giving rise to the total homogenate fraction (TH). A part of this homogenate was centrifuged at 1000 × g for 10 min and the pellet corresponding to nuclei and cellular membranes were discarded. The supernatant was again centrifuged at 2500 × g for 15 min. at 4°C to separate the pellet containing heavy mitochondria. This new supernatant was centrifuged at 37,000 × g for 20 min., giving rise to a peroxisome-enriched pellet (which may also contain some degree of light mitochondria, lysosomes and endosomes) which was gently resuspended in homogenization buffer (PF). The supernatant was saved as cytosol and microsome fraction (CF). Protein concentrations of all fractions were determined by Bradford assay (Bio-Rad, Hercules, CA, USA) and 60 µg of each was loaded on the gels and subjected to Western Blot analysis.

Western Blot

Cells were lysed with specific lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM sodium chloride, 0.5% sodium deoxycholate, 0.5% Triton X-100 and a protease-inhibitor mix). To improve protein extraction, samples were passed 20 times through a 26-gauge syringe needle and incubated on a rotary mixer at for 30 min. at 4°C. After cleared by centrifugation (17,000 × g, 15 min.), protein concentrations were determined by Bradford assay (Bio-Rad). Blots were incubated with the specific primary antibodies MCT2, MCT1, MCT4, Pex14, PMP70, ACOX1, ACOX3 and α-Tubulin. The antibodies were detected by a secondary antibody HRP using an enhanced chemiluminescence system (GE Healthcare, Waukesha, WI, USA).

Immunoprecipitation

22Rv1 cells were transfected with Pex19-YFP using Turbofect *in vitro* transfection kit (Thermo Scientific, Marietta, OH, USA) according to the manufacturer's instructions. For immunoprecipitation of Pex19-YFP, the GFP Trap_M (Chromotek, Planegg-Martinsried, Germany) was used. Transfection with a plasmid containing GFP (GFP-C1) was used as

negative control. After 48 hrs of transfection, cell pellets were incubated in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40 and a protease-inhibitor mix). The lysate was cleared by centrifugation (17,000 × g, 15 min.) and diluted with dilution buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA and a protease-inhibitor mix). Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Ice-cold dilution buffer was used to equilibrate beads and the cell lysates were incubated for 2 hrs at 4°C on a rotary mixer. Beads were washed three times with dilution buffer and resuspended in 3× SDS sample buffer and boiled for 10 min. to elute bound proteins. Immunoprecipitated samples were separated in a 12.5% SDS-polyacrylamide gel and analysed by Western Blot.

Patients' samples and tissue microarray construction

Prostate tissues were obtained from 480 patients with a median age of 64 years old, following radical prostatectomy. Samples, including 203 non-neoplastic, 176 high-grade PIN and 480 neoplastic tissues were used and organized into tissue microarray blocks (TMAs). The clinico-pathological data were assessed for all patients. Haematoxylin and eosin-stained sections for each tumour were examined by two independent pathologists and three 2-mm diameter representative cores from the tumour specimens were cut and placed randomly in TMA recipient blocks. Benign samples were obtained from 12 patients undergoing radical cystoprostatectomy for transitional cell carcinoma of the bladder and 10 metastatic PCa cases were obtained from clinical biopsy samples. The present study was approved by the Hospitals Local Ethical Review Committees.

Immunohistochemistry staining and analysis

Formalin-fixed paraffin-embedded 4-µm sections were prepared from the TMA blocks. IHC technique 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 AMACR (504R-16, Cell Marque), ACOX3 (sc-135435; Santa Cruz Biotechnology) and DBP (a gift from Dr. Gabriele Moller from HelmholtzZentrum, Munich). IHC evaluation was performed blindly by two independent observers that assessed the intensity and the extension of the staining, as previously described [173].

Part of these results were also published in the PhD thesis of Pértega-Gomes N., from the Pathology and Molecular Genetics Doctoral Program at the Institute of Biomedical Sciences, University of Porto, Porto, Portugal.

3.2. The interplay between MCT2 and peroxisomes plays a central role in prostate cancer proliferation

Part of the results from this section will soon be submitted as:

Valença I[1], Correia M[2,3], Máximo V[2,3,4], Islinger M[3] and Ribeiro D[1]. The interplay between MCT2 and peroxisomes plays a central role in prostate cancer proliferation (to be submitted to *Cancers*)

[1]Institute for Research in Biomedicine – iBiMED, Department of Medical Sciences, University of Aveiro, Aveiro, Portugal; [2]i3S-Institute for Research and Innovation in Health, University of Porto, Porto, Portugal; [3]IPATIMUP-Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal; [4]Department of Pathology, Medical Faculty, University of Porto, Porto, Portugal; [5]Neuroanatomy, Center for Biomedicine and Medical Technology Mannheim, University of Heidelberg, Heidelberg, Germany.

Abstract

The lipid reprogramming metabolism contributes directly to malignant transformation and progression. The increased uptake of circulating lipids, the transfer of fatty acids from stromal adipocytes to cancer cells, the *de novo* fatty acids synthesis and the fatty acids oxidation support the central role of lipids in many cancers, including PCa. Fatty acids β-oxidation is the dominant bioenergetic pathway in PCa and recent evidences suggest that PCa take advantage of the peroxisome transport machinery to target MCT2 to peroxisomes in order to increase β-oxidation rates and to maintain the redox balance. Here we show that, in early stages, PCa takes advantage of peroxisomes, upregulating several processes involved in lipid metabolism. Moreover, we show that the presence of MCT2 at peroxisomes leads to alterations in peroxisome dynamics, increasing peroxisome surface and decreasing peroxisome number. Our results furthermore demonstrate that MCT2 promotes PCa migration and, interestingly, that its localization at peroxisomes is required for PCa proliferation.

Introduction

Malignant transformation requires multiple metabolic adaptations to answer energy requirements and support high proliferation rates [169]. Initial evidences reported an increase of glucose uptake and glycolysis in cancer cells, even though oxygen was not limiting, leading to an increased lactate production (Warburg effect) [144]. Despite most studies being focused in glycolysis and glutaminolysis, recent evidences suggest that the reprogramming of cellular lipid metabolism contributes directly to malignant transformation and progression [189]. Several cancers increase the *de novo* lipid synthesis for lipids storage, synthesis of new phospholipids, to e.g. build new cell membranes, production of signaling molecules and escape from drugs and oxidative stress [159, 160]. Besides increased lipogenesis, some cancers also take advantage from fatty acids oxidation for energy production [190, 191].

PCa displays an exclusive metabolic profile: PCa cells consume low rates of glucose and the lipids become the main energy source [154]. The increased uptake of circulating lipids, the transfer of fatty acids from stromal adipocytes to PCa cells, the *de novo* fatty acids synthesis and the fatty acids oxidation, support the general idea that lipids play a central role of lipids in PCa malignancy [155–157]. Although most studies have been focused on *de novo* lipid synthesis, recent studies have shown the role of increased fatty acids β-oxidation in PCa, pointing it as the dominant bioenergetic pathway in PCa [69, 161]. Data showed that PCa induces peroxisomal branched chain fatty acid β-oxidation, by increasing the overexpression and activity of enzymes involved in this pathway [69, 162, 163]. AMACR was shown to be

overexpressed in PCa tissues, comparatively to normal prostate tissues [129, 162, 163]. The strong association of AMACR with PCa led to its recognition as a PCa biomarker [163]. Posteriorly, we and others have shown that not only AMACR, but also key enzymes involved in peroxisomal β-oxidation, including DBP and ACOX3 were overexpressed in PCa (section 3.1. and [69, 192]).

Interestingly, it was observed that one of MCTs (MCT2), that are involved in the transport of monocarboxylates, including lactate, usually associated with glucose metabolism and overexpressed in cancer is mainly present at peroxisomes in PCa cells derived from localized tumour, suggesting a putative role in malignant transformation, through association with an increased β-oxidation level (section 3.1. and [192]). Moreover, tumour cells seem to take advantage from the peroxisomal transport machinery, targeting MCT2 to peroxisomes, via PEX19, probably to ensure higher rates of β-oxidation and to maintain the redox balance (section 3.1. and [182, 192]). Although MCTs are commonly associated with glucose metabolism, MCT2 seems to have a crucial role in malignant transformation of prostate cells. Its expression is more evident in PIN lesions and localized tumour, comparatively to non-tumour cells and metastasis (section 3.1. and [192]). Also, a clear change in peroxisome morphology across prostate malignant transformation was observed, correlated with MCT2's presence at this organelle, providing once more evidence for the involvement of these organelles in tumour initiation and progression (section 3.1. and [192]). Founded on these evidences it was suggested that the localization of MCT2 at peroxisomes is associated with malignant transformation.

In this study, we aimed to further unravel the importance of peroxisome metabolism and dynamics as well as the role of MCT2 in PCa.

Results

Peroxisome metabolism reflects the metabolic switch in prostate cancer malignant transformation and progression

It has previously been demonstrated that PCa induces peroxisomal branched chain fatty acid β-oxidation, increasing the expression of important proteins involved in this pathway (section 3.1 and [69, 192]). In order to further unravel the peroxisome-dependent pathways involved in PCa malignant transformation and progression, we evaluated the expression of key peroxisomal proteins involved in specific pathways, using different cellular models of PCa disease progression: PNT1A, 22Rv1 and PC3 cells. Our results (Fig.12) show an increased expression of the peroxisomal proteins PMP70, ACBD5, CAT, PEX5, PEX19, ACOX1,

ACOX3 in 22Rv1 cells (derived from localized tumour), comparatively to PNT1A (non-tumour cells), suggesting that PCa upregulates the transport of fatty acids (via PMP70), the contact sites with the ER (via ACBD5), the classical peroxisome proliferator-inducible and non-inducible pathways (via ACOX1 and ACOX3, respectively), ROS metabolism (via CAT) and the import of matrix and membrane peroxisomal proteins (via PEX5 and PEX19, respectively) (Fig.12). However, the expression levels of PMP70, ACBD5, CAT, PEX5, PEX19 and ACOX3 decrease in PC3 cells (derived from metastasis), suggesting that the corresponding pathways have a lower relevance with disease progression (Fig.12). Interestingly, the expression of ACOX1 increases with tumour progression, suggesting a more relevant role in metastasis, comparatively with early stages (Fig.12). In contrast, the expression of ACAA1 (responsible for the final thiolytic cleavage in peroxisomal β-oxidation) dramatically decreases in 22Rv1 cells (which may reflect a compensation by the SCPx), being recovered in PC3 cells. As peroxisomes and mitochondria cooperate in many metabolic pathways, we have also analysed the expression of mitochondrial proteins in the same cell models. Our results showed that the expression of CPT1, COXIV, ATP5A/B and TOM20 is increased in 22Rv1 cells, suggesting that PCa increases the transport of mitochondrial proteins (via TOM20), mitochondrial acyl-CoA transport (via CPT1) and energy production (via COXIV, ATP5A and ATP5B) (Fig.12). Once more, the decrease of the expression of these proteins (except TOM20) in PC3 cells, suggest their minor role in metastasis. The expression of VDAC1 is increased in PC3 cells, comparatively with PNT1A and 22Rv1 cells, suggesting a relevant role in metastasis (Fig.12).

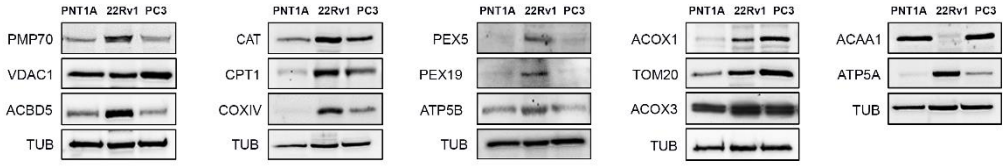

Figure 12. Peroxisome and mitochondria-associated proteins expression profile in PNT1A, 22RV1 and PC3 cells. Western blot analysis, showing the expression levels of PMP70, VDAC1, ACBD5, CAT, CPT1, COXIV, PEX5, PEX19, ATP5B, ACOX1, TOM20, ACOX3, ACAA1, ATP5A. Tubulin was used as loading control.

The presence of MCT2 at the peroxisomal membranes has a direct association with peroxisomal dynamics

We have previously shown that peroxisome morphology significantly changes across prostate malignant transformation, associated with the presence of MCT2 at this organelle (section 3.1. and [192]). In 22Rv1 cancer cells, this organelle presents an unusual morphology, appearing

somewhat elongated and in clusters (section 3.1 and [192]). Electron microscopy analyses have confirmed these results (Fig.13A).

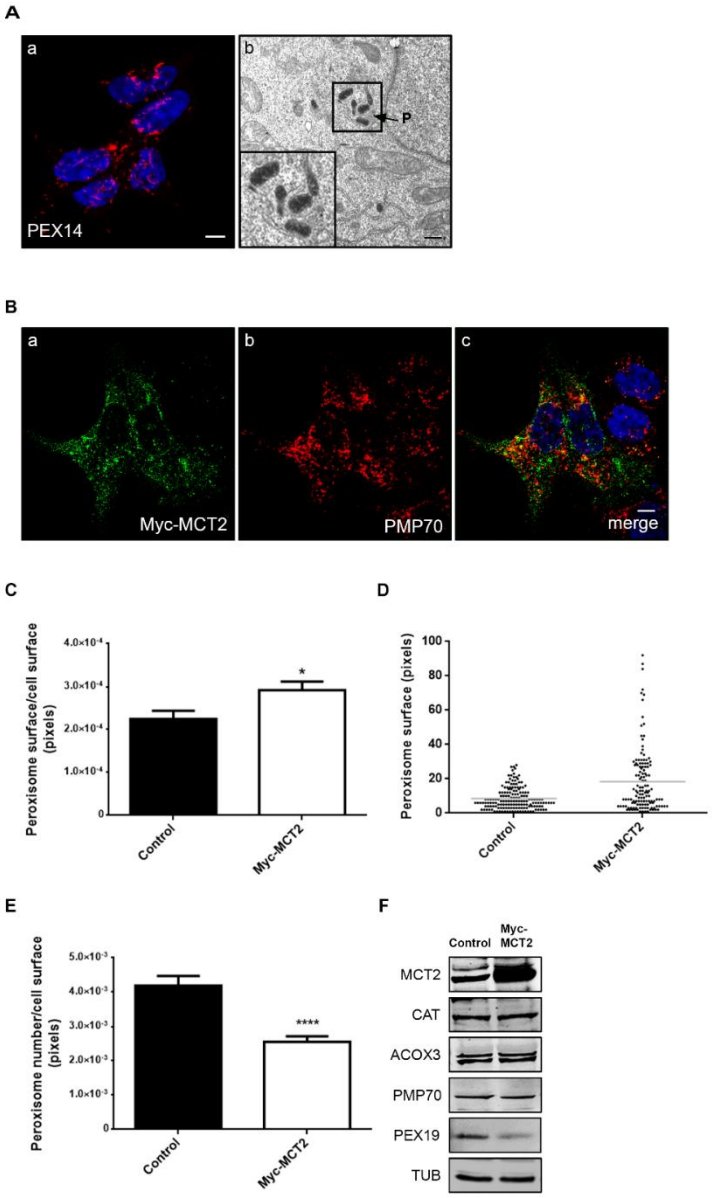

Figure 13. MCT2 overexpression increased peroxisome surface, decreased peroxisome number and did not affect the expression of key proteins involved in β-oxidation in 22Rv1 PCa cells: (A) Peroxisome clusters in 22Rv1 cells. (a) Immunofluorescence of PEX14; (b) DAB-staining based transmission electron microscopy. (P) peroxisomes. (A-C) Peroxisome morphology. (A) MCT2 intracellular localization in 22Rv1 control (a-c) and Myc-MCT2 transfected cells (d-f). (B,C) Results are presented as the mean of peroxisome surface per cell surface and the mean of peroxisome surface per cell, respectively. (D) Peroxisome number. Results are presented as the mean of peroxisome number per cell surface. Nuclei are shown in blue (stained with Hoechst 33258). White bars represent 5 μm and black bars 0.5 μm. Data represent means of three independent experiments and the bars represented SEM of the mean. *p<0.05 ****p<0.0001. (E) Western blot analysis, showing the expression levels of MCT2, CAT, ACOX3, PMP70, PEX19 and TUB in control and transfected cells.

In order to understand the role of MCT2 in peroxisome dynamics in prostate cancer we assessed the effect of MCT2 overexpression in 22Rv1 cells, where MCT2 mainly localizes at peroxisomes. With this propose, 22Rv1 cells were transfected with a Myc-MCT2 construct, which, as expected, was mostly targeted to peroxisomes (Fig.13B). Interestingly, the presence of this overexpressed protein at peroxisomes led to an increase of peroxisome surface, comparatively to non-transfected cells (Fig.13B-D). Fig 13D shows data from a representative transfected single cell, where an increase in the number of bigger peroxisomes can be observed, comparatively to a control cell. Furthermore, we have also noticed a decrease in peroxisome number in transfected cells, comparatively to control cells (Fig.13E). Our results clearly suggest that the presence of MCT2 at the peroxisomal membranes has a direct association with organelle's dynamics.

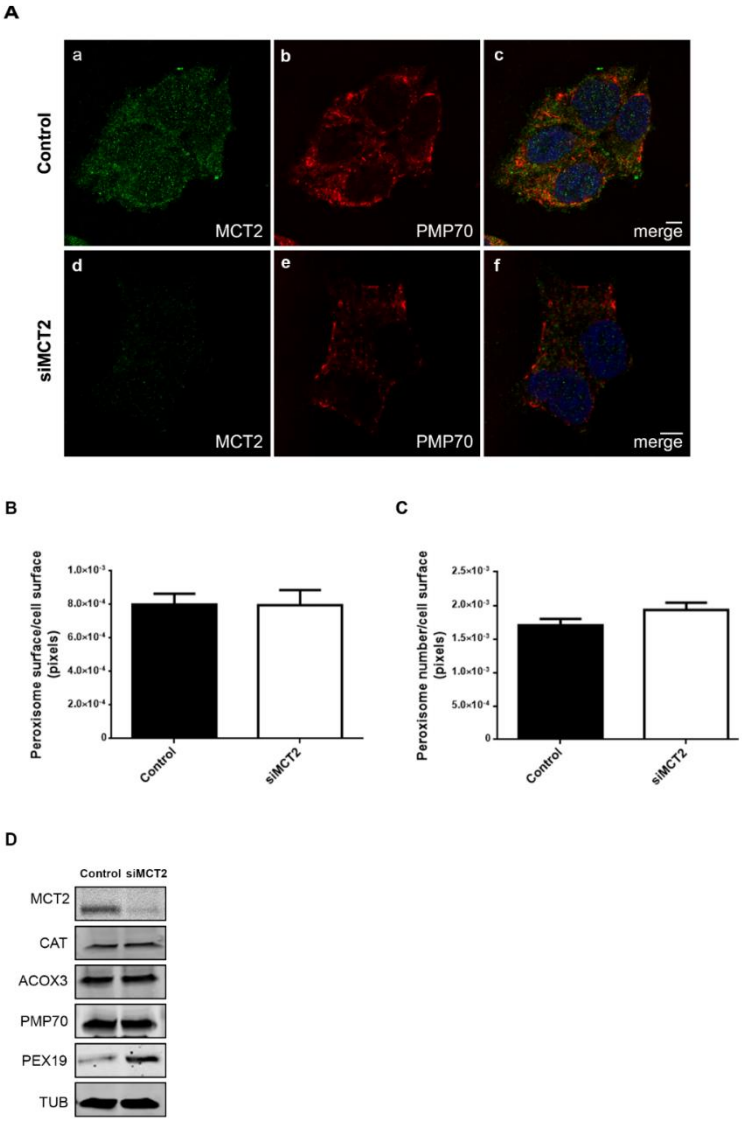

Figure 14. MCT2 knockdown did not interfere with peroxisome dynamics and the expression of key proteins involved in β-oxidation of 22Rv1 PCa cells: (A-B) Peroxisome morphology. (A) MCT2 intracellular localization in 22Rv1 control (a-c) and MCT2 knockdown cells (d-f). Nuclei are shown in blue (stained with Hoechst 33258). Bars represent 5 μm. (B) Results are presented as the mean of

peroxisome surface per cell surface. (C) Peroxisome number. Results are presented as the mean of peroxisome number per cell surface. Data represent means of three independent experiments and the bars represented SEM of the mean. (D) Western blot analysis, showing the expression levels of MCT2, CAT, ACOX3, PMP70, PEX19 and TUB in control and transfected cells.

As we suggested a putative role of peroxisomal MCT2 in malignant transformation, through association with β-oxidation levels (section 3.1. and [192]), we aimed to assess the effect of MCT2 overexpression in the expression of key proteins involved in peroxisomal β-oxidation. The overexpression of MCT2 did not affect the expression of CAT, ACOX3 or peroxisomal surface protein PMP70 (Fig.13F). Interestingly, the overexpression of MCT2 led to a decrease of PEX19 expression, which targets MCT2 to peroxisomes as previously shown (section 3.1. and [192], suggesting a putative regulatory function of MCT2 over PEX19.

We have also analyzed peroxisome morphology and number upon MCT2 knockdown by RNA silencing in 22RV1 cells. Our results showed no significant alterations on peroxisome morphology or number (Fig. 14A-C), as well as on the levels of peroxisome surface protein PMP70 (Fig.14D). These results indicate that the peroxisome unusual morphology typical from these cells, and likely caused by the presence of MCT2 at the organelle's membranes, was not reversed by the transient decrease of MCT2. It is also possible that the low levels of MCT2 that remained after the knockdown are sufficient to maintain this morphology.

We have also analysed the effect of MCT2 knockdown in the expression of key proteins involved in peroxisomal β-oxidation. Our results show that MCT2 knockdown does not interfere with the expression of CAT and ACOX3 (Fig.14D). Remarkably, the expression levels of PEX19 increase with MCT2 knockdown comparatively to control cells, the opposite of which has been observed upon MCT2 overexpression, suggesting once more a putative regulation of MCT2 over PEX19 (Fig.13D).

The localization of MCT2 at peroxisomes is associated with prostate cancer migration and proliferation

We have also evaluated the role of MCT2 in prostate cancer migration and proliferation, through MCT2 overexpression and knockdown in 22Rv1 cells.

MCT2 overexpression led to an increase in 22Rv1 cell motility ($p<0.05$). After 48h of wound creation, transfected 22Rv1 cells increased their migration capacity in about 40%, closing the wound faster than non-transfected cells (Fig.15A-C).

Our results also demonstrate that, upon MCT2 overexpression, there is an increase on cell proliferation ($p<0.05$), comparatively to non-transfected cells. There was an increase of approximately 20% of positive proliferating cells in transfected cells (Fig. 15D-F). As we observed in 22Rv1 cells that the majority of transfected Myc-MCT2 was targeted to

peroxisomes (Fig.13B) we aimed to substantiate that this increased cell proliferation was associated with the presence of MCT2 at this organelle. To that end, we assessed cell proliferation after the knockdown of the chaperone PEX19, inhibiting MCT2 trafficking to the peroxisomal membranes. Interestingly, the overexpression of Myc-MCT2 in the absence of PEX19 did not induce the increase on cell proliferation that had been observed in the presence of this chaperone (Fig.15E and F). These results clearly suggest that the localization at the peroxisomal membranes is essential for MCT2-dependent induction of cell proliferation in this cell line.

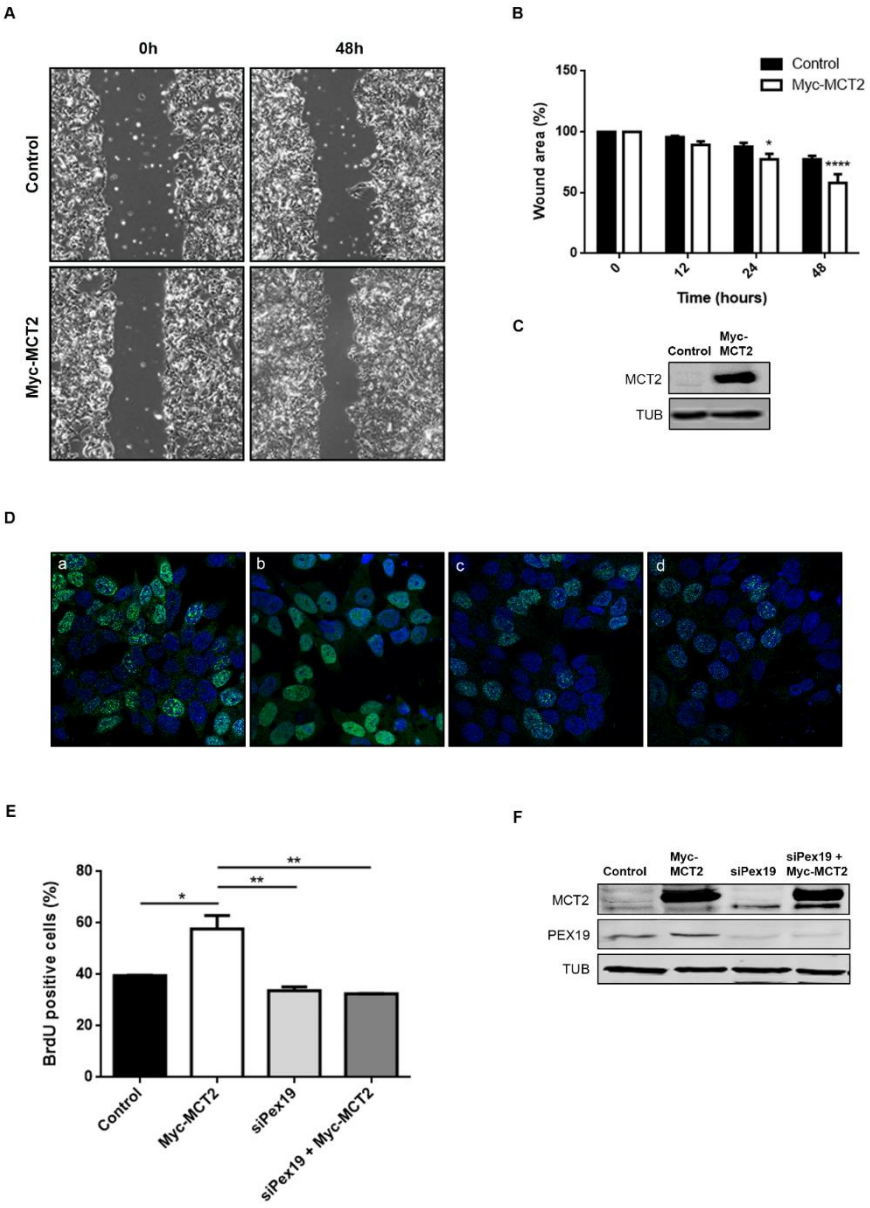

Figure 15. MCT2 overexpression increases 22Rv1 PCa migration and proliferation. (A-C) Cell motility. (A) Representative images of wound healing at 48h after scratch. (B) Cell motility is presented as the percentage of wound area, corresponding to reduction of initial scratch area. (C) Western blot analysis, showing the expression levels of MCT2 and TUB. (D-F) Cell proliferation. (D) Representative images

of proliferating cells, using anti-BrdU antibody (green). Nuclei are shown in blue (stained with Hoechst 33258). (E) Results are presented as percentage of BrdU positive cells. Data were the means of three independent experiments and the bars represented SEM of the mean. *p<0.05 **p<0.01 ****p<0.0001. (F) Western blot analysis, showing the expression levels of MCT2, PEX19 and TUB.

Upon MCT2 knockdown there was not significant differences in cells migration comparatively to control cells (Fig.16A-C). Interestingly, when we assess the effect of MCT2 knockdown on cell proliferation of 22Rv1 cells, we observe a drastic decrease (about 45%), comparatively to control cells, suggesting once more that MCT2 plays a role in PCa proliferation (p<0.05) (Fig.16D-F).

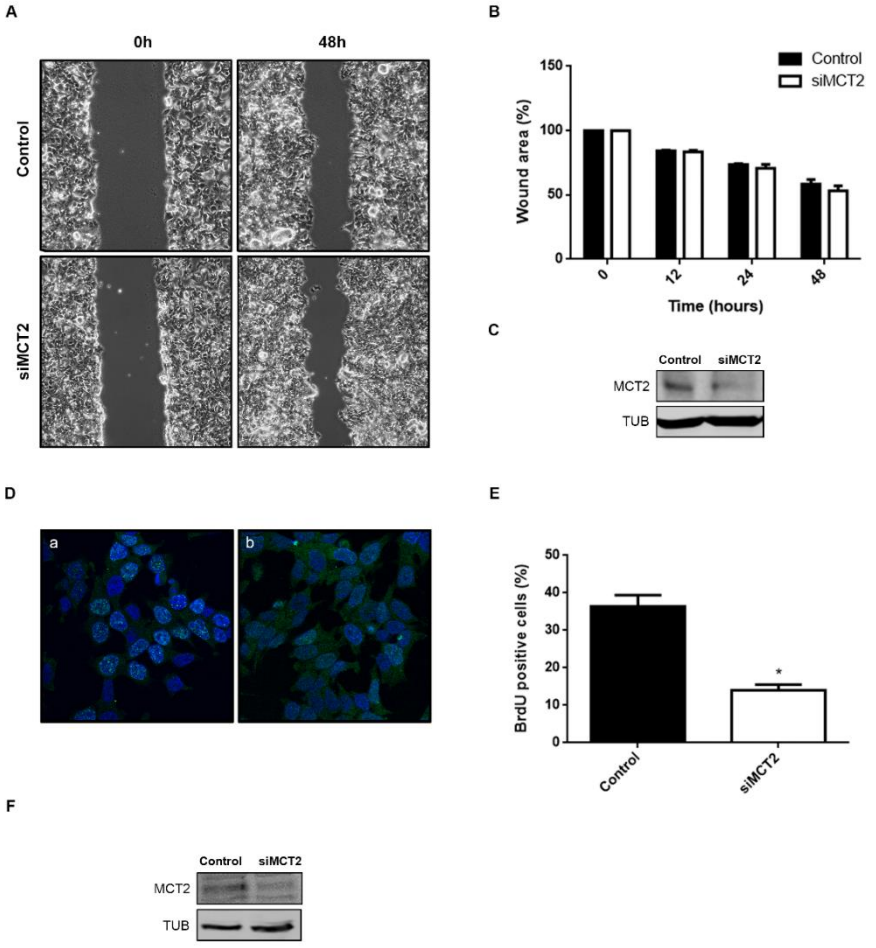

Figure 16. MCT2 knockdown decreases 22Rv1 PCa proliferation. (A-C) Cell motility. (A) Representative images of wound healing at 48h after scratch. (B) Cell motility is presented as the percentage of wound area, corresponding to reduction of initial scratch area. (C) Western blot analysis, showing the expression levels of MCT2 and TUB. (D-F) Cell proliferation. (D) Representative images of proliferating cells, using anti-5-bromo-2´-deoxyuridine (BrdU) antibody (green). Nuclei are shown in blue (stained with Hoechst 33258). (E) Results are presented as percentage of BrdU positive cells. Data were the means of three independent experiments and the bars represented SEM of the mean. *p<0.05. (F) Western blot analysis, showing the expression levels of MCT2 and TUB.

Glucose regulates MCT2 expression and its intracellular localization

Since PCa uses fatty acids as main energy source and take advantage of MCT2, which is usually associated with glucose metabolism (section 3.1. and [161, 192]), we aimed to understand the role of glucose in PCa.

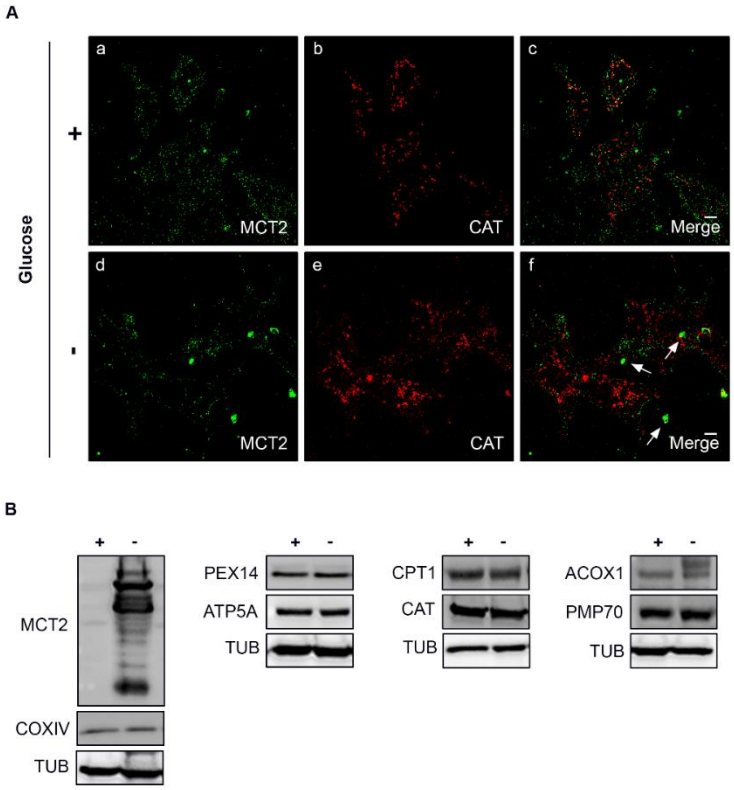

Figure 17. Glucose starvation interfere with intracellular localization and expression of MCT2 in 22Rv1 cells. (A) a–c: MCT2 intracellular localization in 22Rv1 control cells, (a) MCT2, (b) CAT and (c) merge image of a and b; d-f: Clustered MCT2 intracellular localization in starved 22Rv1 cells (arrows), (d) MCT2, (e) CAT and (f) merge image of d and e; Bars represent 5 µm (B) Western blot analysis showing the expression levels of MCT2, COXIV, PEX14, ATP5A, CPT1, CAT, ACOX1, PMP70 and TUB in control cells (+) and in starved cells (-).

We performed a 24h glucose starvation experiment in 22Rv1 cells and assessed the MCT2 intracellular localization and its expression, as well as the expression of peroxisomal and mitochondrial key proteins.

Surprisingly, our results showed that, upon glucose starvation, MCT2 does not localize at peroxisomes and remains clustered in the cell periphery (Fig.17A). MCT2's expression pattern is also altered in glucose starved cells (Fig.17B)

The expression of COXIV, PEX14, ATP5A, CPT1, CAT, ACOX1 and PMP70 seems no to be affected upon glucose starvation, comparatively with control cells (Fig.17B).

ACOX3 overexpression does not affect the expression of key peroxisomal proteins and prostate cancer proliferation

As we have shown that PCa induces peroxisomal branched chain fatty acid β-oxidation (section 3.1. and [192]), we aimed to evaluate the effect of ACOX3 overexpression in the expression of MCT2 and CAT, as well as its effect on cell proliferation of 22Rv1 cells.

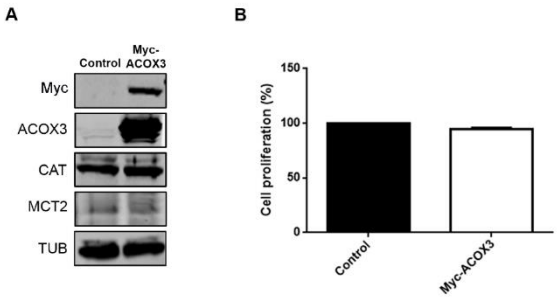

Figure 18. ACOX3 overexpression did not affect the expression of peroxisomal proteins and 22Rv1 cell proliferation. (A) Western blot analysis, showing the effect of ACOX3 overexpression in the expression levels of MCT2, CAT, ACOX3, PMP70, PEX19. TUB was used as loading control. (B) Effect of ACOX3 overexpression in cell proliferation. Results are presented as percentage of cell proliferation. Data were the means of three independent experiments and the bars represented SEM of the mean.

To that end, we have constructed a Myc-ACOX3 plasmid and transfected it in 22 RV1 cells. Our results showed that ACOX3 overexpression did not interfere with the expression of MCT2 and CAT, comparatively to control cells (Fig.18A). Also, cell proliferation was not affected.

Peroxisomal MCT2 localization in liver and cervix cancers

MCT2 expression in human cancers is always less evident than in PCa and reported to be mainly cytoplasmic. We aimed to analyse a putative localization of MCT2 at peroxisomes in some cancers that display high/moderate MCT2 expression [183, 193] and have selected two cancer cell lines derived from liver (HepG2) and cervix (HeLa).

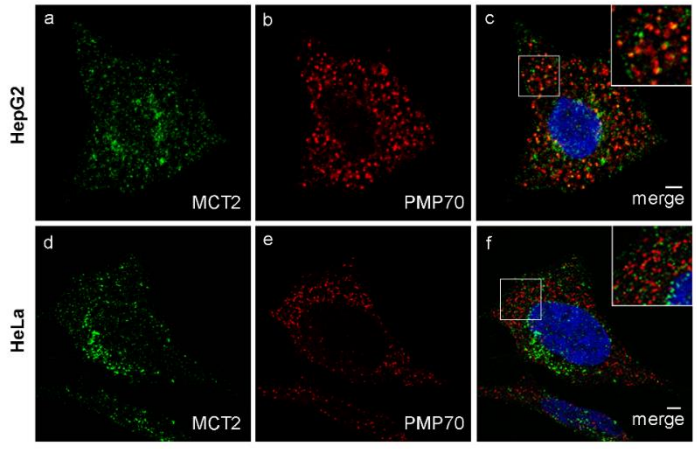

Figure 19. MCT2 localizes at peroxisomes of liver and cervix cancers. (a–c) MCT2 intracellular localization in HepG2 cells, (a) MCT2, (b) PMP70 and (c) merge image of a and b; (d–f) MCT2

intracellular localization in HeLa cells, (d) MCT2, (e) PMP70 and (f) merge image of d and e. Arrows indicate some of the co-localization sites. Nuclei are shown in blue (stained with Hoechst 33258). Bars represent 5 μm

Immunofluorescence analyses showed a cytoplasmic and peroxisomal MCT2 in all the observed cancer cells (Fig.19). However, MCT2 localization was more evident in HepG2, comparatively with HeLa cells.

Discussion

From malignant transformation to progression, PCa displays a dynamic metabolism, remodelling it according to the requirements of each tumour stage. Apparently, lipids support cancer cells demand in early tumour stage and glucose steals the spotlight in late tumour stage. The aberrant PCa lipid metabolism focused the attention on peroxisomes, dynamic organelles that adapt to cellular and/or environmental stimulus, modifying their size, number, morphology and function. We have previously suggested that malignant transformation is directly related to alterations in peroxisome morphology and to the presence of MCT2 at the organelle's membranes, contributing to a redox shuttle system which supports β-oxidation and maintains redox balance (section 3.1 and [192]).

Here, we show that the metabolic switch along PCa initiation and progression is supported by the expression levels of key proteins involved in lipid metabolism. The increased expression of PMP70, ACBD5, CAT, PEX5, PEX19, ACOX1, ACOX3 in 22Rv1 cells, comparatively to non-tumour cells, suggests that PCa take advantage from lipid metabolism, upregulating the peroxisomal transport of fatty acids (via PMP70), the contact sites with ER to transfer fatty acids (via ACBD5) and the classical peroxisome proliferator-inducible and non-inducible pathways (via ACOX1 and ACOX3, respectively). Furthermore, there is also an increase in ROS metabolism (via CAT) as well and in the import of matrix and membrane peroxisomal proteins (via PEX5 and PEX19, respectively), to ensure higher peroxisome metabolic capacity. Although we did not assess the expression levels of mitochondrial β-oxidation enzymes, the increased expression of CPT1, that is involved in the transport of acyl-CoAs to mitochondria, suggests stimulation of this pathway. In PC3 cells, the expression levels of peroxisomal PMP70, ACBD5, CAT, PEX5, PEX19, ACOX3 and mitochondrial CPT1, COXIV and ATP5A/B decrease, suggesting that their respective pathways cease to be relevant with disease progression, in accordance with the switch to the Warburg effect that is observed at this stage [141]. Interestingly, the expression of ACOX1 increases with tumour progression,

suggesting that although late stages rely on Warburg effect, the classical peroxisome proliferator-inducible pathway seems to be also relevant at these stages.

The deregulation of apoptosis and metabolism in many cancers have been associated with upregulation of VDAC1 [194]. Our results showed an increase of VDAC1 expression in PC3 cells. In fact, the association of VDAC1 with the glycolytic enzymes hexokinases is associated with the rapid growth of malignant cells, increased glucose metabolism and inhibition of apoptosis [195, 196].

Our results suggest that MCT2 localization at the peroxisomal membranes directly affects the organelle's dynamics as, upon MCT2 overexpression, we observed an increase of peroxisomal surface and a decrease in peroxisome number. It is, hence, tempting to conclude that MCT2 might be responsible for the elongated and clustered morphologies that are characteristic of the peroxisomes in 22Rv1 cells. MCT2 knockdown did not reverse the organelle's morphologies, perhaps also due to the presence of a residual amount of the protein at the peroxisomal membranes. Our results also show that MCT2 overexpression did not affect the expression of key proteins involved in peroxisomal β-oxidation, suggesting that MTC2's association with increased β-oxidation rates is not related with a specific increase in expression of the involved proteins.

Remarkedly, our results showed an increase of PEX19 expression after MCT2 knockdown and a decrease after MCT2 overexpression, suggesting that MCT2 negatively regulates PEX19. Also, our results from the proliferation experiments showed that the knockdown of PEX19 leads to an increase of MCT2 and PEX19 overexpression leads to a decrease of MCT2 (data not shown). These evidences suggest a reciprocal negative regulation between MCT2 and PEX19 and further studies are needed to address its relevance for PCa.

Importantly, our results show that MCT2 is associated with PCa migration and proliferation. Knockdown of MCT2 in 22Rv1 cells decreased the capacity to proliferate and MCT2 overexpression led to an increase of cell motity and proliferation. Interestingly, the peroxisomal localization of MCT2 seems to be required for PCa proliferation as, upon PEX19 knockdown, MCT2 overexpression was not able to increase the cells' proliferative capacity.

Although, in early stages, PCa relies on lipid metabolism, glucose seems to also play a role, as our results show that glucose deprivation leads to alterations on the MCT2 expression pattern as well as its intracellular localization, suggesting a regulatory role over MCT2. Evidences showed that in glucose starved cervix cancer cells, MCT1 forms stable and functional complexes with its chaperone CD147, being targeted to the plasma membrane to promote migration [197]. However, this mechanism in PCa remains unclear.

The induced peroxisomal branched chain fatty acid β-oxidation and the highjacking of MCT2 by PCa to its benefit, led us to evaluate the effect of ACOX3 overexpression on MCT2 and CAT expression, as well as in 22Rv1 cell proliferation. Our results showed that ACOX3 did

not lead to alterations in these pathways, possibly due to the already high endogenous expression of ACOX3 in these cells.

Our results indicate that MCT2 might also play an important role in other cancers besides PCa. The peroxisomal MCT2 localization in HepG2 and HeLa suggests that these cancers may also target MCT2 to this organelle to ensure high β-oxidation rates and a redox balance. The higher amount of MCT2 at peroxisomes might be associated with the increased β-oxidation levels previously observed in liver and brain cancers [127, 129].

Altogether, our results highlight the importance of the interplay between peroxisomes and MCT2 in PCa, exposing a range of possible targets for PCa therapy.

Materials and methods

Antibodies

In these experiments, the following antibodies were used: MCT2 (sc-50322; Santa Cruz Biotechnology, Santa Cruz, USA), PEX14 (a gift from Dr. Dennis Crane, Griffith University, Brisbane, Australia), CAT (ab88650; Abcam, Cambridge, UK), PMP70 (SAB4200181; Sigma-Aldrich, St. Louis, USA), ACOX1 (a gift from A. Völkl, University of Heidelberg, Germany), ACOX3 (HPA035840, Sigma-Aldrich), PEX19 (Sigma-Aldrich, St. Louis, USA), α-TUB (T9026, Sigma-Aldrich, St. Louis, USA), α/β-TUB (2148, Cell signalling Technology, Beverly, Massachusetts, USA), PEX5 (a gift from Dr. Jorge Azevedo, University of Porto, Portugal), CPT1 (sc-514555, Santa Cruz Biotechnology, Santa Cruz, USA), BrdU (M0744, Dako, Denmark), TOM20 (612278, (BD Bioscience, San Jose, California, USA), COXIV (ab33985, Abcam, Cambridge, UK), VDAC1 (ab15895, Abcam, Cambridge, UK), ACBD5 (HPA012145, Sigma-Aldrich, St. Louis, USA), ACAA1 (a gift from A. Völkl, University of Heidelberg, Germany), ATP5A (612516, BD Bioscience, San Jose, California, USA) and ATP5B (ab5432, Abcam, Cambridge, UK), TRITC (Jackson ImmunoResearch, West Grove, USA) and Alexa 488 (Invitrogen, Life Technologies, Carlsbad, USA), HRP (BioRad, Hercules, California, USA), IRDye 800CW and IRDye 680RD (LI-COR Biotechonology, Cambridge, UK).

Cloning Myc-MCT2 and Myc-ACOX3

The following primer sequences, including sites for *BamHI* and *XhoI*, were used to amplify the coding sequences of MCT2 from human 22Rv1 cells: 5’ CGGGATCCAATGCCACCAATGCCAAGTG 3’(forward) and 5’ CCGCTCGAGTTAAATGTTAGTTTCTCTTTCTG 3’ (reverse). To obtain an N-terminal Myc-tagged MCT2 construct, *BamHI* and *XhoI* were used to clone the cDNA in pCMV-Tag 3a vector (Agilent Technologies, La Jolla, USA). For the construction of Myc-ACOX3, it was used

5’ AGGCTAAGCTTATGGCATCCACTGTGGAAGGA 3’ (forward) and 5’ G’CGTGTCGACCTAGAGCTTCGATTTCAGACTTCC 3’ (reverse) primers sequences to amplify the coding sequences of human ACOX3. The insertion of cDNA in pCMV-Tag 3b vector (Agilent Technologies, La Jolla, USA) was performed with *HindIII* and *SalI* restriction enzymes. Clones were verified by DNA sequencing.

Cell culture and transfection

PNT1A, 22Rv1 and PC3 were seeded in RPMI-1640 (Gibco, Invitrogen, Carlsbad, USA) supplemented with 10% of foetal bovine serum (Gibco, Invitrogen, Carlsbad, USA), 1% of antibiotic (penicillin/streptomycin) (Gibco, Invitrogen, Carlsbad, USA) and incubated at 37°C in an atmosphere containing 5% CO_2. HepG2 and HeLa cells were seeded in DMEM (Gibco, Invitrogen, Carlsbad, USA) supplemented with 10% of foetal bovine serum (Gibco, Invitrogen, Carlsbad, USA), 1% of antibiotic (penicillin/streptomycin) (Gibco, Invitrogen, Carlsbad, USA) and incubated at 37°C in an atmosphere containing 5% CO_2. All cell lines were cultivated under the same experimental conditions and observations were made at about 70% cell confluence, except for wound healing experiments, where it was 90-100%.

In overexpression analyses, 22Rv1 cells were transfected with Myc-MCT2 or Myc-ACOX3 using Lipofectamine 3000 (Invitrogen, Waltham, Massachusetts, USA) and collected after 24h. MCT2 and PEX19 knockdown in 22Rv1 cells was performed using Lipofectamine RNAiMAX (Invitrogen, Waltham, Massachusetts, USA). Two different *SLC16A7* siRNA, S17574 and S17572 (Ambion, Inc, Austin, TX) were incorporated at final concentration of 50 nM. For PEX19 knockdown S11612 (Ambion, Inc, Austin, TX) was used. The transfections were performed according to the manufacturer’s instructions and cells were collected after 48h.

For glucose starvation, 22Rv1 cells were cultured in same conditions as described above and after 24h cells were starved for 24h in RPMI-1640 with no glucose (Gibco, Invitrogen, Carlsbad, USA).

Immunofluorescence and microscopy techniques

Immunofluorescence analyses were performed by seeding cells on glass cover slips that were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 20 min. Afterwards, cells were permeabilized with 0.2% Triton X-100 for 10 min., blocked with 1% BSA solution for 10 min and incubated with primary and secondary antibodies for 1h each. Between each step, cells were washed three times with PBS, pH 7.4. Lastly, cells were stained with Hoechst 33258 (PolySciences, Warrington, FL, USA) and mounted in slides using Mowiol 4-88 containing n-propylgallate. Images were obtained using a Zeiss LSM 510 Meta Confocal setup (Carl Zeiss, Jena, Germany) equipped with a plan-Apochromat 100×/1.4 oil objective and a Nikon 90i

upright microscope mounted with Plan Apo 100×/1.45 NA oil objective for the glucose starvation experiments (Nikon). Image analyses for peroxisome number and surface quantifications were obtained through Spot detector plugin from Icy software [198].

For electron microscopy analyses cells were seeded on glass cover slips and fixed in 4% formaldehyde, 0.05% glutaraldehyde, 2% sucrose in PBS, pH 7.4 for 1h, at room temperature. Following a PBS pH 7.4 rinse, cells were further fixed with 1.5% glutaraldehyde in PBS pH 7.4 for 30 min, at room temperature. After it was performed the DAB staining, following Bonekamp *et al*. [199] and cells were post-fixed in 1% osmium in PBS for 1h on ice and in the dark and then en-bloc stained with 2% uranyl acetate in distilled water for 30 min on ice and in the dark. Cells were finally taken through a graded ethanol dehydration, embedded in EPON resin and polymerized at 60ºC, overnight. 70 nm sections were cut on a UC7 Ultramicrotome (Leica), picked up on formvar coated slot grids and post-stained with uranyl acetate for 5 min and lead citrate for 5 min. Sections were imaged in a H7650 transmission electron microscope (Hitachi, Japan) operated at 100keV and images were recorded on XR41M mid mount AMT digital camera.

The wound healing was monitored in a phase contrast microscopy with a 10x objective in Leica DMI6000 epifluorescence microscope (Germany) equipped with an Orca Flash 4.0v2.0 camera (Hamamatsu, Japan).

Immunoblotting

Cells were lysed with specific lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM sodium chloride, 0.5% sodium deoxycholate, 0.5% Triton X-100 and a protease-inhibitor mix). To improve protein extraction, samples were passed 20 times through a 26-gauge syringe needle and incubated on a rotary mixer at for 30 min. at 4°C. After cleared by centrifugation (17,000 × g, 15 min), protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, California, USA). Blots were incubated with the specific primary antibodies and detected by HRP using an enhanced chemiluminescence system (GE Healthcare, Waukesha, WI, USA) or fluorescent IRDyes.

Cell motility

Cell motility of 22Rv1 cells was assessed using wound healing assay. A total of $7x10^5$ cells were plated 6-well plates and transfected with Myc-MCT2 or MCT2 silencing. Scratching of overexpressed or silenced cells was performed after 24h for MCT2 overexpression and 48h for MCT2 knockdown. The wound healing was monitored each 30 min over 48h.

Cell proliferation

To evaluate the role of MCT2 in proliferation of PCa, 22Rv1 cells were seeded on glass cover slips for 24h and after MCT2 overexpression or knockdown, cell motility was assessed using BrdU incorporation assay. After 24h of cell cultivation, cells were incubated with 10µM BrdU (10280879001, Sigma-Aldrich, St. Louis, USA) for 1h at 37ºC, 5% CO_2. After incubation, cells were fixed with 4% paraformaldehyde for 30 min and incubated in 2M HCl for 20 minutes at room temperature, for DNA denaturation. Then cells were incubated with primary and secondary antibodies for 30 min, stained with Hoechst 33258 and mounted in slides using Mowiol 4-88 containing n-propylgallate. Between each step before denaturation, cells were washed three times with PBS and after denaturation with PBS-T-B (PBS, supplemented with 0.5% Tween 20 and 0.05% bovine serum albumin), pH 7.4. The BrdU-positive cells were counted under the confocal microscope.

Statistical analyses

Statistical analysis was performed in Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, California, USA), using Student's t-test for comparison between two groups and one-way ANOVA, followed by Bonferroni's for multiple comparison; Data are presented as mean ± standard error mean (SEM). P values of ≤0.05 were considered as significant.

4. General Discussion and Future Perspectives

Peroxisomes are versatile and highly dynamic organelles that are involved in a wide range of metabolic pathways, being essential for human health and development. Several studies have associated peroxisomes to severe metabolic disorders as well as age-related disorders, including cancer [99, 103].

Over the years, several studies showed evidences of a direct association between peroxisomes and several types of cancer, including breast, colon, thyroid, colorectal, liver, brain, bladder, kidney, ovarian and PCa [105, 106]. However, the precise role of peroxisomes in cancer is still unknown.

The aberrant lipid metabolism of PCa concentrated the attention on peroxisomes [191]. PCa is the second most frequent cancer and the fifth leading cause of cancer death worldwide in men, mainly due to its late detection that dramatically decreases survival rates [136, 200] .

PCa exhibits a unique metabolic profile, which is remodelled according to tumour stage. Contrasting with other types of cancer, at early stages, PCa relies on lipids to support the cancer cells energetic demands and only switches to the “Warburg effect” at late stages of the disease [141].

Pértega-Gomes *et al*, have shown that one of the MCTs (MCT2), that are involved in glucose metabolism and usually overexpressed in cancer, is overexpressed and localized in clusters in early stages of PCa [175].

Here, we showed for the first time the presence of MCT2 at the peroxisomes of PCa cells, suggesting a possible role for peroxisome-related mechanisms in prostate malignant transformation (section 3.1.). Our results showed that PCa takes advantage of the peroxisomal membrane proteins transport machinery to target MCT2 to peroxisomes. MCT2 has previously been associated with increased rates of β-oxidation and the maintenance of redox balance [182]. Here we suggest that, also in PCa, MCT2 might be associated with increased peroxisomal β-oxidation levels. However, this does not seem to be directly correlated to an increased expression of peroxisomal proteins involved in β-oxidation (section 3.2.). In order to substantiate our results, β-oxidation rates, upon MCT2 knockdown and overexpression, should be evaluated.

Subsequent experiments showed a direct association of MCT2 with PCa migration and proliferation. MCT2 knockdown led to a decrease on proliferation levels and MCT2 overexpression increased the motility and proliferative capacities of PCa cells. Remarkably, our results showed that the MCT2 localization at peroxisomes is essential for PCa proliferation, as the interruption of its targeting to this organelle, upon PEX19 knockdown, led to the inability of MCT2 overexpression to promote PCa proliferation (section 3.2.).

In section 3.1. we showed a clear change in peroxisome morphology across prostate malignant transformation correlated with MCT2 presence at this organelle, providing once

more evidence for the involvement of these organelles in tumour initiation and progression. In non-tumour cells, where no MCT2 was present at peroxisomes this organelle exhibits a regular round morphology. However, in 22Rv1 cells, where MCT2 was mainly observed at peroxisomes, this organelle appears elongated and in clusters. In PC3, the highly metastatic model, peroxisomes appear similar to the ones in the non-tumour cells. As peroxisome dynamics and morphology play important roles in cell pathology, and defects on these machineries may lead to significant implications in health and disease [86] , we aimed to study the effect of MCT2 in peroxisome morphology and number in cells derived from localized tumour. Interestingly our results showed a direct association of MCT2 with peroxisome morphology and number, pointing a role for MCT2 on peroxisome morphology changes (section 3.2.). Whether the 22RV1 cells atypical peroxisome phenotype is beneficial for cancer cells is not yet clear, however, evidences of peroxisomes cluster in other cell models, suggest that clusters might represent functional units of peroxisomes, which interact and cooperate via contact sites [201]. To elucidate the role of peroxisome clusters in PCa, it would be important to manipulate peroxisome dynamics (promoting peroxisomal fragmentation and elongation) and evaluate β-oxidation rates, as wells as PCa proliferation and migration.

Our results have shown an increase in the expression of several proteins, involved in important peroxisomal pathways (PMP70, ACBD5, CAT, PEX5, PEX19, ACOX1 and ACOX3) in 22RV1 cells, as well as in prostate cancer tissues (AMACR, ACOX3 and DBP) (sections 3.1. and 3.2), comparatively to non-tumour cells or BT. We have also observed an increase the mitochondrial ATP5A/B and COXIV, comparatively to non-tumour cells. Furthermore, the increased levels of CPT1 in these cells suggests that also mitochondrial β-oxidation is upregulated in PCa. To validate our results, the expression of mitochondrial β-oxidation proteins should be assessed.

Additionally, we assessed the expression levels of peroxisomal and mitochondrial proteins in metastasis-derived cancer cells. Our results showed a decrease in the expression of peroxisomal PMP70, ACBD5, CAT, PEX5, PEX19, ACOX3 and mitochondrial proteins CPT1, COXIV, ATP5A/B, suggesting that the pathways that these proteins are involved in cease to be relevant with disease progression, being in accordance with the switch to Warburg effect that is observed in this stage [141]. Despite the switch to Warburg effect, metastasis seem to also induce the classical peroxisome proliferator-inducible pathway, reflected in the increased expression of ACOX1, comparatively to non-tumour cells and cells derived from localized tumour (section 3.2.). Also, the increased expression of MCT1 and 4 in metastasis substantiate the switch to glycolytic metabolism (section 3.1.).

In this study we demonstrated that MCT2 is targeted to peroxisomes, through interaction with PEX19 (section 3.1.). In addition to MCT2-PEX19 interaction, our results suggest a reciprocal negative regulation of MCT2 and PEX19 (section 3.2.), raising important questions: could

PEX19 be downregulated when MCT2 is abundant in the cell, as its transport to the peroxisomes is less required? Could PEX19 be upregulated to ensure the targeting of MCT2 to peroxisomes when MCT2 is limited? Further experiments should be performed in order to answer these questions.

Furthermore, our study suggests that MCT2 is regulated by glucose, since upon its deprivation, MCT2 expression pattern and intracellular localization are altered. As De Saedeleer *et al* reported that, in glucose starved cervix cancer cells, MCT1 forms stable and functional complexes with its chaperone CD147, being targeting to plasma membrane to promote migration [197], the expression of the standard MCT2 chaperone, Gp70, should be assessed under glucose starvation, as well as its interaction with MCT2. Also, PCa migration, under this condition, should be evaluated to verify if MCT2 is able to promote cell migration.

Notably, our results show that MCT2 may also play an important role in other cancers beyond PCa. The peroxisomal MCT2 localization in HepG2 and HeLa cells suggests that these cancers might also rely on β-oxidation, targeting MCT2 to peroxisomes to ensure high β-oxidation and a redox balance. To understand and elucidate the role of the peroxisomal MCT2 in these cancers, the same approaches as those used in section 3.1. and 3.2., should be applied.

The contradictory evidences have been hampered the understanding of peroxisomes' role in cancer. Although some tumours seem to be favoured by the absence of peroxisomes, this organelle has also been shown to contribute to cancer progression [105, 106]. To understand the role of peroxisomes in cancer, it is imperative to consider the intra- and inter-tumour heterogeneity, as well as the differences between cancer cells and tissues in distinct tumour stages. Nevertheless, our results have highlighted an important role for peroxisomes in PCa and suggest MCT2 as a possible target for the development of novel therapies against this disease.

5. Final Remarks

Concluding remarks

With this work, we provided clear evidences that the interplay between peroxisomes and MCT2 plays an important role in prostate malignant transformation.

We demonstrated that MCT2 localizes mainly at peroxisomes in PCa cells and that PCa take advantage of the peroxisomal transport machinery to target this protein to to peroxisomes via Pex19. We also showed that the increased MCT2 expression from non-malignant to malignant cells is directly correlated with its peroxisomal localization and with the increased expression of key proteins involved in peroxisomal β-oxidation.

We have also demonstrated that the presence of MCT2 at peroxisomes leads to alterations in peroxisome dynamics, increasing peroxisome surface and decreasing peroxisome number. Furthermore, we demonstrate that MCT2 promotes PCa migration and, remarkably, that its localization at peroxisomes is required for PCa proliferation.

Moreover, our results show that PCa, in early stages, takes advantage from the peroxisomal lipid metabolism, upregulating the peroxisomal transport of fatty acids, the contact sites with ER to transfer fatty acids, the classical peroxisome proliferator-inducible and non-inducible pathways, ROS metabolism and the import of matrix and membrane peroxisomal proteins, to ensure higher peroxisome metabolic capacity.

Notably, our results show the presence of MCT2 in liver and cervix cancer cells, suggesting that MCT2 may also play an important role in other cancers besides PCa.

Altogether, our results highlight the importance of the interplay between peroxisomes and MCT2 in PCa, exposing a range of possible targets for PCa therapy.

6. References

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