UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Prostate cancer cells metabolism on the interplay of androgenic regulation and metabolic environment

Henrique José Matos Morão Mingote Cardoso

Tese para obtenção do Grau de Doutor em
Biomedicina
(3º ciclo de estudos)

Orientador: Prof. Doutora Sílvia Socorro
Co-orientador: Prof. Doutora Patrícia Madureira

Covilhã, Janeiro de 2020

«Sei que tenho um cancro e que um dia me vai vencer.
Mas esse dia não chegou e até lá
Tenciono continuar a aproveitar cada momento.
Tive várias derrotas na vida,
Mas de todas as vezes caí de pé.
É preciso nunca deixar de viver.

A vida continua e continuarei a vivê-la,
Todos os dias, sem nunca me distrair

Eu provavelmente morrerei da doença
Mas o que nunca acontecerá é a doença matar-me»

Passados 5 anos de ter colocado esta citação na dissertação, reafirmo e dedico: a todos os investigadores que continuam a acreditar que um dia daremos mais esperança a todos os lutadores desta doença. "Juntos, somos mais fortes, seremos o céu que abraço o mundo"

Dedico às pessoas que amo e me possibilitaram hoje estar aqui!

Agradecimentos

O espaço que tenho nesta secção não é suficiente para agradecer a todas as pessoas que ao longo deste Doutoramento, direta ou indiretamente, me ajudaram a terminar esta fase da minha vida. Por isso se esquecer alguém não deixo de ter um agradecimento profundo.

A vida é feita de oportunidades e de lutas e por isso quero expressar o meu profundo agradecimento à minha orientadora Professora Doutora Sílvia Socorro. Sempre acreditou nas minhas capacidades, deu-me o suporte e apoio fundamental, mesmo quando não correu bem, ou ouvi um não como resposta. Quero também agradecer por toda a dedicação prestada, principalmente nas difíceis correções que toda a tese exigiu. De todas as vezes que fiquei frustrado e me deu-me uma palavra de incentivo, um muito obrigado, não me deixou desistir.

À Professora Doutora Patrícia Madureira queria agradecer pela aceitação da co-orientação deste projeto, pelas opiniões relevantes que deu, bem como a disponibilidade para ajudar quando foi necessário. Quero ainda agradecer à Doutora Vilma Sardão por todo o apoio prestado na realização e interpretação dos resultados do Seahorse.

A todas as pessoas do Centro de investigação em Ciências da Saúde, em especial à Sofia Duarte, Margarida Carrilho pela disponibilidade que sempre demonstraram.

Agradeço ainda aos meus colegas do laboratório que de uma forma ou outra me foram ajudando ao longo destes anos no laboratório Ana Manuela Silva, Ricardo Marques, Luís Rato, Carlos Gaspar, Margarida Grilo, Luís Espínola, Gonçalo Silva, Sara Gonçalves, Inês Mateus, Mariana Feijó, Joana Monteiro, Luís Brás, Catarina Serra, Lara Fonseca. Um agradecimento especial à Doutora Cátia Vaz e Doutora Sara Correia porque tudo o que aprendi no laboratório há uns anos atrás, sem elas não tinha sido possível. Um agradecimento pela partilha de conhecimento que foram feito ao longo destes anos. Um agradecimento também à Micaela Almeida por sempre acreditar nas minhas capacidades e pelos momentos de partilha e frustrações. Por último, mas não menos importante quero agradecer ao Tiago Carvalho e à Marília Figueira, sem vocês chegar aqui tinha sido bem mais difícil. Pelas noitadas que me ficaram a ajudar, pelas vezes que me levantaram nos momentos difíceis, e pela felicidade das vitórias conseguidas. Um muito obrigado pela amizade!

Não posso também fugir de agradecer à loucura que ao longo deste processo aconteceu, que foi a criação da inSci! Quero agradecer às minhas sócias que tiveram a compreensão de me ajudar na empresa quando eu não consegui conciliar com o resto das tarefas, cresci muito com vocês e espero assim continuar. Um obrigado Margarida Gonçalves, Cátia Vaz, Marília Figueira e Micaela Almeida.

De seguida quero agradecer à amizade especial de algumas pessoas fundamentais ao longo deste tempo, que não posso enumerar aqui todas, mas um especial obrigado Duarte Diogo e Óscar Nunes, obrigado por me empurrarem sempre para lutar!

Nenhuma casa se constrói sem alicerces! A minha família foi tudo mais que isso, deu-me o suporte todos os dias, estou eternamente grato. Aos meus pais que me deram esta educação que tanto me orgulho, e por serem um exemplo. À minha avó, que sempre foi a minha segunda mãe, que esteve ao meu lado desde que nasci e nunca deixou de estar lá para quando mais precisei. À minha irmã, que longe ou perto esteve sempre comigo, com a palavra certa de força e amizade, e porque trouxe duas princesas que tanto me fizeram sorrir e deixar com mais força para a próxima etapa. À minha Prima Odete que é uma força que me protege sempre com o seu abraço e ao Tio Humberto que sei que gostaria de ver esta tese, mas estará sempre a olhar por mim. Quero também agradecer à Daniela, pela paciência de me aturar, sei que fui insuportável em alguns dias, mas acima de tudo, foste a única pessoa que nunca me deixou ter um discurso derrotista, partilhaste comigo sorrisos, choradeiras, loucuras, mas acima de tudo ensinas-me a saber saborear a vida com a máxima intensidade! Foste o meu apoio, deste-me sempre a palavra certa no momento certo, e fez-me sempre feliz com todo esse amor que sobressai dos teus olhos!

Por último gostaria de agradecer à FCT pelo financiamento (SFRH/BD/111351/2015).

List of publications

Publications included in this thesis:

- **Cardoso HJ**[#], Vaz CV[#], Carvalho TM, Figueira MI, Socorro S. Tyrosine Kinase Inhibitor Imatinib Modulates the Viability and Apoptosis of Castrate-Resistant Prostate Cancer Cells Dependently on the Glycolytic Environment. Life Sci. 2019 1;218:274-283. doi: 10.1016/j.lfs.2018.12.055.
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- **Cardoso HJ**, Figueira MI, Carvalho TM, Vaz CV, Madureira PA, Oliveira PJ, Sardão VA, Socorro S. Glutaminolysis is a metabolic route essential for survival and growth of prostate cancer cells, and a target of 5α-dihydrotestosterone regulation. Submitted to Cellular Oncology. 2019

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Resumo

O cancro da próstata (PCa, "*prostate cancer*") é um dos cancros que mais frequentes nos homens, representando a quinta maior causa de morte por cancro na população masculina. Numa fase inicial da doença, a fase conhecida como sensível aos androgénios, a sobrevivência e proliferação das células tumorais dependem grandemente da ação estimuladora dos androgénios. Esta dependência é a característica que permite a utilização da terapêutica de privação androgénica (ADT, "*androgen-deprivation therapy*"), a qual tem por base a redução dos níveis circulantes de androgénios ou o bloqueio das suas ações. A administração contínua desta terapia leva a que as células de PCa se tornem resistentes ao tratamento, sendo capazes de sobreviver e originar metástase, ainda que na ausência ou sob a influência de níveis baixos de androgénios. Este estado corresponde à fase da doença designada como PCa resistente à castração (CRPC, "*castrate-resistant prostate cancer*"), a qual apresenta elevada mortalidade e cujas opções de tratamento têm maiores limitações.

Nos últimos anos, uma quantidade substancial de informação relevante tem demonstrado que as células cancerosas têm a capacidade de reprogramar o seu metabolismo optimizando a sobrevivência e potencial metastático. Os estudos de Warburg foram pioneiros a demonstrar que as células tumorais usam predominantemente a glicólise para obtenção de energia, em detrimento da fosforilação oxidativa, produzindo assim grandes quantidades de lactato. Esta descoberta abriu portas para anos mais tarde, se vir a considerar a adaptação metabólica como um "*hallmark*" do cancro. Atualmente, é aceite que, quer a respiração mitocondrial quer a glicólise, sustentam a sobrevivência e crescimento das células cancerosas. No caso do PCa, os tumores primários diferem de outros tipos de cancros pelo facto de serem menos glicolíticos, característica que tem alimentado a ideia de que na fase inicial as células de PCa usam predominantemente ácidos gordos e glutamina como fonte de energia. Efetivamente, tem sido demonstrado que a glicólise apenas se encontra sobreativada nas fases mais avançadas da doença, predominantemente no CRPC. No entanto, a compreensão de como cada uma das diferentes vias metabólicas sustenta a sobrevivência e crescimento das células do PCa ainda necessita de alguma clarificação. Os androgénios, para além das suas reconhecidas funções como os principais responsáveis pela sobrevivência e crescimento do PCa, têm, igualmente, vindo a ser apontados como importantes reguladores metabólicos, modulando, essencialmente, a glicólise e a utilização de lípidos. Ainda assim, existem muitos aspetos ligados à ação dos androgénios na regulação do metabolismo do PCa que permanecem por esclarecer.

Apesar dos esforços feitos nos últimos anos para o desenvolvimento de novos fármacos para o tratamento do PCa, nomeadamente, para o CRPC, estes compostos têm demonstrado uma duração limitada dos benefícios clínicos e da sobrevivência. Seguindo a tendência observada noutros tipos de cancros, também no caso do PCa e CRPC, têm sido testadas

abordagens terapêuticas tendo como alvo o metabolismo energético. No entanto, existem lacunas importantes no conhecimento existente no que diz respeito ao metabolismo específico das células do PCa, as quais devem ser colmatadas para assim se a aumentar a eficácia e evitar a resistência metabólica de muitas das terapias usadas.

A presente tese teve como objetivo principal a clarificação do papel dos androgénios e do "ambiente metabólico" na modulação do metabolismo do PCa, e de que modo esta relação pode afetar o "destino" ("*cell fate*") das células tumorais. Este trabalho estabeleceu pela primeira vez de que modo as células do PCa dependem das diferentes vias metabólicas (glicólise, glutaminólise e metabolismo lipídico). Foi ainda demonstrado que as células de CRPC apresentam uma taxa metabólica mais elevada, sendo mais glicolíticas que as células sensíveis à ação dos androgénios, o que foi particularmente evidente no caso das células PC3, as quais também demonstraram uma maior capacidade de oxidação da glutamina. As células LNCaP, as quais são sensíveis aos androgénios, exibiram uma maior capacidade para usar ácidos gordos como "combustível" para a mitocôndria. Estes resultados demonstraram assim uma capacidade e dependência diferencial no uso de fontes de energia entre as células sensitivas aos androgénios e as que mimetizam o CRPC. De seguida, foi determinada qual a importância da glutaminólise para a sobrevivência e crescimento das células do PCa, assim como os efeitos dos androgénios na regulação do metabolismo da glutamina. O tratamento das células do PCa com 5α-dihidrotestosterona (DHT, 10 nM) potenciou o metabolismo da glutamina nas células do PCa, enquanto que a inibição da atividade da glutaminase diminui a viabilidade celular e migração, aumentando a apoptose. Estes efeitos foram particularmente evidentes nas células de CRPC. Para além disso, o tratamento simultâneo com o inibidor da glutaminase (BPTES) e o antiandrógeno (bicalutamida) teve um efeito sinérgico na supressão da viabilidade das células LNCaP, o que indica os potenciais benefícios de se atuar simultaneamente ao nível do recetor de androgénios e metabolismo da glutamina como forma de tratamento do PCa.

O papel dos androgénios na regulação do metabolismo lipídico e a influência destas hormonas e do colesterol-LDL na modulação do destino das células do PCa foram outro dos focos desta dissertação. A DHT aumentou a expressão da síntase de ácidos gordos e da carnitina palmitoiltransferase 1A nas células de PCa sensíveis aos androgénios. O enriquecimento em colesterol-LDL aumentou a viabilidade, proliferação e migração das células do PCa de forma dependente da presença da DHT. Os resultados desta abordagem *in vitro* sustentam os dados clínicos e epidemiológicos que ligam a obesidade e colesterol com o PCa, e implicaram, pela primeira vez, a ação dos androgénios nesta relação.

Por fim, investigou-se de que modo diferentes concentrações de glucose podem afetar a resposta das células de PCa a terapias anti-cancro. Com este propósito, foi usado um inibidor do recetor tirosina cinase, o imatinib, tendo-se testado os seus efeitos em dois modelos celulares de CRPC. A maior disponibilidade de glicose aumentou os efeitos do imatinib na supressão da sobrevivência e crescimento das células de CRPC. Para além disso, o tratamento com imatinib estimulou o metabolismo glicolítico destas células. No geral,

demonstrou-se que a hiperglicemia, a principal alteração observada no soro dos pacientes diabéticos, potenciou os efeitos do imatinib nas células CRPC, o que aumenta a curiosidade acerca da eficácia deste fármaco no tratamento de pacientes diabéticos na fase de CRPC.

Em conclusão, os principais resultados desta tese confirmaram assim a ação crucial dos androgénios na regulação do metabolismo das células do PCa. Estes efeitos foram essenciais para a obtenção de energia por parte das células de PCa e no desencadeamento dos processos de migração e proliferação celular. Outro resultado inovador da presente tese foi a identificação da inter-relação entre colesterol e androgénios na indução da sobrevivência das características de invasão das células do PCa. Esta dissertação demonstrou ainda a flexibilidade das células de PCa no uso de diferentes metabolitos energéticos, contribuindo assim para uma melhor compreensão do papel dos lípidos e glutamina no PCa. Foram igualmente revelados alguns dos mecanismos moleculares subjacentes ao papel de suporte do metabolismo na sobrevivência e crescimento das células cancerosas. No global, a informação e os resultados obtidos nesta tese suportam a existência de uma ação cúmplice do "ambiente metabólico" e dos androgénios na orquestração da reprogramação do metabolismo do PCa, e no desenvolvimento do cancro. Investigação adicional sobre esta relação poderá vir a ser uma base fundamental para o desenvolvimento de novas abordagens terapêuticas para o PCa.

Palavras-chave

Androgénios, cancro da próstata, cancro da próstata resistente à castração, glicólise glutaminólise, imatinib, metabolismo lipídico

Resumo Alargado

O cancro da próstata (PCa, "*prostate cancer*") é um dos cancros que mais frequentes nos homens, representando a quinta maior causa de morte por cancro na população masculina. Vários fatores de risco têm vindo a ser associados ao desenvolvimento desta doença, o que inclui fatores endógenos como a idade, antecedentes familiares, fatores genéticos, etnia, hormonas, inflamação e o stress oxidativo, e também vários fatores exógenos, nomeadamente, adieta, reduzida atividade física, obesidade, fatores ambientais e ocupacionais, e tabagismo. Numa fase inicial da doença, a fase conhecida como sensível aos androgénios, a sobrevivência e proliferação das células tumorais dependem grandemente da ação estimuladora dos androgénios. Esta dependência é a característica que permite a utilização da terapêutica de privação androgénica (ADT, "*androgen-deprivation therapy*"), a qual tem por base a redução dos níveis circulantes de androgénios ou o bloqueio das suas ações. A administração contínua desta terapia leva a que as células de PCa se tornem resistentes ao tratamento, sendo capazes de sobreviver e originar metástase, ainda que na ausência ou sob a influência de níveis baixos de androgénios. Este estado corresponde à fase da doença designada como PCa resistente à castração (CRPC, "*castrate-resistant prostate cancer*"), o acontece em média 38 meses após o inicio da administração da ADT. Os mecanismos subjacentes ao desenvolvimento do CRPC são complexos, e ainda não totalmente conhecidos. Contudo, atualmente, é aceite que o desenvolvimento da resistência à castração resulta de mudanças adaptativas na via de sinalização do recetor de androgénios (AR, "*androgen receptor*"). Estas alterações resultam de diversas modificações na sinalização mediada pelo AR, nomeadamente, em consequência da ocorrência de mutações, da existência de transcritos de RNA alternativos ou de amplificação genómica, assim como no aumento da produção intra-prostática de androgénios, ou na desregulação da expressão de co-reguladores do AR, entre outros aspectos. O CRPC representa uma fase da doença, normalmente letal, na qual a falha da ADT não consegue ser ultrapassada com eficácia. As alternativas terapêuticas que têm surgido e que têm vindo a ser utilizadas, continuam a apresentar muitas limitações, e os benefícios em termos de progressão da doença e aumento da sobrevivência são ainda relativamente modestos. É o caso das combinações de fármacos como o doxetaxel ou mitoxantrona, e que têm vindo a ser usados de forma isolada ou em combinação com outros agentes, contudo o beneficio clinico tem sido pouco evidente. A metastização para outros órgãos como o osso, pulmões e nódulos linfáticos é comum e, inevitavelmente, acaba por acontecer.

Nos últimos anos, uma quantidade substancial de informação relevante tem demonstrado que as células cancerosas têm a capacidade de reprogramar o seu metabolismo optimizando a sobrevivência e potencial metastático. Os estudos de Warburg foram pioneiros a demonstrar que as células tumorais usam predominantemente a glicólise para obtenção de

energia, em detrimento da fosforilação oxidativa, produzindo assim grandes quantidades de lactato. Esta descoberta abriu portas para anos mais tarde, se vir a considerar a adaptação metabólica como um "*hallmark*" do cancro. Atualmente, é aceite que, quer a respiração mitocondrial quer a glicólise, sustentam a sobrevivência e crescimento das células cancerosas. O aparecimento de metástase está, igualmente, associado à readaptação metabólica resultante da pressão do microambiente tumoral e de uma disponibilidade de nutrientes limitada. A reativação de diversas vias metabólicas, sendo de referir a glicólise, glutaminólise, e o metabolismo lipídico e de outros aminoácidos para além da glutamina, tem vindo a ser descrita como um fenómeno comum em diversos tipos de cancro. No caso do PCa, os tumores primários diferem de outros tipos de cancros pelo facto de serem menos glicolíticos, característica que tem alimentado a ideia de que na fase inicial as células de PCa usam predominantemente ácidos gordos e glutamina como fonte de energia. Efetivamente, tem sido demonstrado que a glicólise apenas se encontra sobreativada nas fases mais avançadas da doença, predominantemente no CRPC. Para além disso, a desregulação do metabolismo do PCa encontra-se relacionada com a sobre ativação de diversos oncogenes, assim como à perda de função de genes supressores de tumor. No entanto, a compreensão de como cada uma das diferentes vias metabólicas sustenta a sobrevivência e crescimento das células do PCa ainda necessita de alguma clarificação. Os androgénios, para além das suas reconhecidas funções como os principais responsáveis pela sobrevivência e crescimento do PCa, têm, igualmente, vindo a ser apontados como importantes reguladores metabólicos, modulando, essencialmente, a glicólise e a utilização de lípidos. Ainda assim, existem muitos aspetos ligados à ação dos androgénios na regulação do metabolismo do PCa que permanecem por esclarecer.

Apesar dos esforços feitos nos últimos anos para o desenvolvimento de novos fármacos para o tratamento do PCa, nomeadamente, para o CRPC, estes compostos têm demonstrado uma duração limitada dos benefícios clínicos e da sobrevivência. Seguindo a tendência observada noutros tipos de cancros, também no caso do PCa e CRPC, têm sido testadas abordagens terapêuticas tendo como alvo o metabolismo energético. No entanto, existem lacunas importantes no conhecimento existente no que diz respeito ao metabolismo específico das células do PCa, as quais devem ser colmatadas para assim se a aumentar a eficácia e evitar a resistência metabólica de muitas das terapias usadas.

A presente tese teve como objetivo principal a clarificação do papel dos androgénios e do "ambiente metabólico" na modulação do metabolismo do PCa, e de que modo esta relação pode afetar o "destino" ("*cell fate*") das células tumorais. Este trabalho estabeleceu pela primeira vez de que modo as células do PCa dependem das diferentes vias metabólicas (glicólise, glutaminólise e metabolismo lipídico). Foi ainda demonstrado que as células de CRPC apresentam uma taxa metabólica mais elevada, sendo mais glicolíticas que as células sensíveis à ação dos androgénios, o que foi particularmente evidente no caso das células PC3, as quais também demonstraram uma maior capacidade de oxidação da glutamina. As células LNCaP, as quais são sensíveis aos androgénios, exibiram uma maior capacidade para usar

ácidos gordos como "combustível" para a mitocôndria. Estes resultados demonstraram assim uma capacidade e dependência diferencial no uso de fontes de energia entre as células sensitivas aos androgénios e as que mimetizam o CRPC. Para além disso, foi demonstrado que altas concentrações de glucose inibem a atividade glicolítica promovendo o metabolismo lipídico. Este resultado demonstra a capacidade de adaptação metabólica das células de PCa em função da disponibilidade de glucose. De seguida, foi determinada qual a importância da glutaminólise para a sobrevivência e crescimento das células do PCa. O tratamento das células do PCa com 5α-dihidrotestosterona (DHT, 10 nM) potenciou o metabolismo da glutamina nas células do PCa, traduzido pelo aumento do consumo da glutamina, resultante do aumento da expressão do transportador de glutamina (ASCT2) e da glutaminase, a enzima mitocondrial que converte a glutamina em glutamato. Os efeitos da DHT na regulação da expressão do ASCT2 e da glutaminase foram observados, de igual modo, na próstata de rato, transpondo-se estes resultados para um modelo *in vivo*. A inibição da atividade da glutaminase através da utilização de um inibidor específico, o BPTES, diminui a viabilidade celular e migração, aumentando a apoptose. De salientar que estas alterações foram acompanhadas por um aumento pronunciado da expressão do oncogene c-Myc, o qual tem vindo a ser indicado como um regulador central da glutaminólise. Os efeitos do BPTES foram particularmente evidentes nas células de CRPC. Para além disso, o tratamento simultâneo com o inibidor da glutaminase e o antiandrógeno bicalutamida teve um efeito sinérgico na supressão da viabilidade das células LNCaP, o que indica os potenciais benefícios de se atuar simultaneamente ao nível do AR e metabolismo da glutamina como forma de tratamento do PCa. A inibição da glutaminase apresentou ainda efeitos na resposta metabólica das células de PCa, afetando a glicólise e o metabolismo lipídico, embora de forma diferencial nas células sensíveis aos androgénios e nas CRPC. Estes resultados demonstraram que a inibição de uma via metabólica nas células cancerosas pode induzir alterações importantes noutras vias, o que deve ser tomado em consideração quando se pretende inibir o metabolismo com objetivos terapêuticos.

O papel dos androgénios na regulação do metabolismo lipídico e a influência destas hormonas e do colesterol-LDL na modulação do destino das células do PCa foram outro dos focos desta dissertação. A DHT aumentou a expressão da síntase de ácidos gordos e da carnitina palmitoiltransferase 1A nas células de PCa sensíveis aos androgénios. A estimulação da expressão da sintase de ácidos gordos pela DHT manteve-se em condições de depleção de lípidos, no entanto a presença de LDL aboliu os efeitos da DHT inibindo a expressão da sintase de ácidos gordos. Foi ainda confirmado que o efeito da DHT na regulação dos níveis desta enzima depende da ação do fator de transcrição SREBP-1. O enriquecimento em colesterol-LDL aumentou a viabilidade, proliferação e migração das células do PCa de forma dependente da presença da DHT. Para além disso, a mesma conjugação (LDl(+)/DHT(+)) aumentou o conteúdo de lípidos nos corpos lipídicos. Por outro lado, a inibição do recetor de LDL e da carnitina palmitoiltransferase 1 suprimiu os efeitos da LDL sobre a viabilidade das células de PCa. Os resultados desta abordagem *in vitro* sustentam os dados clínicos e epidemiológicos

que ligam a obesidade e colesterol com o PCa, e implicaram, pela primeira vez, a ação dos androgénios nesta relação.

Por fim, investigou-se de que modo diferentes concentrações de glucose podem afetar a resposta das células de PCa a terapias anti-cancro. Com este propósito, foi usado um inibidor do recetor tirosina cinase, o imatinib, tendo-se testado os seus efeitos em dois modelos celulares de CRPC. A maior disponibilidade de glicose aumentou os efeitos do imatinib na supressão da sobrevivência e crescimento das células de CRPC. Para além disso, o tratamento com imatinib estimulou o metabolismo glicolítico destas células, aumentando o consumo de glucose, a produção de lactato, assim como a expressão e a atividade da enzima lactato desidrogenase. No geral, demonstrou-se que a hiperglicemia, a principal alteração observada no soro dos pacientes diabéticos, potenciou os efeitos do imatinib nas células CRPC, o que aumenta a curiosidade acerca da eficácia deste fármaco no tratamento de pacientes diabéticos na fase de CRPC.

Em conclusão, os principais resultados desta tese confirmaram assim a ação crucial dos androgénios na regulação do metabolismo das células do PCa. Estes efeitos foram essenciais para a obtenção de energia por parte das células de PCa e no desencadeamento dos processos de migração e proliferação celular. Outro resultado inovador da presente tese foi a identificação da inter-relação entre colesterol e androgénios na indução da sobrevivência das características de invasão das células do PCa. Esta dissertação demonstrou ainda a flexibilidade das células de PCa no uso de diferentes metabolitos energéticos, contribuindo assim para uma melhor compreensão do papel dos lípidos e glutamina no PCa. Foram igualmente revelados alguns dos mecanismos moleculares subjacentes ao papel de suporte do metabolismo na sobrevivência e crescimento das células cancerosas. No global, a informação e os resultados obtidos nesta tese suportam a existência de uma ação cúmplice do "ambiente metabólico" e dos androgénios na orquestração da reprogramação do metabolismo do PCa, e no desenvolvimento do cancro. Investigação adicional sobre esta relação poderá vir a ser uma base fundamental para o desenvolvimento de novas abordagens terapêuticas para o PCa.

Palavras-chave

Androgénios, cancro da próstata, cancro da próstata resistente à castração, glicólise glutaminólise, imatinib, metabolismo lipídico

Abstract

Prostate Cancer (PCa) is one of the most common cancer in men and represents the fifth leading cause of cancer deaths. In an initial phase of PCa, the so-called androgen-sensitive stage, PCa cells are extremely dependent on androgens actions to survive and proliferate. This condition allows the effectiveness of androgens deprivation therapy (ADT) that reduce the circulating levels of androgens or block their action. The continuous administration of ADT renders PCa cells resistant to treatment, becoming capable of survive and metastasize even in the absence or very low circulating levels of androgens. At this moment, it is reached the stage of castrate-resistant prostate cancer (CRPC), a condition with high mortality rates and treatment limitations.

In the last years, a substantial amount of data showed that cancer cells have the ability of reprogramming metabolism to survive and metastasize. Warburg studies were pioneer showing that tumour cells predominantly use glycolysis for obtaining energy, in detriment of oxidative phosphorylation, with the production of high amounts of lactate. These findings opened the door to the metabolic adaptation being considered a hallmark of cancer. Currently, it is accepted that mitochondrial oxidative phosphorylation and glycolysis, support tumour cells survival and growth. Primary PCa cases differ from other cancer types by the fact that is less glycolytic, and the idea that predominantly use fatty acids and glutamine as energy substrates has been gaining consistency. Indeed, glycolysis is only overactivated in more advanced stages of the disease, in CRPC. However, the understanding of how each metabolic pathway sustains PCa cells survival and growth still is incomplete. Besides the recognized functions as the main drivers of PCa survival and growth, androgens have been indicated as metabolic regulators in PCa, modulating glycolysis and cell lipid handling. Nevertheless, there are several issues in the role of androgens controlling PCa metabolism that need to be clarified.

Although efforts have been made in recent years to develop new drugs for PCa treatment, namely, for CRPC, they have shown limited duration of clinical and survival benefits. Following the trend observed in other cancer types, treatment approaches targeting metabolism also have been explored in the case of PCa and CRPC. However, there are important knowledge gaps in the understanding of PCa cells metabolism that should be fulfilled to improve its efficacy and to avoid resistance and bypassing metabolic pathways.

The main goal of this thesis was to clarify the role androgens and the metabolic environment in shaping PCa metabolism, and how this interplay can affect PCa cell fate. The present thesis first established the PCa cells dependency on the different metabolic pathways (glycolysis, glutaminolysis and lipid metabolism). It was demonstrated that CRPC cells have higher metabolic rates being more glycolytic than the androgen-sensitive cells, especially the PC3 cells, which also showed a higher capacity to oxidize glutamine. Androgen-responsive

LNCaP cells displayed a higher capacity for using fatty acids as mitochondrial fuels. These findings allowed to demonstrate a differential dependency and capacity of fuel use between androgen-sensitive and CRPC cells. Next, we determined the relevance of glutaminolysis for PCa cells survival and growth and the effect of androgens in the regulation of glutamine metabolism. Treatment of PCa cells with 5α-dihydrotestosterone (DHT, 10nM) potentiated glutamine metabolism in PCa cells, whereas the inhibition of glutaminase activity diminished cell viability and migration, and increased apoptosis, particularly in the CRPC. Moreover, co-treatment with glutaminase inhibitor BPTES and the anti-androgen bicalutamide had a synergic effect suppressing LNCaP cells viability, which highlights the benefit of co-targeting androgen receptor and glutamine metabolism in PCa treatment. Glutaminolysis inhibition also had an impact on glycolysis and lipid metabolism.

The role of androgens in regulating lipid metabolism and the influence of these hormones and LDL-cholesterol modulating PCa cells fate were evaluated. DHT upregulated the expression of fatty acid synthase and carnitine palmitoyltransferase 1A in androgen-sensitive PCa cells. LDL-cholesterol enrichment increased PCa cells viability, proliferation, and migration dependently on DHT. This *in vitro* approach supports clinical and epidemiological data linking obesity and cholesterol with PCa, and first implicated androgens in this relationship.

Finally, we investigated the effect of different glucose availability on the PCa cells response to therapy. For this purpose, it was used the receptor tyrosine kinase inhibitor imatinib and two cell line models of CRPC. Higher glucose availability improved the effectiveness of imatinib suppressing survival and growth of CRPC cells Moreover, imatinib treatment stimulated the glycolytic metabolism of CRPC cells. Overall, it was showed that hyperglycemia, the main serum alteration in diabetic patients, potentiated the effects of imatinib in CRPC cells, which raises the curiosity about the efficacy of this drug for treatment of castration-resistant diabetic patients.

In conclusion, the main findings of this thesis confirmed the crucial actions of androgens in regulating the metabolism of PCa cells. These effects were pivotal for PCa cells obtaining energy and triggered proliferation and metastasis. Another innovative result of the present thesis was the identification of the cholesterol and androgens interplay in inducing survival and invasiveness features of PCa cells. Moreover, this dissertation demonstrated the flexibility of PCa cells using different energy sources and contributed to a better understanding of the role of lipids and glutamine in PCa. The molecular mechanism underlying the metabolic support of cancer cell survival and growth were highlighted. Overall, the information gathered in this thesis supports the metabolic environment and androgens as “co-authors” orchestrating the reprogramming of PCa and cancer development. Further research on this interplay could be a basis for the development of new treatment approaches for PCa.

Keywords

Androgens, glutaminolysis, lipid metabolism, glycolysis, imatinib, castrate-resistant prostate cancer, prostate cancer

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2-DG	2-deoxy-D-glucose
3PO	3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one
ABHD5	Alpha-beta Hydrolase Domain Containing 5
ACAT	Acyl-CoA cholesterol acyl transferase
ACC	Acetyl-CoA carboxylase
Ac-DEVD-pNA	Ac-DEVD-pNA
ACLY	Adenosine triphosphate citrate lyase
ADT	Androgen-deprivation therapy
AKT	Protein kinase B
ALDO	Aldolase
ALT	Alanine aminotransferase
AMF	Autocrine motility factor
AMPK	AMP-activated protein kinase
AR	Androgen receptor
ASCT2	Sodium dependent antiporter, the alanine, serine, cysteine, glutamine transporter
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BPH	Benign prostatic hyperplasia
Ca^{2+}	Calcium
CaMKK2	Calcium/calmodulin-dependent protein kinase kinase 2
CKI	Cyclin-dependent kinase inhibitor (CKI
COX-2	Cyclooxygenase-2
CPT1	Carnitine palmitoyltransferase 1
CPT1A	Carnitine Palmitoyltransferase 1A
CRPC	Castrate-resistant prostate cancer
CYP17	Cytochrome P
CZ	Central zone
DGAT	Diglyceride acyltransferase
DHT	5-α-Dihydrotestosterone
DTT	DiThioThreitol
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
FA	Fatty acid
FABPm	Fatty acid binding protein

FASN	FA synthase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDG	[18F]fluorodeoxyglucose
G6PD	Glucose-6-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
GLN	Glutamine
GLS	Glutaminase
GLUT	Glucose transporter
HGPIN	High-grade PIN
HIF-1	Hypoxia-inducible factor 1
HK	Hexokinase
HK-2	Hexokinase-2
HMGCR	HMG-CoA reductase
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1
IDO	Indoleamine-2,3-dioxygenase
IL	Interleukin
JNK	c-Jun N-terminal kinase
LD-FBS	Lipid Depleted FBS
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LDLr	Low density lipoproteins receptor
LMW-E	Low molecular weight isoform of cyclin E
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MCT	Monocarboxylate transporter
MCT4	Monocarboxylate transporter 4
MICs	Metastasis-initiating cells
miR-22	MicroRNA-22
MMPs	Matrix metalloproteinases
MSR1	Macrophage scavenger receptor 1
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2M
MTT	1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan
MUFAs	Monounsaturated fatty acids
ORCHX	Orchidectomised
OXPHOS	Mitochondrial oxidative phosphorylation
P5C	Δ^1-pyrroline-5-carboxylate

P5CR	Pyrroline-5-carboxylatereductase
PAP	Prostatic acid phospathase
PBS-T	PBS containing 0.1% (w/v) Tween-20
PC	Phosphatidylcholine
PCa	Prostate cancer
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PDK	Pyruvate dehydrogenase kinase
PDK1	Pyruvate dehydrogenase kinase-1
PE	Phosphatidylethanolamine
PEP	Phosphoenolpyruvate
PER	Proton efflux rate
PFA	ParaFormAldehyde
PFK	Phosphofructokinase
PFK1	Phosphofructokinase 1
PFK15	1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase
PHI	Phosphohexose isomerase
PI3K	Phosphoinositide-3-kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PKM	Pyruvate kinase
PKM2	Pyruvate kinase isozyme M2
PKM2	Pyruvate kinase isozyme M2
PLA2	Phospholipase A2
PLD	Phospholipase D
PMSF	PhenylMethylSulfonyl Fluoride
pNA	p-Nitro-Aniline
PRODH	Proline dehydrogenase
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
PVDF	PolyVinylidene DiFluoride
PZ	Peripheral zone
RIPA	radioimmunoprecipitation assay buffer
RNASEL	Ribonuclease L
ROS	Reactive oxygen species
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SARDH	Sarcosine dehydrogenase

SCAP	SREBP-cleavage activating protein
SCD	Stearoyl-CoA desaturase
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
SQLE	Squalene monooxygenase
SRB	Sulforhodamine B
SREBP	Sterol regulatory element-binding protein
STAT3	Signal transducer and activator of transcription 3
TCA	Tricarboxylic acid
TNF-α	Tumor necrosis factor-α
TRAMP	Transgenic adenocarcinoma of mouse prostate
TZ	Transition zone
VEGF	Vascular endothelial growth factor
WB	Western Blot
α-KG	α-ketoglutarate

Chapter 1

Prostate Cancer: from aetiology to therapy

1.1. Onset and progression of prostate cancer

The prostate is the major accessory gland of the male reproductive tract, being a pyramid-shaped organ located posterior to the symphysis pubis, anterior to the rectum, and inferior to the bladder. The prostate gland is enclosed by a fibrous capsule containing smooth muscle cells and innumerous veins and nerves [1,2]. Studies by McNeal and colleagues described the prostate as a single gland with different histological zones, namely, the central zone (CZ), the transition zone (TZ), and the peripheral zone (PZ) (Fig. 1.1.). An alternative anatomical classification divides the prostate in an anterior lobe, a dorsal lobe, a lateral lobe and a ventral lobe [3]. The Mcneal classification is based on different embryologic origins and can be distinguished by histology, anatomic landmarks, biological functions, and susceptibility to pathologic disorders [1,4-6]. The PZ is enfolded around the outer portion distally and represents 70-75 % of the prostate, being recognized as the most susceptible zone for the development of prostate cancer (PCa) [3,7,1]. Towards the base, the PZ surrounds the CZ and the TZ (Fig. 1.1.) [1,4-6]. The PZ contains the ducts of urethra, in the mild-prostate, to the prostatic apex [5]. The TZ is situated in bilateral regions in the middle to the base of the gland located near the prostatic urethra, constituting about 5% of the prostate in young adults [5]. In older men the TZ is commonly augmented, a condition compatible with the so-called benign prostatic hyperplasia (BPH). The CZ has a conical structure, with the broader portion at the base of the prostate and the apex at the verumontanum (glandular-stroma tissue) (Fig. 1.1.). It is composed by ducts diverging from the mid-prostate to the prostatic

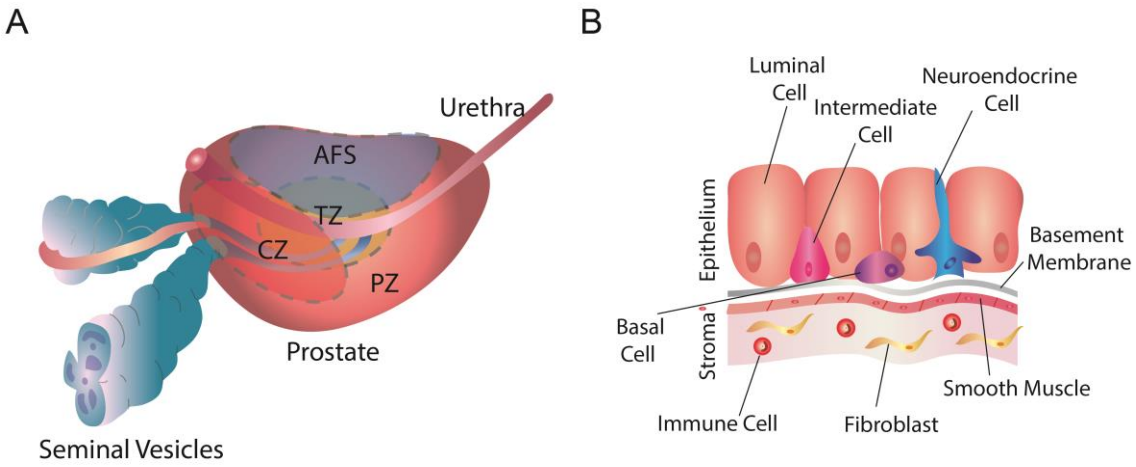

Figure 5.1. Schematic representation of prostate anatomy and prostate epithelium. **(A)** The anterior and apical surfaces are bounded by anterior fibromuscular stroma (AFS). In addition, three main zones can be identified in prostate anatomy, the transition zone (TZ) that surrounds proximal prostatic urethra, the central zone (CZ) that surrounds the ejaculatory ducts, and the peripheral zone (PZ), which encompasses approximately 70-75 % of the prostate gland. **(B)** Detail of prostate epithelium composed by the columnar luminal cells that produce the prostatic secretions, including the prostate-specific antigen. Basal cells are located on the base of epithelieum in contact with the basement membrane. A third type of cells, the neuroendocrine cells, are located among the epithelial cells and seem to be involved in the regulation of secretory activity and prostate cell growth. Prostate epithelial cells maintain contact with stroma, including fibroblasts and several types of immune cells.

base. The CZ has not been identified as a site of origin for any disease. It accounts for 25% of the glandular prostate in young adults and its size decreases gradually in older men [5,8]. The prostate gland also includes a fourth non-glandular region that comprises the fibromuscular stroma (Fig. 1.1.), which covers the urethra in the anteromedial prostate [9,3]. The supporting stroma is a mixture of collagenous fibrous tissue and smooth muscle fibers, that does not contain glandular tissue and is not associated with prostate pathology [9].

The glandular prostatic epithelium (Fig. 1.1.) is composed of acini and ducts constituted by three types of cells, epithelial luminal cells, basal cells and neuroendocrine cells. The majority of luminal cells, are columnar luminal cells active in the production of prostatic secretions and responsible for main prostate functions. Luminal cells also produce the prostate-specific antigen (PSA), a secretory product that has been used over the year in PCa screening and disease monitoring [3]. Luminal cells express typical markers such as the androgen receptor (AR) and PSA. PSA is produced by ductal epithelial cells and normally secreted into the lumen, being removed with ejaculation. The population of basal cells is present on the base of the prostate gland in contact with the basement membrane [3]. Neuroendocrine cells comprise no more than 1% of the epithelial cells and can secret neurosecretory products that promote prostate growth [10,11]. It was proposed a cell hierarchy in prostate epithelium pointing that epithelial and neuroendocrine cells are originated from stem cells [12]. Prostate stem cells are a heterogenous small population confined to the basal compartment, uncommitted stem cells that differentiate to form semi-committed progenitors, or multiple populations of stem cells [13,14].

The main function of the prostate gland is to produce a set of semen constituents, including PSA, citrate, choline and proteolytic enzymes that provide a good environment for the spermatozoa, crucial to support sperm motility, survival and delivery to the female reproductive tract. These secretions represent 30 % of the semen volume and have a basic pH that neutralizes the acidic environment of the duct deferent and female vagina [15-17].

The human prostate is small in childhood, but in puberty rises in size to approximately 20-25 g. During the period of prostate growth increased rate of cell proliferation over death occurs, which is regulated by hormones, mainly androgens. At peripheral tissues, including prostate, testosterone is metabolized to 5α-dihydrotestosterone (DHT), a powerful androgen that binds to the AR, strongly enhancing its activity as a transcription factor [18,19]. The AR upon activation regulates the expression of several genes involved in prostate cell proliferation and development [20-23]. From around 30 years old, the prostate weight rises slowly and typically upon reaching 50 years of age present the onset of BPH nodules [24-26].

PCa is the most common cancer in men and represents the fifth leading cause of cancer deaths. In 2018, 1.3 million new cases of PCa were diagnosed and 359,000 associated deaths were reported worldwide [27]. In Portugal, statistics indicate that the mortality by PCa has been growing, and that, only in 2017, 1796 men's lost their life in consequence of this disease [28]. PCa is a heterogeneous and multifactorial disease arising from the contribution of several factors. Several transformations can be identified from PCa onset to the establishment of prostate adenocarcinoma and development of metastatic PCa (Fig. 1.2.). The prostatic epithelium can be damaged due to inflammation, infection and/or exposure to carcinogens [29]. The first identifiable histological alteration is the prostatic intraepithelial neoplasia (PIN). PIN lesions are asymptomatic and characterized by the thickening of the epithelial cells in the PZ of prostate, and loss of distinct basal and secretory layers [30-32]. The development of PIN is characterized by the contact of inflammatory cells with prostate cells. The High-grade PIN (HGPIN) lesions can be considered as the precursors of PCa [30,31,33]. PIN and HGPIN lesions are frequently found in PCa patients [34,35]. In fact, HGPIN

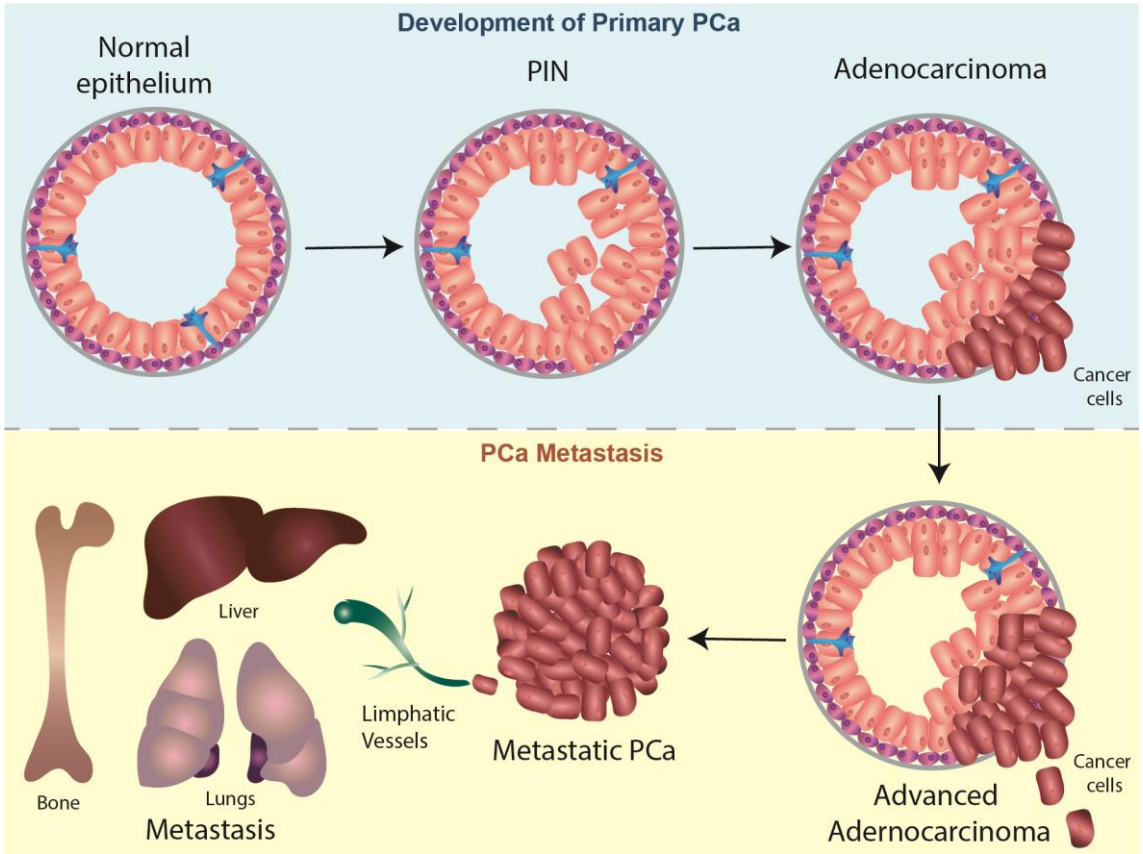

Figure 1.6. Schematic representation of the proposed model of the cellular events associated with development and progression of PCa. The pathophysiology of prostate comprises multiple steps and starts with the appearance of pre-malignant lesions and the transformation of prostate epithelial cells, which in turn may generate cancer progenitor cells and the prostate intraepithelial neoplasia (PIN) emerges. In turn, PIN lesions progress to localized prostate adenocarcinoma, which then becomes locally invasive carcinoma as the basal cell layer is degraded and cancer cells invade the basal lamina. The constant activation of distinct growth signalling pathways in PCa cells, including the activation of AR, leads to the development of local and invasive carcinoma and ultimately to the metastization to different organs, including liver, lungs and bones.

and PCa present many similarities. HGPIN also shows moderate high levels of PSA and abnormal proliferation, with basal and luminal cells presenting dysplastic features including nuclear hyperchromasia, nuclear crowding and prominent nucleoli [36,37]. Other inflammatory alteration described to be associated with PCa is the proliferative inflammatory atrophy (PIA). PIA is characterized by the presence of inflammatory and proliferating epithelial cells with a stromal atrophy and increased oxidative stress, most likely derived from the inflammatory cells signals or malignancy itself [38]. The induction of inflammatory processes, and consequently the appearance of PIA and PIN lesions, is related with a decreased number of basal cells, accumulation of luminal cells, hyperplasia and appearance of somatic mutations [39]. PIA lesions may give rise directly to carcinoma, but are frequently associated with the development of HGPIN [29,40,30]. Thereafter, it is possible the development of localized PCa followed by the establishment of advanced prostate adenocarcinoma (Fig. 1.2.). Although initially confined to the prostate, PCa spreads to adjacent lymph nodes and distant organs such as the liver, brain, lungs and bones [41-43] (Fig. 1.2.). PCa cells undergo epithelial to mesenchymal transition (EMT) and are capable of colonizing other organs, mainly the bone marrow [41]. In fact, the highest incidence of PCa metastases are detected in bone, with rearrangement of the bone matrix, severe pain and frequent fractures [44,43].

Overall, PCa is a complex pathology involving several possible processes that determine the initiation of disease, which are not totally understood. In a classical view epithelial luminal cells are considered the principal cells in the origin of PCa, whereas basal cells (Fig. 1.1.) are viewed as a barrier to protect luminal cells from oncogenic stimulus [45]. In fact, reports establish epithelial cells as more responsive to oncogenic stimulus, whereas basal cells are resistant [46]. Nevertheless, basal cells stay in direct contact with the stroma that can receive, for example, cytokines, which can drive cancer signalling to the epithelial cells [46]. Moreover, basal cells can also intermediate the processes underlying the development of metastatic PCa, because they present higher expression levels of genes involved in the EMT compared with the epithelial luminal cells and, thus, are highly responsive to oncogenic signalling [47].

Prostate stem cells also have been implicated in the malignant transformation of prostatic tissues [48]. Prostate stem cells can be reactivated and differentiate into intermediate epithelial cells that can originate PCa initiators cells [49-51]. In addition, the activity of molecules produced and secreted by stromal cells, and the cross-talk between cancer cells and the stroma have been identified as crucial aspects in carcinogenesis. This includes the secretion of growth factors and cytokines by stromal cells, which modulate the behavior of cancer cells. The stroma also may sustain invasiveness of PCa by releasing products capable of digesting the extracellular matrix [52], which favors the spread of cancer cells, invasion and migration to other tissues.

The histological patterns of PCa are distinguished by the Gleason grading system [53,54], which has been modified over the years, but continues to constitute the grading

system commonly used to classify and stage PCa. The Gleason grading method is entirely based on the architectural arrangements of prostatic carcinoma. The histologic patterns are categorized into five basic grade patterns at relatively low magnification. Gleason patterns 1 and 2 should no longer be assigned by needle core biopsy [53]. Gleason pattern 3 normally have well-formed, individual glands of various sizes including branching glands [53]. Gleason pattern 4 includes poorly-formed, fused, and cribriform glands [53]. Gleason pattern 5 consists of sheets of tumour, individual cells, and cords of cells [53]. These five basic grade patterns are used to generate a histologic score that ranges from 2 to 10. Currently, the subtypes of prostatic carcinoma are classified as, glandular neoplasms (acinar, intraductal, ductal adenocarcinoma), neuroendocrine carcinoma, basal cell carcinoma, squamous neoplasms (squamous cell carcinoma and adenosquamous carcinoma), or urothelial carcinoma [55]. Intra-acinar and/or intraductal adenocarcinoma has some similarities with HGPIN, but have more cytological atypia [55]. Typically they are characterized by intraductal spread of advanced prostatic carcinoma, but may also reflect a preinvasive carcinoma derived from HGPIN [56]. The ductal adenocarcinoma is located in the periurethral area, often protruding into the urethra, and normally presents a mass arising from verumontanum [55]. Its clinical stage often is more advanced than that of typical acinar adenocarcinoma, and can mestastasize to the penis, testis and lung [57,58]. Neuroendrocrine carcinoma is classified as an acinar or ductal adenocarcinoma without a detectable neuroendocrine morphology [55]. Basal cell carcinoma is extremely rare and include adenoid cystic/cribriform patterns and small solid nests with palisading [55]. Squamous cell and adenosquamous carcinoma approximately arise in patients with prostatic acinar adenocarcinoma subsequent to hormone therapy or radiotherapy [55,59]. These tumours are usually large and originated in the periurethral glands or prostatic acini as well as from the lining basal cells [55,59]. Prostatic urothelial carcinoma is originated from the urothelium of the prostatic urethra or the prostatic ducts. Commonly prostatic urothelial carcinoma is presented with bladder carcinoma [55]. This comprehensive characterization of PCa and the underpinning molecular alterations help clinicians selecting the best treatment approaches.

Over the years, several large-scale genomic studies have identified recurrent changes on DNA copy number, chromosomic rearrangements, mutations and gene fusions in PCa. The most common genomic alterations in PCa are translocations involving androgen-regulated promoters and E26 transformation-specific, such *ERG [60]. TMPRSS2:ERG* fusion is present in ~50% of localized PCa [60]. Mutations are also common in PCa and can result in genomic rearrangements including alterations on the expression of several important genes [61-64]. The gain-of-function mutations in oncogenes and the deletion of tumor suppressor genes are also implicated in prostate carcinogenesis [65]. AR mutations, but also gain of function of AR coactivators, and loss of AR corepressors are genomic alterations in the AR pathway mostly associated with PCa development [66-68]. A frequent mutation is found in SPOP, a component of E3 ubiquitin–protein ligase complex, that is associated with the stabilization of oncogenic substrates (AKT, JNK) promoting prostate tumorigenesis [69,70]. The amplification of the

oncogene *c-MYC* is also detected in 50% of prostate tumors [71]. On the other hand, loss of PTEN and TP53 proteins is frequent in PCa and correlates with poor prognosis [67,72]. A combination of alterations in several genes can concur to exacerbate proliferation, disrupt apoptosis and alter the metabolic profile of prostate cells towards malignant transformation [73-75].

1.2. Risk factors

In the last years, a substantial amount of epidemiologic studies have identified several biologic and lifestyle factors influencing the appearance and progression of PCa. Overall, the risk factors that contribute to the development of PCa (Fig. 1.3.) can be divided in two main classes [76,77]: endogenous (age, family, genetic factors, ethnicity, hormones, inflammation and oxidative stress) and exogenous (dietary factors, physical inactivity, obesity, environmental factors, occupation, smoking).

Nevertheless, the only total risk factors intimately associated with the incidence of PCa are age, African-American race, and a genetic risk [78,77]. Moreover, age is considered the highest risk factor for the development of PCa [79,80]. PCa is more prevalent in men older than 40 years of age, and the incidence of diseases strongly increases after 55 [81,82]. Race and family history seem to contribute to the development of PCa and, in fact, a higher prevalence of disease is found in African descents [78,83]. Contrastingly, PCa incidence and mortality rates are lower amongst Asian, American Indian, and Hispanic men [84,85]. This difference in PCa risk associated with the populations ethnicity has been attributed to genetic variants, lifestyle factors and also the hormone levels, including androgens [86-88]. Considering the familiar antecedents, men whose first-degree family members (brother or father) had PCa, are at a two-three higher risk of being diagnosed with PCa, and the risk is nearly nine times higher for men with both [89,90]. In fact, some studies identified genetic factors involved in prostate carcinogenesis [91,92]. Genome-wide analysis and gene linkage studies also revealed several susceptibility genes in PCa. Ribonuclease L (*RNASEL*), involved in immune defense mechanisms, is one of the most promising susceptibility genes discovered, and modifications in this gene expression seem to be related to the progression and poor prognosis of PCa [93-96]. Other genes like *HPC2/ ELAC2*, macrophage scavenger receptor 1 (*MSR1*), *BRCA1 and 2*, or mutations in the X chromosome, that encodes for the AR, are among the genetic factors that can contribute for PCa development [97-101].

Hormones, more precisely androgens are other risk factors in PCa. Several studies have reported that PCa cells growth is dependent on androgens levels [102,20]. High serum and intraprostatic androgens concentrations have a positive association with PCa risk [103,104]. Also, mutations and polymorphisms in genes associated with androgen biosynthesis have been significantly linked with the onset of PCa, namely on those encoding for the enzymes involved in DHT synthesis, such as 5α-reductase or CYPs [105-108]. Currently, it is widely accepted that androgens and AR signalling have a central role in PCa development and

progression. Due to its importance this issue will be explored in a specific section of this chapter below.

Several epidemiologic studies have suggested that chronic inflammation or sexually transmitted infectious agents also have a role on the pathogenesis of some types of PCa [109,110]. In fact, a persistent inflammation, or prostatitis are significantly associated with PCa [110,111]. Chemical or physical trauma, transmitted pathogens, urine reflux are enhancers of prostatitis development, and consequently positively related with PCa [112-114]. Several genes involved in inflammation like cyclooxygenase (*COX-2*), interleukins or tumor necrosis factor-α (TNF-α) have also been associated with PCa risk [115-118]. Sexually transmitted diseases, like gonorrhea and syphilis, are among the principal simulators of the inflammatory process and intimately related with PCa [119].

Exogenous factors like lifestyle or dietary factors have also been implicated in the aetiology of PCa. Obesity, a growing public health problem in our society nowadays has been strongly linked with an increased risk of PCa development [120,121]. Obesity lead to metabolic disorders related with the deregulation of hormone synthesis and action, and inflammation [121,122]. Several molecules secreted by the adipose tissue like cytokines and adipokines (e.g. leptin and adiponectin) [123,124] have been shown to deregulate prostate tissue homeostasis. Adipokines, for example, have been related with the progression of PCa

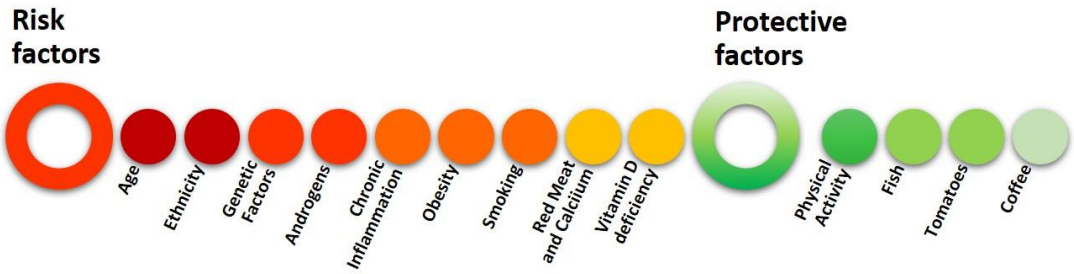

Figure 1.7. The diverse exogenous and endogenous risk factors contributing to prostate carcinogenesis, and the protective factors against prostate malignancy. Colour grade indicates the strength of association (dark colours mean stronger evidence).

by driven angiogenesis, cell proliferation, metastasis, and alterations in sex-steroid hormone levels [125]. Also, it was demonstrated that the periprostatic adipose tissue has a paracrine role in prostate cells, directly prompting the development and progression of PCa. Periprostatic adipocytes secrete the CCL7 chemokine that, acting through the CCR3 receptor present in cancer cells, induces migration and invasion of PCa, an effect that is empowered in obesity [126]. More recently, the same authors showed that a bidirectional crosstalk exists between adipocytes and tumor cells, with PCa cells inducing lipolysis of adipocytes, and the release of exogenous fatty acids that can be taken by tumor cells promoting invasion [126,127]. However, additional studies and further research efforts are still needed to clarify the relationship between obesity and PCa.

Hyperglycemia and insulin deregulation are other metabolic disorders that have significant, but controversial, association with PCa. Some studies have shown a higher risk for

PCa onset linked to high glucose levels, whereas others studies verified that hyperglycemia or type II diabetes have a protective role in PCa [128-130]. Diets highly enriched in red meats and excessive calcium ingestion have been positively associated with PCa, and related with a higher risk of lethal forms of PCa [131-133]. Smoking also has a negative association with PCa, and smokers are at higher risk of PCa-related mortality [134]. Vitamin D deficiency is another agent that has been linked to increasing PCa risk [135,136].

On the other hand, several other diet products have been identified as having a protective role in PCa (Fig. 1.3.). This is the case for diets rich in fish, or tomato-based products and coffee [137-140]. Also, physical activity has been described as having an inverse association with PCa, and has been associated with improved survival and decreased rates of PCa progression [141,142].

1.3. From androgen-sensitive to castration-resistant prostate cancer

Testosterone circulating in the blood stream is metabolized in the prostate to DHT by the activity of 5α-reductase. DHT has more affinity to AR than testosterone, and its binding to this receptor induces the dissociation of heat-shock proteins, and subsequent receptor phosphorylation and dimerization [143,144]. AR dimers bind to the androgen-response elements in the promoter regions of target genes, which upon recruitment of co-regulatory proteins (co-activators or co-repressors) form the AR-transcriptional complex regulating gene transcription [144]. The AR transcriptional complex regulates the expression of several genes that encode for protein network involved in PCa growth, with cancer cells being extremely dependent on androgens actions to survive and proliferate [145-148]. This represents an initial phase of PCa, the so-called androgen-sensitive stage, which can be controlled by the application of androgens deprivation therapy (ADT) aiming to reduce the circulating levels of androgens or blocking their action [149,150].

ADT can be achieved mechanically by orchiectomy or chemically with the use of anti-androgenic drugs [151-154]. The continuous administration of ADT renders PCa cells resistant to treatment, becoming capable to survive and metastasize even in the absence or very low levels of androgens [155,156]. At this stage, it is reached the castrate-resistant prostate cancer (CRPC), which develops on an average of 38 months upon administration of ADT. The 2015 guidelines indicate that two conditions should be followed for the establishment of CRPC: 1. serum testosterone level of the castrated patient has to be lower than 1.7 nmol/l; and 2. PSA expression levels should have increased twice in a row from an interval of 1 week or more than 3 consecutive measurements with the lowest value increased >50% and >2 g/l, and more than 2 increases in novel lesions based on bone scanning or soft tissue lesions [157,158].

The molecular mechanisms and the intracellular pathways underlying the development of CRPC remain a challenge for researchers and clinicians. The understanding of

the mechanisms that support the acquisition of the androgen-resistant phenotype is a key element to improve treatment. Currently, the existent knowledge establishes that the mechanisms driving CRPC include the adaptative capacity of the AR-pathway, modifications on the expression of AR coregulator proteins, as well as changes in AR-associated cell signaling pathways (Fig. 1.4.).

The amplification of the *AR* gene is one of the most recognized events in CRPC development. The AR gene amplification, rarely observed in untreated patients, is quite common in CRPC patients. It causes an overexpression of the AR protein that allows activation of the AR pathway even when androgens levels are extremely reduced (Fig. 1.4.) [159,160]. Also, post-translational modifications of the AR protein have been found in CRPC samples like acetylation, methylation ubiquitination, sumoylation and phosphorylation [161-163]. However, their impact on AR activity and driven of CRPC remains to be clarified.

Furthermore, various of the identified *AR* gene mutations have been linked with the establishment of CRPC (Fig. 1.4.) [164,165]. Mutations regularly found in CRPC cases are more frequent in the ligand-binding domain [166-169] and have been shown to affect AR specificity, allowing receptor activation by other molecules than androgens. The ACT>GCT mutation responsible for changing Thr877 to Ala in the hormone-binding domain is frequently detected in CRCP patients and is one of the most recognized examples of how a single mutation can alter the AR specificity [170,171]. LNCaP cells harboring this mutation displayed increased growth in response to androgens but also to non-androgenic steroids (Fig. 1.4.) [170,171].

Additionally, several AR splice variants have been shown to be involved in PCa resistance to ADT [172]. These variants are typical of resistance conditions being non-detectable in men not receiving any therapy [173,174]. It is the case of AR-Vs variants, which are characterized by lacking the ligand-binding domain, and thus are unable to bind androgens ligands. Among the AR-Vs, the AR-V7 is the most studied AR variant in PCa and has been associated with advanced stages and reduced survival in patients with CRPC [175]. The AR-V7 is a constitutively active variant of AR and its action has been related with repression of tumour-suppressor genes [176-179], being a driving force in tumour growth. This is a feature also identified in patients. It was found that the AR-V7 variant can regulate androgen-responsive genes, namely target oncogenes that contribute to the development of CRPC [180]. The AR-V7 was also shown to circumvent anti-androgen-chemotherapy treatment in CRPC [181-184].

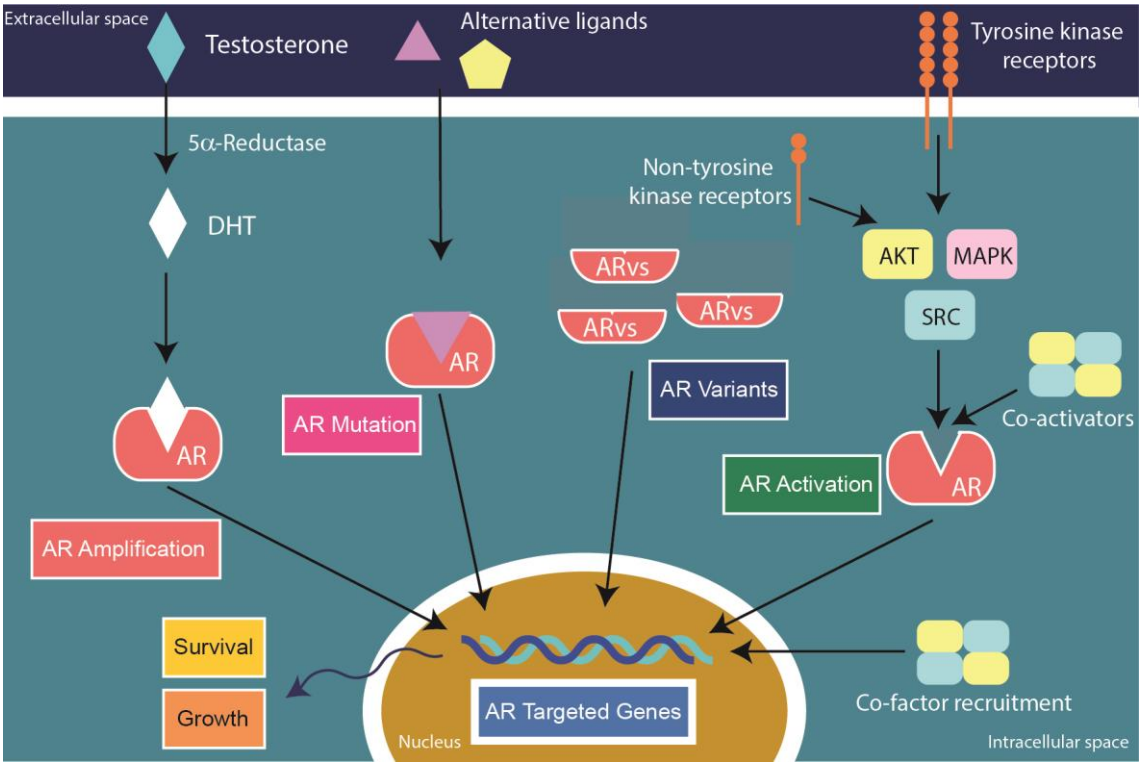

Figure 1.8. Molecular pathways associated with the development of CRPC. The amplification of the AR gene with the enhanced sensitivity to androgens is the most common event occurring in CRPC. The augmented production of DHT due to increased activity of 5α-reductase has also been implicated. Moreover, mutations in the receptor ligand-binding domain may allow that other molecules, like estrogens, corticosteroids, or even anti-androgens can bind to and activate the AR pathway. On the other hand, several AR variants, like the ARvs, devoid of the ligand-binding domain have been connected with treatment resistance. Furthermore, the AR can be activated by others receptors, like the receptor tyrosine kinases), which when overactivated can phosphorylate AR activating it, through AKT, SRC and/or the mitogen-activated protein kinase (MAPK) pathways. Finally, it can be mentioned the deregulated actions of AR co-activators inducing transcription or recruiting other transcription factors enhancing the transactivation of AR-regulated genes.

AR regulation of transcription depends on its interaction with other co-factors responsible to co-activate or co-repress receptor function (Fig. 1.4.). At this moment, there are >150 different co-activators or co-repressors of AR [185]. AR co-activators recruit other transcription factors to initiate transactivation of AR-regulated genes enhancing receptor activity. Mostly, these co-regulators are enzymes that modulate other proteins, but can also be chaperones and regulators of the RNA splicing machinery [186,187]. Some of these AR co-activators, like ARA70, Tip60, TIF2 or SCR1 have been shown to be up-regulated in CRPC samples [188-190]. Moreover, inflammatory signals like, IL-4 and 8 could promote the activity of coactivators of AR, consequently activating the AR and regulating the development of CRPC [191,192].

On the other hand, co-repressors inhibit the transcription of AR-regulated genes, and the alteration of co-repressors like SMRT seems to be involved in progression of advanced stages of PCa [193].

Other androgen-independent pathways capable of cross-talk with the AR have been shown to be activated during ADT, modulating cell survival and promoting the development of CRPC (Fig. 1.4.). The PI3K/AKT/mTOR pathway is the best studied in the context of

progressing CRPC. Activation of PI3K/AKT/mTOR leads to increased cell proliferation and survival. AKT activity is increased in CRPC cell lines compared to androgen-dependent cells [194,195]. The loss or AR signaling by enzalutamide treatment was shown to drive PTEN loss and, thus, increased AKT signaling [196,197]. On the other hand, PI3K inhibition in the absence of PTEN contributed to increasing AR levels [197]. In this way, the PI3K/AKT/mTOR and AR signaling interact and compensate each other in response to ADT therapy. Proteins with kinase activity and downstream effectors in intracellular signaling like SRC and MAPK are also increased in CRPC [198,199]. SRC and MAPK family kinases have been shown regulate the AR signal and the development of CRPC [200,199]. MAPK is related with the HER2/Neu pathway and can directly phosphorylate AR [200,201]. Also, SRC family members were shown to directly phosphorylate AR with its subsequent activation [199,202]. The overexpression of epidermal growth factor receptor in CRPC cell lines was capable of activating the transcription of AR-target genes independently of androgens [203,200,204,205].

The increased intra- and extra-prostatic production of androgens have been indicated as another mechanism underlying the development of CRPC. Currently, it is accepted that the transition to CRPC is related with increased activity of 5α-reductase, which enhances DHT biosynthesis and actions, and, thus, maintain AR signaling active even when androgens circulating levels are low [206-209]. Recently, it was also verified that the androgens production in CRPC cases is higher than that observed in hormone sensitive patients [210]. This growing source of androgens can also be sustained by *de novo* steroidogenesis from cholesterol within tumor tissues or by its conversion in the adrenal glands [211,212].

Other evidence pointed out that CRPC develops from a reserve of stem cells that do not depend on the presence of androgens but can respond to this hormone [213]. In fact, a minority sub-population of cells in prostate tumors that do not express the AR have been identified as PCa stem or progenitor cells [214]. These cells are drug-resistant and can continue proliferating upon ADT, existing as a darwinian selection of resistant-cancer cells [214,215]. It is currently known that mutations in prostate stem cells can generate PCa stem cells that are associated with resistance to PCa treatment [216,214,217].

Finally, the progression of PCa is also related with a metabolic adaptation. Like other cancer cell types, PCa cells have the ability of reprogramming metabolism and alterations on glycolysis, fatty acid metabolism and other metabolic pathways have been characterized as features accompanying the progression of disease [218]. It is a matter of fact that non-neoplastic prostate cells produce, accumulate, and secrete high levels of citrate, which is essential to sperm function [219]. This ability of PCa cells to accumulate and secrete citrate is a consequence of the reduced flux of this metabolite to the mitochondria for metabolization into the tricarboxylic acid (TCA) cycle [220,221]. Contrastingly, PCa cells produce very low levels of citrate [221,222]. Interestingly, increasing evidence has shown that glycolysis, fatty-acid and glutamine metabolism are the principal sources of energy in PCa cancer cells, and that this metabolic activity is exacerbated in CRPC [73,223,224]. The details

of PCa cells metabolism and its impact in progression of the disease will be further explored in a chapter 3.

1.4. Prostate cancer diagnosis and therapy

The principal methods to detect the initial phases of PCa include, the digital rectal examination, PSA serum levels and transrectal ultrasound guided biopsy followed by histologic examination [225]. Rectal examination is a systematic inspection to screen dysfunction in male and remains an indicator for PCa biopsy if an irregularity or nodule is detected [226]. However, the performance of digital rectal examination to detect initial stages of PCa is limited. Historically, the human prostatic acid phosphatase (PAP) was the first serum biomarker for PCa screening, but it showed insufficient sensitivity [227,228]. Later on, PSA was considered the ideal biomarker, which, despite the known limitations, still is the most used diagnosis marker [227,229,230]. It is known that 15 % of men with normal or low levels of PSA had PCa [231,232]. On the other hand, PSA can generate false positives, as benign diseases and prostatitis are also known to increase its levels [233,234]. In the last years, to improve the use of PSA ceras screening method, several derivatives of its measurement were implemented, such as total vs free-PSA, PSA density, PSA age-adjusted or PSA velocity [235-238].

Collection of biopsy materials is a fundamental and standard piece for diagnosis of PCa, eliminating the presence of false positives and false negatives by PSA screening [239]. The heterogeneity, multifocal nature, and the complex mechanisms driving PCa progression, have made difficult the identification of sensitive and accurate biomarkers. Nevertheless, molecular markers like *PCA3* gene, *TMPRSS2:ERG* fusion gene, *PTEN*, *AR*, microRNAs, metabolic molecules, or circulating tumor cells and exossomes, are promising biomarkers that should be used more frequently in the diagnosis of PCa [240-242].

Imaging techniques based on the detection of cancer metabolites are also important tools helping the diagnosis of PCa, but cannot be used as primary techniques due to their still limited accuracy. PET/CT based on increased tumors glycolysis and enhanced incorporation of [18F]fluorodeoxyglucose (FDG) has been used in several types of cancer. However, it showed modest results with limited sensitivity in PCa, which displays a high glycolytic profile only in advanced stages [243]. [11C]acetate uptake is another diagnostic tool for the detection of tumour cells, which is related with the overexpression of fatty acid synthase (FASN), a key enzyme in lipid synthesis in cancer cells [244,245]. Despite the fact that PCa displays increased FASN expression, PET/CT scanning based on detection of [11C]acetate also showed poor results in PCa diagnosis because it is not a cancer-specific tracer [246]. C-choline PET/CT showed better results and has been identified as useful for advanced stages of PCa [247]. Magnetic resonance imaging is also used for detection, localization and definition of local extent of PCa and is recommended as an additional imaging in patients with negative

biopsy and suspected primary PCa [248]. Treatment approaches for PCa differ according with the stage of the disease.

Radical prostatectomy is common for treatment of localized PCa, and encompasses removing the prostate gland and if necessary the surrounding tissue [249,250]. Radiotherapy is also used at this stage with the objective of eradicating local PCa before it metastasizes [251,252]. ADT is also used for treatment at this stage together with prostatectomy in patients with high risk to develop metastasis [252]. ADT suppresses the production of testosterone or the action of androgens by inhibiting the AR. ADT also is the first-line treatment for metastatic PCa. However, several adverse effects of ADT are known, such as decreased bone mineral density, metabolic changes, and sexual dysfunction [253,254]. First-generation anti-androgens used for several years include bicalutamide, flutamide and nilutamide. Bicalutamide is the most common ADT therapy as a nonsteroidal anti-androgen for PCa treatment and has less hepatotoxicity compared with flutamide or nilutamide [255]. To improve the efficacy and minimize adverse side-effects of treatment the second-generation of anti-androgens was developed, such as enzalutamide, a competitive AR antagonist, that is administered orally. It binds to the receptor ligand-binding domain and inhibits AR translocation to the nucleus [256]. Enzalutamide treatment was shown to improve patient's survival and its use in combination with other drugs is in phase of evaluation[257,258].

As mentioned above, within 38 months of administrating ADT, the CRPC emerge, and other treatment options have to be envisaged, namely chemotherapy. Mitoxantrone was the first cytotoxic chemotherapy approved for the treatment of CRPC [259]. Next, Docetaxel, an inhibitor of microtubules disassembling, showed a positive effect increasing overall survival in mCRPC patients in approximately 21 months [260-262]. In the last years, several agents based in docetaxel have been developed. Cabazitaxel is a taxane microtubule dynamics inhibitor by promoting tubulin assembly and blocking its dissociation. It has shown good activity in docetaxel-resistant PCa [259]. In clinical trials, the combination of cabazitaxel and prednisone resulted in longer progression-free survival and overall survival [263]. Moreover, to overcome the taxane resistance an early switch between docetaxel and cabazitaxel showed an improvement in the overall survival [264]. However, the clinical benefit of cabazitaxel administration is still very limited in CRPC treatment, increasing survive modestly, displaying toxicity and some studies also reported patients death [263,265]. In the effort of trying to find alternatives to ADT and taxanes, radium-223 was also approved for bone metastasis PCa improving the survival in these patients [266].

The continued production of androgens in CRPC makes the inhibition of intra-tumoral androgen synthesis an alternative strategy for treatment with clinical benefits. At this level, the inhibition of cytochrome P (CYP17), which converts pregnanes into steroid hormones, by abiraterone acetate showed a preventive action in PCa growth [267,268]. Nowadays, the combination of abiraterone acetate with other therapies is being tested.

Immunotherapy has emerged as a promising treatment approach in several types of cancer, and diverse immune-based therapies are currently in test or being investigated. The application of immunotherapy, i.e targeting the immune system to control cancer growth also has been studied in PCa. Immunotherapy with Sipuleycel-T was recently approved for the treatment of CRPC [269]. The treatment is composed by antigen-presenting cells cultured in the presence of recombinant PAP linked to granulocyte-macrophage colony-stimulating factor for maturation of antigen-presenting cells [269]. Clinical benefits were shown with this therapy, but it has high costs associated [270].

Despite the advances made in the last years, CRPC treatment still is a puzzling issue for clinicians. Only further research efforts deepening the knowledge of the molecular pathways underpinning the development of PCa and CRPC, may lead to the establishment of new and effective therapies, alone or in combination.

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Chapter 2

The peculiarities of cancer cell metabolism: a route to metastasization and a target for therapy

This Chapter was submitted published as:

Carvalho TM[#], **Cardoso HJ**[#], Figueira MI, Vaz CV, Socorro S. The peculiarities of cancer cell metabolism: a route to metastasization and a target for therapy. Eur J Med Chem. 2019 1;171:343-363. doi: 10.1016/j.ejmech.2019.03.053

[#] Contributed equally

Abstract

The last decade has witnessed the peculiarities of metabolic reprogramming in tumour onset and progression, and their relevance in cancer therapy. Also, it has been indicated that the metastatic process may depend on the metabolic rewiring and adaptation of cancer cells to the pressure of tumour microenvironment and limiting nutrient availability. The present review gatherers the existent knowledge on the influence of tumour microenvironment and metabolic routes driving metastasis. A focus will be given to glycolysis, fatty acid metabolism, glutaminolysis, and amino acid handling. In addition, the role of metabolic waste driving metastasization will be explored. Finally, we discuss the status of cancer treatment approaches targeting metabolism. This knowledge revision will highlight the critical metabolic targets in metastasis and the chemicals already used in preclinical studies and clinical trials, providing clues that would be further exploited in medicinal chemistry research.

Keywords: cancer; metabolism; metastasization; therapy; tumour microenvironment; metabolic waste

2.1. Introduction

Despite the enormous advances that have been made in the comprehension of tumour cell biology and development of innovative therapeutic strategies, 90% of cancer-associated deaths occur in consequence of metastasis, since the majority of patients with advanced metastatic disease do not respond to the current therapies [1]. This is mainly due to the complexity of tumour development, existing more than 200 distinct cancers "entities" depending on cell origin, mutations and genetic variability, and on the crosstalk with the tumour microenvironment [2], which greatly compromises the effectiveness of any therapy.

The metastatic process involves the dissemination of tumour cells from the primary tumour and seeding and development of new colonies in distant tissues. Although not completely understood, this is a multi-step process called the invasion-metastasis cascade triggered by the continuous cell proliferation, angiogenesis, accumulated genetic alterations and activation of complex pathways in the primary tumour [2,3]. More recently, the capacity of cancer cells to reprogram energy metabolism was reintroduced into the puzzling complexity of tumour development and progression [4], and has been explored in the context of therapy.

The initial considerations about metabolic reprogramming in cancer were stated by Otto Warburg and colleagues during the early twentieth century, which demonstrated that tumour cells display increased glucose consumption and enhanced glycolytic capacity (Fig. 2.1.) in detriment of the use of mitochondrial oxidative phosphorylation (OXPHOS) even in the presence of high oxygen concentrations [5]. Actually, both OXPHOS and glycolysis are recognized pathways supporting tumour cells survival and growth, and it is known that the

lactate resulting from the enhanced glycolytic activity can promote mitochondrial metabolism and can be used to generate alanine and glutamate [6,7]. The lactate exported to the extracellular space contributes to the acidification of microenvironment and remodelling of extracellular-matrix promoting tumour dissemination, and can also be taken up by neighbouring cells and used as a metabolic fuel enhancing cell survival and proliferation [6].
Nevertheless, cancer cells reliance on other metabolic pathways has been gaining increasing relevance. It has been widely accepted that tumour cells use glutamine as a substrate for energy production, maintaining the tricarboxylic acid (TCA) cycle and being important also for redox homeostasis, and nucleotide and fatty acid (FA) synthesis [8]. Concerning lipids, these are crucial targets in cancer cell biology, as the *de novo* lipid synthesis is necessary for membrane assembly in cell division. Citrate, a key metabolite of the TCA cycle, is a precursor for lipid biosynthesis and is also taken up and metabolized by cancer cells [9]. Also, FA consumption through ß-oxidation is important as an energy source, contributing to generate NADH and/or NADPH, supporting ATP production and redox balance, and other biosynthetic reactions [6].

More challenging is the role of the metabolic waste products (e.g. acetate and ketone bodies) that have been emerged as active molecules capable of modulating cancer cells metabolism, and that can also be used as a bioenergetic source for tumour cells [10].
Also, cannot be ignored the experiments that evidence the metabolic cooperation among cancer cells themselves, as well as with other surrounding cells, in which cells transfer catabolites to each other, allowing tumour cells survival in a nutrient-deficient environment [6].

Finally, it has been demonstrated that tumour cells suffer metabolic adjustments during tumour progression and metastasization, which are crucial to their adaptation and survival in "hostile" microenvironments with nutrient and oxygen fluctuations and with high pressure from a variety of extracellular signals [11]. Nevertheless, a deep understanding of all the metabolic strategies adopted by cancer cells during the metastatic process still is a challenge.

The present review addresses the influence of tumour microenvironment and the metabolic alterations known to be involved in the metastatic process, namely, those concerning glucose, lipid, glutamine and non-essential amino acids metabolism. The role of metabolic waste products driving metastasization also will be discussed. Lastly, the review summarises the therapeutic approaches employed for treatment of metastatic and non-metastatic cancers having metabolism as a target. It is expected all gathered information will provide insight into the molecular mechanisms underlying metabolic alterations driven metastasis and highlight important targets that would be exploited in medicinal chemistry in the design of new compounds.

2.2. Generalities of the metastatic process

The sequence of events occurring in the invasion-metastasis cascade starts with the dissemination of a single cell or tumour clusters in a process that resembles, at molecular and cellular levels, the epithelial-mesenchymal transition (EMT) occurring in embryogenesis [2,12]. The EMT enables cancer cells to acquire multiple malignant features crucial to the development of metastatic capacity, as the loss of adhesion and alteration of cell morphology. These changes mainly result from the diminished expression of cytokeratins and E-cadherin (an intercellular adhesion molecule and key marker of the epithelial phenotype), and increased expression of E-cadherin repressors (e.g. SNAIL), and N-cadherin and vimentin, both well-known markers of the mesenchymal phenotype [13-15]. The altered expression of these molecular players triggers cell detachment and the acquisition of a motile phenotype, which together with the increased ability to degrade components of the extracellular matrix, through the increased the expression of matrix metalloproteinases (MMPs), promotes the invasion of tumour cells to the surrounding tissue [14].

The metastatic process progresses with the intravasation of tumour cells through the nearly blood vessel endothelial cells to the circulation, were the circulating tumour cells, exposed to a hostile environment, will gain immune and physical protection conferred by the platelets and neutrophils actions [2]. Circulating tumour cells may be arrested on the vessel surface and overflow thought the blood vessels walls by different mechanisms depending on the tissue: i) proliferating in the lumina of the vessels leading to endothelial wall rupture and directly accessing the tissue parenchyma [16]; ii) passing in a passive way; or iii) passing through the blood vessels walls by a process termed transendothelial migration, which depends on bioactive factors released from the cancer and immune system cells that promote an increase on vascular permeability [17].

The metastatic process ends with tumour cells colonization in the host tissue, which may regain epithelial characteristics and form a secondary tumour [14]. This process, as the initial activation of the EMT, is dependent on the interaction of tumour cells with the nearby stroma and modulated by environmental factors.

2.3. The pressure of tumour microenvironment to cancer cell dissemination and metastasis

The tumour microenvironment is composed by different types of noncancerous cells, such as endothelial cells, adipose cells, fibroblasts and immune cells [18], which cooperate or compete in their roles and “objectives”. Also, for this reason, the tumour metabolic microenvironment is characterized by low levels of oxygen (hypoxia to anoxia), acidosis, and a high-enrichment in waste products [19,20]. Therefore, cancer cells remain in pressure for having a favourable microenvironment allowing their survival and dissemination to different places in the human body [2].

2.3.1. Hypoxia

Hypoxia is a well-known event in neoplastic tissue due to the high oxygen consumption rates of highly proliferative cells and the limiting capacity of vasculature to supply cancer cells with enough amounts of oxygen. This causes insufficient intracellular oxygen concentrations, which have been related to several hallmarks of cancer, namely, the metabolic reprogramming, with a switch in metabolism from OXPHOS to glycolysis [21,22]. Over the years, the hypoxia-inducible factor 1 (HIF-1) has been characterized as the "master orchestrator" of hypoxia-associated events in malignancies. HIF-1 acts as a transcription factor regulating the expression of a panoply of genes that have the consensus HIF-1 binding sites [23]. Under hypoxia-associated low glucose levels, HIF-1 promotes the expression of key regulators of glycolytic metabolism (Fig. 2.1.), namely, glucose transporters (GLUTs), hexokinase (HK), and lactate dehydrogenase (LDH) [24-26].

Moreover, hypoxia is also responsible for driving angiogenesis, resistance to treatment, and genetic instability associated with cancer progression [27]. HIFs promote angiogenesis by rising the phenomenon of perfusion, and modulating the expression of multiple angiogenic factors, such as vascular endothelial growth factor (VEGF), stem cell factor, placental growth factor and platelet-derived growth factor (PDGF) [28-32]. In a HIF-dependent manner, hypoxia also promotes the phenomenon of extravasation and EMT [33,34], since it contributes to the remodelling of extracellular matrix, for instance by regulating the activity of collagen-modifying enzymes [35,36], the expression of cadherins, and the degradation of cytoplasmatic membrane through MMPs, which globally increases the motility of cancer cells [37-41]. On the other hand, hypoxia also modulates the immune surveillance promoting the accumulation of macrophages [42,43], which have been shown to have a role stimulating fibrosis and creating new vessels, since they produce growth factors like VEGF, PDGF, or tumour growth factor-α and -β [44-46]. In sum, the hypoxic microenvironment is intimately linked with cancer invasion and metastasization, mostly by the HIF-1 activity stimulating angiogenesis, EMT and cell mobility, as well as the high glycolytic profile.

2.3.2. pH

Tumour microenvironment is generally acidic, whereas inside the cells an alkaline pH is maintained. The main sources of H^+ ions are ATP and CO_2, and extracellular acidosis also occurs due to the acceleration of glycolysis, and compensatory mechanisms of other pathways, such as glutaminolysis, FA metabolism and ketogenesis [47]. Several lines of evidence showed that acidosis activates several oncogenic signalling pathways increasing migration and invasiveness of cancer cells [48-50]. Lower pH has also been shown to prompt the destruction of the extracellular matrix. Proteases such as MMPs and cathepsins have been shown to have their activity increased at low pH [51].

Dissemination of cancer cells to different organs is driven by the contribution of lymphatic system, and is usually depends on VEGF actions. Expression levels of this growth factor are highly regulated by hypoxia as already discussed. However, several reports

demonstrated that also an acid environment promotes VEGF expression and new blood vessels growth [52,50].

In addition, some studies demonstrated that the Na^+/H^+ exchanger 1 is upregulated in cancer cells enabling the expulsion of H^+ ions to the extracellular space [53-55]. Inside the cell, alkaline conditions are essential to maintain cell proliferation through glycolysis and the high activity of limiting enzymes in this process [56,57]. The H^+ gradient established between inside and outside the cell was also implicated in drug resistance, as it difficult drugs passage across the cell membrane [58,59]. Moreover, acidosis also is capable of inhibiting the differential immune responders, such as natural killer and $CD8^+$ T lymphocytes [60-63]. Taken together, the acidic metabolic environment surrounding tumours endow cancer cells with a set of features routing to metastasis and resistance to therapy.

Finally, a set of diverse metabolites and metabolic waste products generated by glycolytic, amino acid and FA metabolism can be engaged in the promotion of metastasis. The specific actions of these players will be discussed in the following sections.

2.4. Glucose metabolism in association with cancer metastasis

Increased use of glycolysis and production of lactate even in the presence of enough oxygen to maintain OXPHOS is a feature observed in many cancers types, mainly as a result of the overexpression of glycolytic genes (Fig. 2.1.) [64]. The augmented glycolytic activity is generally accepted to support the high division rate of cancer cells by rapidly providing ATP [65]. Although less efficient compared with OXPHOS (2 ATP in glycolysis vs. ~36 ATP in OXPHOS), glycolysis generates ATP faster than OXPHOS [66] and sustains the high energy demand of tumour cells. This higher glycolytic rate also has been related with amino acid, lipid, and nucleic acid metabolism, with several metabolites of glycolysis entering the biosynthetic pathways [67]. Overall, the enhanced glycolytic flux supplies cancer cells with the energy substrates and raw material that allow rapid turnover of cell constituents and the high rate of cell division.

The molecular programming of metastasis promotes tumour progression through cell migration and anchorage-independence, and not necessarily enhanced proliferative activity [68]. However, several studies have corroborated the idea that aberrant cancer metabolism, namely, the enhanced aerobic glycolysis, not only provides energy for rapid tumour cells growth, but it also helps cancer cells against anoikis and provides advantages for metastasization [69]. Thus, besides supporting the exacerbated cancer cell proliferation, aerobic glycolysis may promote metastasis by leading to ATP generation, and modulating the pH of tumour microenvironment, as well as, several signalling pathways. All these alterations are crucial for anti-anoikis and anchorage-independent expansion [70].

2.4.1. Glucose transporters

Several glycolytic genes (Fig. 2.1.) are overexpressed in tumours and associated with malignancy and aggressiveness. Firstly, it can be mentioned the facilitative GLUTs, a family of 14 transmembrane proteins responsible for the uptake of glucose, and other hexoses, from the extracellular space [71]. Several isoforms of these transporters, namely GLUT1, GLUT3, GLUT4 and GLUT5 have been reported as contributors for progression, poor prognosis, and invasion in various types of cancer such as colorectal, non-small cell lung, cervix, breast, pancreatic, gastric and head and neck squamous cell carcinomas [72-77]. Overexpression of the above-mentioned members of the GLUTs family was found to be closely related with the hallmarks of the metastatic process, namely, lymphatic permeation, venous invasion, and lymph node and hepatic metastasis [74]. Moreover, GLUT1 was shown to increase MMP-2 expression and activity, which was crucial for the development of metastasis [73].

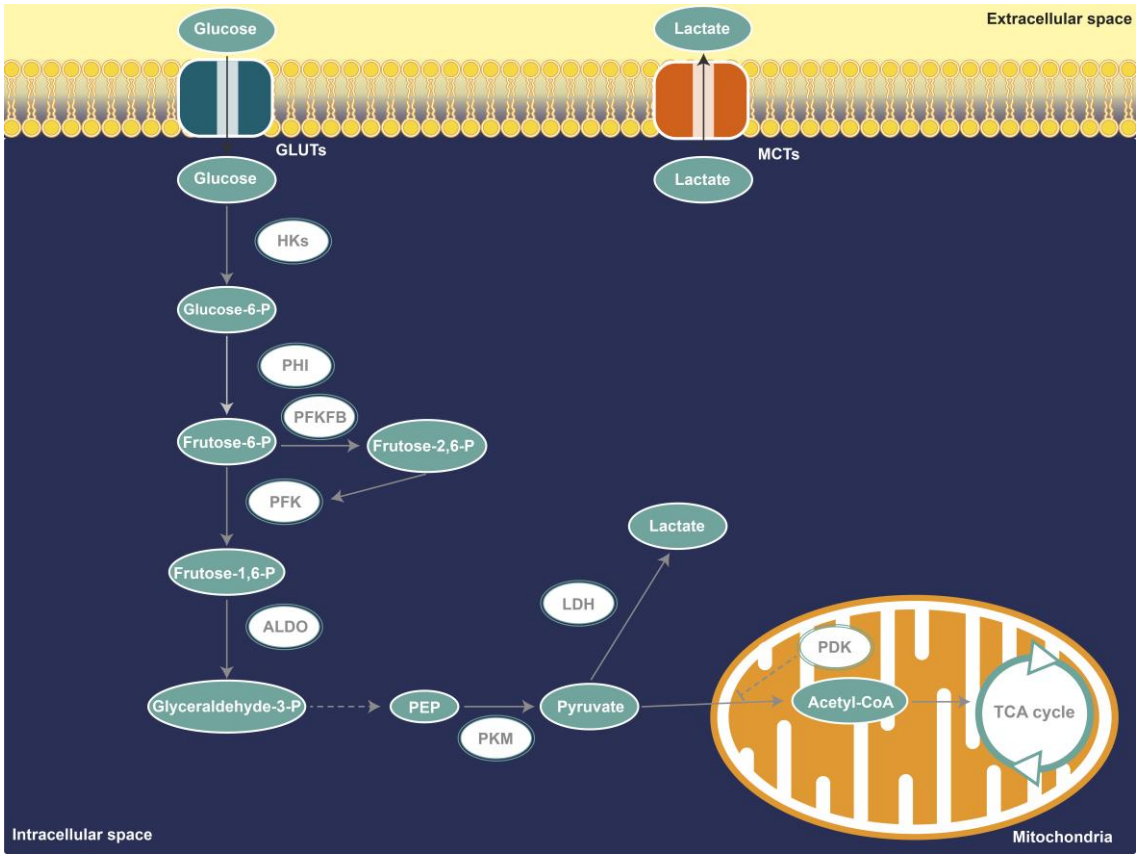

Figure 2.1. Glycolytic metabolism in cancer cells. Glucose enters into the cell through glucose transporters (GLUTs). In the cytoplasm, glucose undergoes several modifications mediated by a tandem activation of enzymes involved in glycolysis. The product of glycolysis, pyruvate, enters the mitochondria where it is decarboxylated, generating acetyl-CoA that is the starting point for the tricarboxylic acid (TCA) cycle to produce energy for the proliferating cells. Cancer cells preferentially use the conversion of pyruvate into lactate, by the activity of lactate dehydrogenase (LDH). Then, lactate can be exported to the extracellular space by monocarboxylate transporters (MCTs) to be used by neighbourhood cells. Targets known to be involved in the metastatic process are highlighted with white letters and ovals. Legend: ALDO, Aldolase; HKs, Hexokinases; PDK, Pyruvate dehydrogenase kinase; PEP, Phosphoenolpyruvate; PFK, Phosphofructokinase; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PHI, Phosphohexose isomerase; PKM, Pyruvate kinase.

2.4.2. Glycolytic enzymes

Inside the cell, glucose is metabolized to glucose-6-phosphate by HK in the first step of glycolysis (Fig. 2.1.). HK2, the embryonic isoform of HK, has been correlated with advanced tumour stages, increased risk of recurrence and harmful clinical outcomes in breast cancer, pancreatic cancer and neuroblastoma patients [78-81]. HK2 also seems to have a role in the emergence of metastasis. The *in vitro* knockdown of HK2 in a pancreatic ductal adenocarcinoma cell line or its overexpression in neuroblastoma cells led, respectively, to decreased or increased tumour growth and incidence of lung metastases in a mice xenograft model [82,83].

Phosphohexose isomerase (PHI) is another glycolytic enzyme that has been demonstrated to stimulate the metastatic process by its action as the extracellular autocrine motility factor (AMF) [84]. In non-malignant cells, PHI/AMF endorse cell motility during neural growth, lymphocyte maturation and embryo implantation, although in cancer it can promote invasion and metastasis [85-87]. PHI is exported to the extracellular space through non-classical pathways and it binds to the membrane receptor gp78 activating intracellular downstream molecules [88,89]. This results in relocalization of small GTPases, crucial regulators of actin dynamics [90]; augmented expression of integrins, which translocate to the cell surface regulating cell adhesion and activating the MMP-2 [91,92]; stimulation of EMT by NK-kB activation; upregulation of SNAIL and downregulation of E-cadherin [93,94]. Serum PHI/AMF has been proposed as an useful diagnostic marker for gastrointestinal, kidney and bladder cancers [95,96]. High levels of PHI/AMF are linked with metastasis in colorectal carcinoma, esophageal squamous cell carcinoma and lung cancer [97-99]. Overexpression of PHI/AMF correlates with NIH-3T3 fibroblasts transformation and enhances the metastatic ability of human colon cancer cells [88]. The mechanism involves cytoskeletal reorganization through the activation of RhoA and Rac1 GTPases, expression of integrins and stimulation of EMT [88]. PHI/AMF can also bind endothelial cells that express gp78 stimulating angiogenesis and vascular permeability, thus promoting cancer cell intravasion [100,101]. Suppression of PHI/AMF led to reverse mesenchymal-epithelial transition in lung fibrosarcoma and endometrial cancer cells [102,103]. Besides PHI/AMF, also its receptor, known as gp78 or AMFR, has been directly associated with metastasis, namely, in the case of colorectal carcinoma, esophageal squamous cell carcinoma and lung cancer [97-99,104].

A specific isoform of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB) the PFKFB3 also displayed increased expression levels in cancer and has been linked with metastasis [105]. This enzyme has a regulatory role and fuels glycolysis by producing fructose-2,6-biphospate (Fig. 2.1.), an allosteric activator of the glycolytic enzyme phosphofructokinase 1 (PFK1) (Fig. 2.1.). Moreover, PFKFB3 can support cancer by permitting cell cycle progression from G1 to S phase [106]. PFKFB3 inhibition in endothelial cells responsible for producing tumour blood vessels led to normalization of their proliferation and maturation, resulting in diminished cancer cell invasion, intravasion and metastasis [107].

To the glycolytic targets listed above should be added aldolase A (ALDOA), a glycolytic enzyme responsible for the conversion of fructose-1,6-biphosphate to glyceraldehyde 3-phosphate (Fig. 2.1.) and dihydroxyacetone phosphate. Various studies have demonstrated the association of ALDOA with the metastatic phenotype in several cancer types, namely, osteosarcoma, lung squamous cell carcinoma, hepatocellular carcinoma, renal cell carcinoma and pancreatic carcinoma [108-113]. Moreover, recent findings suggest that ALDOA acts as an inducer of EMT by upregulating N-cadherin and vimentin and downregulating E-cadherin [110,114].

Also, the embryonic isoform of pyruvate kinase isozyme M2 (PKM2) has been linked with tumour aggressiveness and metastasis. Elevated PKM2 levels were associated with poor prognosis in gallbladder cancer, hepatocellular carcinoma, tongue cancer, esophagus cancer, cervix cancer and human hilar cholangiocarcinoma, and also with advanced tumour stages and metastasis in colorectal and pancreatic cancers [115-121]. PKM2 appears to promote metastasis by directly or indirectly supporting cell migration, angiogenesis and chemoresistance. Knockdown of PKM2 diminished cell migration in human colon cancer, lung cancer, and hepatocellular carcinoma cells [115,120,122]. This enzyme also has been shown to bind the tumour endothelial marker 8 on the surface of endothelial cells promoting angiogenesis [123]. Moreover, PKM2 activity has been linked with EMT, since it was shown to regulate the transcriptional activity of ß-catenin triggering augmented expression of SNAIL and vimentin, and decreased expression of E-cadherin [124,125]. High PKM2 serum levels were correlated with poor response to 5-fluorouracil in colorectal cancer patients, suggesting its role in chemotherapy resistance [126]. On the other hand, targeting PKM2 with pharmacological approaches and RNA interfering technology has been found to change cancer cell metabolism towards OXPHOS, and suppress tumour invasion, migration and metastasis [127,122].

Lactate dehydrogenase is a crucial enzyme responsible for the last step of glycolysis converting pyruvate in lactate (Fig. 2.1). The accumulated lactate in consequence of the high glycolytic activity of cancer cells is exported to the extracellular space acidifying the tumoral microenvironment, which favours tumour growth and metastasis [128]. Accordingly, multiple studies have shown that diminished expression of LDHA represses the invasive and metastatic phenotype of cancer cells [129-131]. Moreover, LDHA downregulation increased E-cadherin expression and decrease focal adhesion kinase, MMP-2 and VEGF, which are key factors for EMT and angiogenesis [129,132].

Lastly, pyruvate dehydrogenase kinase-1 (PDK1) that works as an inhibitor of pyruvate dehydrogenase (Fig. 2.1.), the enzyme responsible for the conversion of pyruvate to acetyl-coA. This is an important step for OXPHOS, and PDK1 is crucial linking glycolytic metabolism and mitochondrial phosphorylation activity. PDK1 was observed to be important for the emergence of breast and liver metastasis, where the conversion of pyruvate to lactate is increased and oxidative metabolism is concomitantly reduced [133,134]. Interestingly, HIF-1 and angiogenesis are required in malignant process and metastasis, being diminished by

mitochondrial activation as a result of PDK1 inhibition, which corroborates the crucial role of this enzyme in tumour progression and metastatic process [135].

2.5. Lipid metabolism pathways promoting cancer metastasis

Altered lipid metabolism and mainly FA biosynthesis and oxidation (Fig. 2.2.), seem to be a central characteristic in the metastatic process. Enhanced use of FA might supply proliferating cells with the building blocks for assembly of new cell membranes or other cellular structures, signalling metabolites and substrates for FA oxidation, which ensures the fulfilment of the increased energy needs associated with cancer progression [136]. Both FA biosynthesis and oxidation contribute significantly to cancer cell proliferation, but also metastasis.

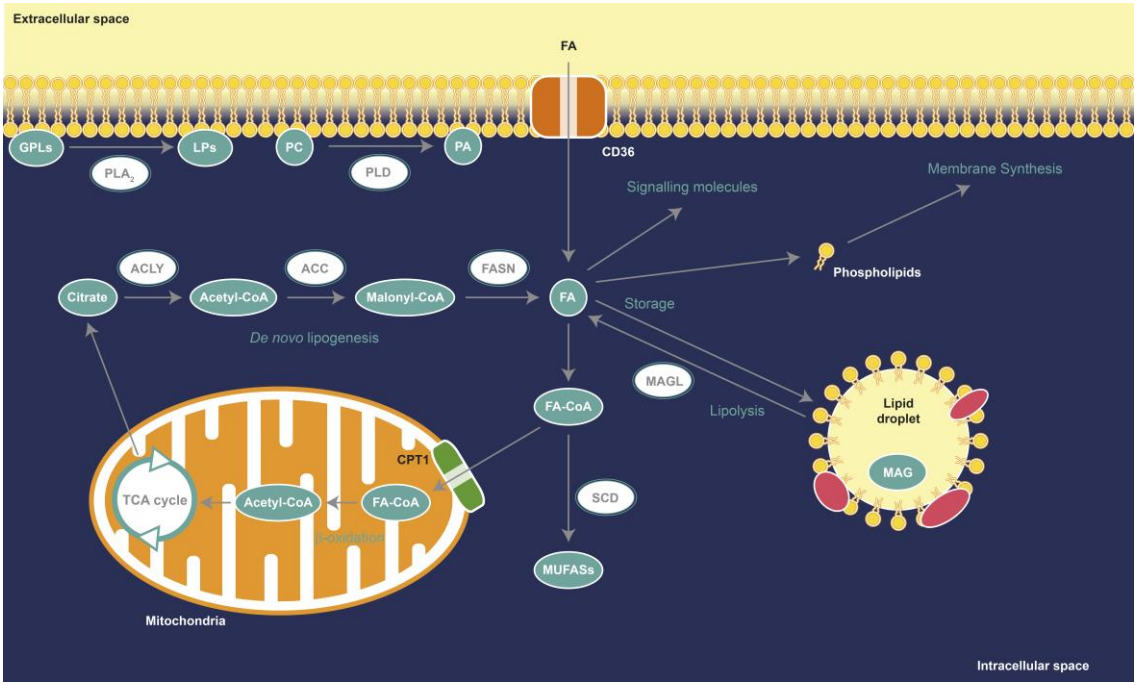

Figure 2.2. Fatty-acid (FA) uptake, oxidation and biosynthesis in the interplay of carcinogenesis and metastasization. FA uptake occurs through the CD36 transporter. Intracellular FA supply proliferating cells with signalling metabolites, generate phospholipids for membrane synthesis, or, if not used, can be stored in lipid droplets. Upon cell needs more energy substrates, lipid droplets are a source for lipolysis, for example, through the activity of monoacylglycerol lipase (MAGL) that converts monoacylglycerols to FA. FA oxidation starts with conjugation with carnitine by carnitine palmitoyltransferase 1 (CPT1), allowing its transport to the mitochondria generating acetyl-CoA. This product of FA ß-oxidation can enter the tricarboxylic acid (TCA) cycle producing citrate. Citrate is the fuel for the de novo synthesis of FA over the activation of adenosine triphosphate citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and FA synthase (FASN), which culminates with the activity of stearoyl-CoA desaturase (SCD) to produce monounsaturated fatty acids (MUFAs). Phospholipase A2 (PLA2) and phospholipase D (PLD) are enzymes involved in the catabolism of cell membrane lipids and associated with cancer cells ability to migration and invasion. Targets known to be involved in the metastatic process are highlighted with white letters and ovals.

2.5.1. Fatty acid synthesis

FA synthesis is an anabolic process that depends on the tandem activation of adenosine triphosphate citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and FA synthase (FASN) (Fig.

2.2.). Firstly, ACLY is responsible for *de novo* FA synthesis as a crucial enzyme that converts citrate to oxaloacetate and cytosolic acetyl-CoA. ACLY expression was correlated with progressive stages and presence of lymph node metastasis in lung cancer, and high expression of ACLY was found in gastric, bladder and breast tumour cases when compared with patient adjacent normal tissues [137-140]. Moreover, a novel study demonstrated that ACLY is a crucial interacting protein of the low molecular weight isoform of cyclin E (LMW-E) in the cytoplasm [141]. ACLY is fundamental for the LMW-E mediated migration, transformation and invasion of breast cancer cells both *in vitro* and *in vivo* [141]. LMW-E augmented ACLY enzymatic activity, which increased lipid droplet formation providing cells with the building blocks that support aggressive progression [141]. Other aggressive types of cancer namely ovarian, colorectal cancer and melanoma also have this particular relationship [141]. In opposition, the microRNA-22 (miR-22) targeting ACLY was evidenced to suppress cancer cell proliferation and invasion in osteosarcoma, prostate, cervical and lung cancer cells [142]. Moreover, the level of intracellular lipid droplets and cellular triglycerides and cholesterol were markedly decreased after miR-22 action [142], which demonstrated the role of this miRNA modulating ACLY activity, and the importance of lipid metabolism in metastasization.
ACC is the limiting enzyme in FA synthesis, which carboxylates acetyl-CoA to form malonyl-CoA (Fig. 2.2.). Enhanced phospho-ACC expression was associated with worse overall survival in node-positive patients with head and neck squamous cell carcinoma [143]. Furthermore, in hepatocellular carcinoma patients, increased ACC1 expression was markedly associated with several aggressive clinicopathological characteristics, namely vascular invasion and poor differentiation [144]. ACC1 upregulation was also notably correlated with poorer overall survival and disease recurrence in hepatocellular carcinoma patients [144].

FASN is an important enzyme in the production of saturated FA. It uses one acetyl-CoA and adds sequentially seven malonyl-CoA molecules to form the 16-carbon palmitate. Several reports demonstrated that FASN is overexpressed and strongly correlated with tumour progression [145-149]. FASN expression promoted peritoneal metastasis of ovarian cancer by the induction of EMT [145]. EMT mediated by FASN actions was also described in prostate and breast cancer cells [149,146]. Moreover, FASN overexpression provided metastatic advantages on colorectal cancer cells and was also a key player in the maintenance of glioma stem cells stemness being associated with tumour progression and invasion in glioblastoma [150,147]. Inhibition of FASN expression repressed the invasion and migration of hepatocellular carcinoma cells and retinoblastoma, suggesting the contribution of FASN to metastasis [151,152].

Stearoyl-CoA desaturase (SCD) is the last enzyme involved in the *de novo* synthesis of FA (Fig. 2.2.) and catalyses the rate limiting step in the formation of monounsaturated FA (MUFAs). SCD introduces the first double bond in the cis-delta-9 position of several saturated fatty acyl-CoAs. SCD1 expression was directly related with the tumour-node-metastasis stage, grade of tumour, and lymphatic metastasis in clear cell renal cell carcinoma whereas SCD1 knockdown contributed to the suppression of the aggressive phenotype in tumour cells [153].

SCD1 activity was also implicated in the EMT, and migration and invasion of colon cancer cells [154].

2.5.2. Lipid catabolism

As above mentioned, also the lipid catabolic metabolism contributes to cancer metastasis. Firstly, monoacylglycerol lipase (MAGL), known as the rate-limiting enzyme for the breakdown of monoacylglycerols, converts monoacylglycerols to FA (Fig. 2.2.). Prostate cancer cell aggressiveness, ovarian tumour growth and ovarian cancer cell migration can be impaired by inhibiting MAGL with chemical inhibitors as JZL184, or using shRNA probes [155,156]. Moreover, administration of JZL184 to several malignant colorectal cancer cell lines suppressed and altered the expression of EMT markers [157]. Accordingly, MAGL expression and activity was shown to induce EMT in hepatocellular carcinoma [158]. Thus, MAGL has been indicated as part of a gene signature related to EMT and stem-like properties of cancer cells [157].

Other important enzymes in lipid catabolism are the phospholipase A2 (PLA2) and phospholipase D (PLD). PLA2 catalyses the hydrolysis of glycerophospholipids to produce lysophospholipids and FA (Fig. 2.2.). PLD hydrolyses phosphatidylcholine (PC) to yield phosphatidic acid (PA) and free choline. Human epithelial ovarian cancer ascites simulated migration and invasion of human epithelial ovarian cancer cells and promoted metastasis *in vivo* in a PLA2-dependent manner [159]. PC and phosphatidylethanolamine (PE) comprise part of the bulk of cell membrane lipids, and several lines of evidence have shown that PLD has a crucial role in cell migration, being also important for invasion and metastasis [160,161]. Active PLD augmented lymphoma cell metastasis, whereas inactive PLD2 isoform inhibited metastasis, MMP-2 expression, and glioma cell invasion [162]. In breast and lung cancer, silencing PLD2 affected negatively the ability of invasiveness, which indicates some dependency of cell invasion on PLD2 [163]. Moreover, overexpression of PLD2 or PLD1 enhanced cell invasion in Matrigel [163,164]. Ablation of PLD1 in the tumour environment suppressed the activation of Akt and MAPK signalling pathways by VEGF, resulting in integrin-dependent cell adhesion, and migration on extracellular matrices attenuated the neovascularization and tumour progression [165]. Moreover, mice lacking PLD1 or treated with the PLD1 inhibitor FIPI showed fewer lung metastasis than wild-type counterparts [165].
Carnitine palmitoyltransferase 1 (CPT1) catalyses the first and rate-limiting step of FA oxidation. CPT1 conjugates FA with carnitine transporting them to the mitochondria (Fig. 2.2.), where the acylcarnitines undergo FA oxidation and are oxidizide to carbon dioxide. CPT1A is the most widely expressed isoform being detected in the liver and in several other tissues. CPT1B is predominately expressed in the muscle whereas CPT1C is the main isoform in the brain [166,167]. Several studies suggested the involvement of CPT1 in the metastatic process. In triple-negative breast cancer a high expression of FA oxidation enzymes, together with the generation of high levels of ATP, was implicated in the metastatic process. FA oxidation inhibition led to reduced transwell migration potential and inhibition of Src

autophosphorylation, a common pathway upregulated in metastasis [168]. Similarly, CPT1A knockdown resulted in decreased tumour growth. All these findings suggest that CPT1A expression is correlated and promote tumour distant metastasis. Furthermore, high-grade glioblastoma was associated with the aberrant expression of some regulators of FA metabolism, namely CPT1A and CPT1C [169]. CPT1A also had the ability to promote migration and anoikis resistance in colorectal cancer and alveolar rhabdomyosarcoma cells, thus favouring the metastatic process and cancer progression [170,171].

2.5.3. Fatty acid uptake

Lastly, a mention to the cell surface receptor CD36 (Fig. 2.2.), which is responsible for the uptake of FA from the extracellular space. CD36 is upregulated or amplified in several types of human metastatic tumours [172]. Knockdown or overexpression of CD36 in oral squamous cell carcinoma cells led to increased or decreased appearance of metastasis, respectively [173]. Moreover, FA deprivation reduced the *in vitro* migration of breast cancer cells even in the presence of glutamine and glucose, suggesting that FA metabolism takes the crucial role in the metastatic process [174]. Recent findings highlighted the stimulating identification of metastasis-initiating cells (MICs) in different types of cancers and demonstrated that they could be distinguished from non-metastatic subpopulations based on their lipid handling properties [175-177,173]. The internalization of FA is a crucial process of quiescent MICs that eventually might promote metastasis upon their dissemination [173]. These MICs displayed high expression of CD36 and have been shown to be responsible for initiating metastasis in experimental models of human melanoma and breast cancer [173]. MICs are so sensitive to circulating fat levels, that a high-fat diet or stimulation with palmitic acid strongly increases the metastatic potential of MICs [173]. Moreover, FA uptake by CD36 promoted EMT in liver cancer cells, increasing their migration and invasion in *in vitro* assays [178].

The polyunsaturated FA consumed through the diet also might have relevant effects on cancer dissemination and metastasis. This type of FA have been correlated with aggressiveness of cancer cell lines and demonstrated to accumulate within both normal-appearing and cancer breast tissue of human patients and animal models [179]. Though, while omega-6 FA, namely linoleic acid increased tumour growth and progression, omega-3 FA such as eicosapentaenoic acid and docosahexaenoic acid had the opposite effect [180-182]. This important piece of data strongly emphasizes how a controlled diet can be a relevant aspect in cancer patients. Also, supports that appropriate management of a diet can be envisaged as a complementary approach in therapy.

2.6. Glutaminolysis and non-essential amino acids in the metastatic process

Besides participating as the building blocks of proteins, amino acids exert several roles as metabolic drivers, energy source and mediators of cell signalling. Furthermore, these molecules can affect the "behaviour" of cancer disease, since amino acids supply might affect tumorigenesis, invasion and the metastatic process [183].

2.6.1. Glutamine

Firstly, glutamine that is the most abundant amino acid in the bloodstream. However, glutamine was found to be markedly reduced in tumour environment due to increased uptake and utilization as an energy source [184]. The use of glutamine in cellular metabolism begins with its conversion to glutamate by glutaminase (GLS, Fig. 2.3.) [185], which is enhanced in the case of cancer cells and under the control of c-Myc oncogene [186]. Glutamate has a wide range of functions, including the supply of TCA cycle by its conversion to α-ketoglutarate by glutamate dehydrogenases (GDH1 and GDH2). GDH1 expression was associated with tumour size, tumour stage, lymph node metastasis and liver metastasis in colorectal cancer [187]. GDH1 expression also has been reported to be increased in metastasis of gallbladder cancer and murine hepatocarcinoma [188,189]. Recently, the mechanistic of GDH1 driven metastasis started to be disclosed. It was reported that the GDH1 product α-ketoglutarate activates calcium/calmodulin-dependent protein kinase kinase 2 signalling, which contributed to modified energy production conferring resistance to anoikis and tumour metastasis in human lung cancer xenograft models [190].

GLS expression was upregulated in highly-invasive ovarian cancer cells, which depended more on glutamine metabolism than the low-invasive counterpart cells [191]. Similarly, the expression of GLS was show to modulate the metastatic potential of B16F10 melanoma cells [192], SNAIL-induced EMT and tumour metastasis growth [193]. Accordingly, glutamine deprivation decreased invasiveness of ovarian cancer cells while feeding cells with α-ketoglutarate restored their invasiveness ability [191]. Moreover, distinct approaches targeting glutamine metabolism, such as GLS gene silencing, glutamine deprivation, or GLS inhibitors (e.g. 968 and BPTES) inhibited cancer cell proliferation and migration, and metastasis [194,195,193]. Either via GDH1 or GLS, glutamine incorporation into the TCA cycle supported the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) leading to promotion of proliferation, migration and invasion in cancer cells [196]. Other reports also showed that GLS inhibition reduced breast and lung tumour cell proliferation and invasiveness by decreasing glutathione, an important antioxidant that confers protection against reactive species[197,198].

2.6.2. Proline

Another important amino acid is proline (Fig. 2.3.), which can be obtained from the interconvertion of glutamate via the intermediate metabolite Δ^1-pyrroline-5-carboxylate (P5C) that is produced from glutamate by pyrroline-5-carboxylate synthase [186]. The proline cycle is characterized by the production of proline in the cytosol, from P5C with the oxidation of NADH or NADPH by pyrroline-5-carboxylatereductase (P5CR). In the mitochondria proline is converted back to P5C by proline dehydrogenase (PRODH). ATP production via PRODH is essential to sustain cell proliferation and the development of tumour cell spheroids *in vitro* [199]. Furthermore, inhibition of PRODH decreases the appearance of breast cancer metastasis in mouse models [199]. Proline metabolism seems to be increased in a metastatic cell line derived from MCF10A human epithelial breast cells relative to nonmetastatic MCF10A-derived cell lines [200]. Moreover, increased proline metabolism has been observed in esophageal squamous cell cancer and correlated with invasiveness and resistance to oxidative stress [201]. This relationship suggests that an increased capability to accumulate proline is necessary for highly metastatic cells survive under stressful conditions. In addition, collagen, that accounts for 80% of the extracellular matrix proteins, is composed by about 25% proline and might function as an external supply of proline, since proteolytic enzymes remodelling the extracellular matrix has been suggested to trigger mesenchymal-like behaviour [202].

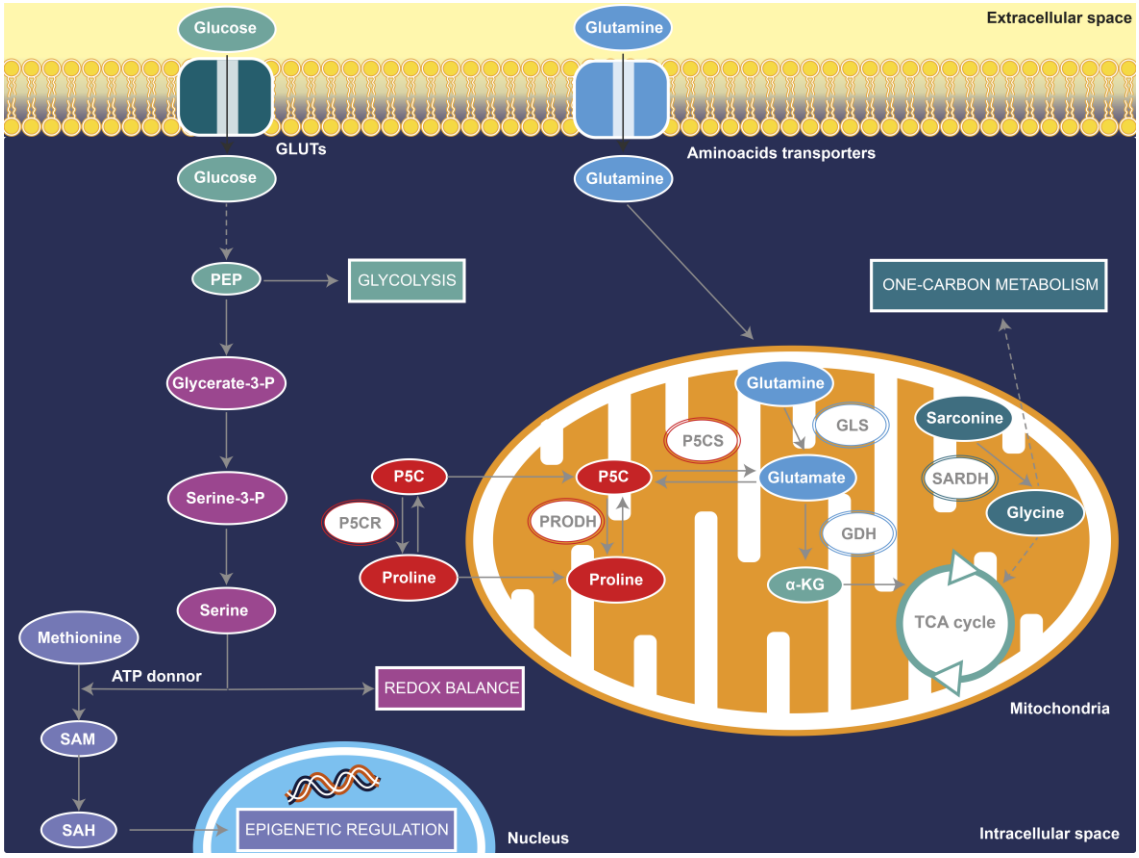

Figure 2.3. Glutaminolysis and non-essential amino acids in cancer cells at the interplay of one-carbon metabolism, glycolysis and redox balance. The uptake of glutamine occurs through amino acids transporters at cell membrane. In mitochondria glutamine is converted to glutamate by glutaminase

(GLS). Glutamate is converted to α-ketoglutarate by glutamate dehydrogenases (GDH) and can enter the tricarboxylic acid (TCA) cycle. Proline can be obtained from the conversion of glutamate via the intermediate metabolite Δ1-pyrroline-5-carboxylate (P5C) that is produced from glutamate by pyrroline-5-carboxylate synthase (P5CS). The proline cycle is characterized by the production of proline in the cytosol from P5C with the oxidation of NADH or NADPH b pyrroline-5-carboxylatereductase (P5CR). In the mitochondria proline is converted back to P5C by proline dehydrogenase (PRODH). Serine is generated through the glycolytic pathway from the intermediate compound 3-phosphoglycerate. Serine has been shown contributing to redox balance and epigenetic regulation. Epigenetic regulation is susceptible to de novo ATP synthesis from serine via tetrahydrofolate cycle, since ATP is important for the conversion of methionine to S-adenosylmethionine (SAM), and consequently SAM is a substrate for methyl transfers to various biomolecules that yields S-adenosylhomocysteine (SAH). a substrate used for DNA methylation. Sarcosine is an intermediate in the one-carbon metabolism of glycine, through the activity of sarcosine dehydrogenase (SARDH). Glycine can be an intermediary of TCA cycle or one-carbon metabolism.

2.6.3. Serine

Serine synthesis is related to the glycolytic pathway at the intermediate compound 3-phosphoglycerate, which is converted to serine via O-phosphoserine. Besides the role in redox balance, serine contributes to the epigenetic regulation in tumour cells. DNA/RNA methylation is partially susceptible to *de novo* ATP synthesis from serine via tetrahydrofolate cycle, since ATP is important for the conversion of methionine to S-adenosylmethionine (SAM), a substrate for DNA methylation [203]. SAM-dependent DNA methylation can influence stem cell pluripotency and survival, and the same mechanisms might be involved in EMT [204,205]. Furthermore, serine also may contribute to metastasis through an extrinsic cell mechanism, since an exogenous source of serine triggers osteoclast-mediated bone resorption, which is essential for osteolytic bone metastasis [206].

2.6.4. Sarcosine

Lastly, sarcosine, an intermediate in the one-carbon metabolism of glycine, has been studied in prostate cancer and its levels are correlated with malignancy [207]. Supplementation with exogenous sarcosine or knockdown of sarcosine dehydrogenase were enough to promote an invasive phenotype in benign prostate epithelial cells and led to intravasation and migration [207-209]. Thus, it can be possible that sarcosine modulates the invasiveness of prostate cancer cells by the one-carbon metabolism, playing a critical role in DNA methylation and epigenetic regulation [210]. More recently, a microarray study showed that sarcosine modulates prostate cancer cells growth *in vitro* and *in vivo* by directly changing the expression pattern of cell proliferation, cell cycle and apoptosis genes networks [211]. Besides prostate cancer, sarcosine was also linked to breast cancer cases. Low expression of sarcosine dehydrogenase was correlated with malignancy and invasiveness features in breast cancer patients, as well as with poor prognosis and reduced survival [212].

2.7. The role of metabolic waste driving metastasization

Complementarily to the previous sections, mainly describing transporters and enzymes related to the regulation of metabolic pathways, this topic will detail the influence of waste products of tumour cell metabolism towards metastasization.

Although glucose is the primary energy substrate for many types of cancer cells, it is established that in stressful conditions transformed cells can rapidly adapt metabolism to the use of alternative fuel sources, including lactate, ketone bodies and acetate among others [67,213].

Lactate is the last product of glycolysis and its overproduction by cancer cells has been known to i) promote acidification of the tumour microenvironment; ii) suppress the immune system; iii) drive the remodelling of the extracellular matrix; and iv) stimulate cell migration and angiogenesis [214]. Altogether these changes concur supporting the metastatic process. Following the known feature of cancer cells as highly-glycolytic and robust producers of lactate, numerous studies have described the high concentration of lactate in primary tumours. This is the case of head and squamous cell carcinomas, cervical cancers and rectal adenocarcinomas in which, the high-lactate content was predictive of high incidence of distant metastasis [215-219]. Indeed, several *in vitro* and *in vivo* studies support the implicating role of lactate in metastasization. In the case of head and neck squamous carcinoma, hepathoma cell lines and glioma lactate increased cell motility in a concentration-dependent manner, likely by modulating expression of cytoskeleton proteins [220-222]. Lactate administration to breast cancer mice xenografts led to the formation of lung metastasis without impacting primary tumour growth [223]. As mentioned above, the export of lactate lowers the extracellular pH due to the co-transport of H^+, which has been directly linked with the promotion of metastasis. Oral administration of bicarbonate or a buffering agent, 2-imidazole-1yl-3-ethoxycarbonylpropionic acid, increased tumour pH markedly diminishing the metastasis process in mice [224,225]. Other mechanisms linked with lactate actions promoting metastasis include the augmented expression of VEGF, HIF-1, and transforming growth factor-ß2, as well as the activation of MMPs and cathepsin in consequence of diminished pH [226,222,195].

Another side-product of glycolysis related with promotion of metastasis is methylglyoxal, which was shown to interact with the molecular chaperone Hsp90 by glycation, permitting transcriptional activation of oncogenes like c-Myc [227]. Furthermore, increased incidence of metastasis was observed in colorectal cancer cell lines when methylglyoxal was added to the cell culture medium, or the knockdown of methylglyoxal-detoxifying glyoxalase was performed [228].

Ketone bodies are produced in the liver under starvation or low food intake, low-carbohydrates diets, intense and prolonged exercise, alcoholism or non-controlled diabetes. After hepatic production, these natural mitochondrial fuels are shuttled through blood stream to other organs where are converted back to acetyl-coA, which can be re-used to supply

mitochondria when nutrients sources are limited [229]. Moreover, enriching mitochondria with ketone bodies produces more energy than lactate and diminishes oxygen consumption [230]. The actually proposed theory is that catabolic fibroblasts in the tumour stroma, have a mitochondrial dysfunction producing ketone bodies that are released in the microenvironment. These metabolites can be reutilized by adjacent cancer cells, fuelling the mitochondrial processes for OXPHOS, to supply anabolic tumour growth [231]. MDA-MB-231 human breast cancer cells overexpressing the crucial enzymes for utilization of ketone bodies have shown enhanced tumour growth and metastatic ability [231]. Furthermore, ketone and lactate were shown to function as chemo-attractants promoting cancer cell migration and fuelling lung metastasis [223].

Lastly, acetate that can also be used as an energy source fuelling mitochondria towards cancer progression and metastasis [232,233]. Acetyl-coA synthetase is an important enzyme that converts acetate into acetyl-CoA. In murine models of liver cancer lacking acetyl-coA synthetase, animals exhibited reduced tumour burden [234]. Brain metastasis also displayed moderate to high expression of acetyl-coA synthetase [234]. Moreover, brain metastasis used acetate as a bioenergetic substrate, which was sufficient to fulfil the energy needed to maintain migration and invasion processes [234].

Thus, considering all the discussed evidence, it becomes clear that the so-called metabolic waste products might have a crucial role as regulator molecules promoting tumour growth, migration and invasion under stressful or scarce resources conditions.

2.8. Therapeutic approaches targeting metastasis through metabolism

The specificities of cancer cells metabolism, and the metabolic alterations that have been identified towards cancer progression and metastasis have greatly nurtured the interest in developing novel anti-cancer therapies that target energy and metabolic pathways (Table 2.1.).

2.8.1. Glycolytic approach

Glucose is the most abundant nutrient in the bloodstream and is a preferred energy substrate used by cancer cells [235]. Thus, several drugs that interfere with glycolytic metabolism have been investigated as anti-cancer agents. This includes GLUT-targeting drugs, which are under clinical investigation, namely the flavonoids silibinin (also called silybin) and dehydrosilybin, and a small molecule known as WBZ117 [236-238]. WBZ117 is a small-molecule inhibitor of GLUT1 leading to downregulation of glycolysis inducing cell-cycle arrest and inhibiting both breast and lung cancer cell growth *in vitro* and *in vivo* [239]. Other GLUTs inhibitors used in preclinical studies in different cancer models, or in clinical trials of phase I or II are fasentin, Bay876, STF-31, apigenin, and ritonavir (Table 2.1.).

The inhibition of HKs with 2-deoxy-D-glucose blocks the first step of glycolysis (Fig. 2.1.) and has been shown to have tolerable toxicity in patients with glioma when treated with fractioned radiotherapy [240]. Nowadays, the interest in targeting HKs has been mainly focused on HK2 isoform, which is often over-expressed by tumour cells [241]. Encouraging preclinical and clinical results have been demonstrating that 3-bromopyruvate and methyl jasmonate can inhibit HK2 exerting anti-cancer effects in several tumour models [242,243]. However, the specificity of 3-bromopyruvate for HK2 is restricted, as this compound also exhibited inhibitory activity for other enzymes, including glyceraldehyde-3-phosphate dehydrogenase and LDH [244]. Indeed, this is a common problem faced by research in this area, that is the lack of specificity of inhibitors, which mainly occurs because of the structural similarities between members of related protein families or by the existence of multiple, sometimes dozens, isoforms in a specific family. Also, for this reason other HK inhibitors have been developed, namely, clotrimazole, bifonazole, and ionidamine (Table 2.1.), which is in clinical trials [245-247].

Still considering the inhibition of glycolytic metabolism, another promising molecule is 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO). This molecule is responsible for the inhibition of PFKFB3 (Fig. 2.1.), leading to reduced cell proliferation and suppression of glycolytic flux, with diminished glucose uptake and decreased the intracellular concentrations of lactate, ATP, NAD+ and NADH [248,249]. Also, a 3PO derivative known as 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15) was selected for pre-clinical evaluation of anti-metabolic and anti-neoplastic activities [250]. PFK15 showed to have acceptable pharmacokinetic properties provoking a rapid induction of apoptosis in malignant cells, diminishing glucose uptake and suppressing tumour growth [249,251].Other molecules able to inhibit PFKFB3 activity (e.g. N4A, YN1 and YZ9) have been tested in preclinical studies in different cancer models, namely cervical cancer (Table 2.1.) [252].

The molecular players at the final steps of glycolysis also have been envisaged as therapeutic targets, namely, those involved in the production of pyruvate. The TLN-232 molecule is an inhibitor of PKM2 (Fig. 2.1.), which has entered in clinical development and has been evaluated as a therapeutic approach in patients with recurrent melanoma or metastatic renal cell carcinoma [253]. Moreover, other two compounds (shikonin and alkannin) were shown to inhibit the effects of PKM2 in breast and lung cancer cell lines (Table 2.1.) [254]. Although the preliminary results have been encouraging, other available studies also suggested that some tumours are PKM2-independent, which can hamper the interest of targeting PKM2 as a therapeutic strategy [255,256].

Several molecules that inhibit LDH have shown anticancer effects. Examples include the natural product gossypol and its derivatives, the FX11 compound, AT-101, oxamate and the gallic acid derivative galloflavin (Table 2.1.), with some compounds of this list already tested in clinical trials for prostate, lung and brain tumours treatment [257-262,131]. At the bottom line of glycolytic flux, have to be considered the high lactate production and the acidification of tumour microenvironment by the exportation of lactate through the activity

of MCTs (Fig. 2.1.) [263]. These molecular players have been associated with the metastatic process and cancer progression [264] and identified as targets for inhibition. In this sense, several inhibitors have been developed and are on ongoing tests. AZD3965, atorvastatin and simvastatin are among the most promising candidates, and all of them are already in clinical assays as inhibitors of MCT1 in several types of malignancies (Table 2.1.) [265-269].

2.8.2. Targeting lipid metabolism

Since high rates of proliferation endorse a marked demand for generation of new membrane phospholipids, targeting lipid synthesis also has represented an important approach for anticancer therapy. The most "attractive" targets in this process are ACLY, ACC and FASN enzymes (Fig. 2.2.). Concerning ACLY and ACC, no evidence exists of compounds targeting these enzymes being tested in clinical trials. Nevertheless, some chemicals targeting ACLY and ACC are being tested in preclinical models, such as BMS-303141, SB-204990 and cucurbitacin B for ACLY and ND-646, ND-654, soraphen A and BAY ACC002 for ACC (Table 2.1.) [270-278]. FASN inhibitors such as cerulenin, C75 and orlistat have been shown to promote apoptosis in several cancer cell lines and retard tumour growth in numerous cancer xenograft models (Table 2.1.) [279]. Furthermore, triclosan, G28UCM, EGCG, fasnall and IPI-9119 are other FASN inhibitors tested in preclinical studies in various tumour models (Table 2.1.) [280-293]. TVB-2640 was the only FASN inhibitor compound that reach the stage of clinical trials (Table 2.1.) [294-296].

On the reverse side of the coin, it has been shown that FA have a role as an energy source for cancer cells. Therefore, blockage of FA oxidation has emerged as a logical promising therapeutic approach. Several preclinical studies using etomoxir as a CPT1 inhibitor have been performed in numerous cell line and animal cancer models as summarized in Table 2.1. Also, etomoxir was tested into the clinical setting with encouraging anti-cancer results, but due to hepatoxicity observed in patients with congestive heart failure it is no longer under clinical trials [297]. Therefore, more alternatives are needed as CPT1 inhibitors. Perhexiline and ranolazine are two candidates already used for antianginal treatments [298], which have emerged with promising results in preclinical studies in malignancies such as prostate and cervical cancer (Table 2.1.) [299-301]. However, the anticancer potential of perhexiline and ranolazine remains to be investigated in clinical trials.

2.8.3. Amino acid-dependent pathways

In line with the assumption that cancer cells depend on glutamine for proliferation and survival, the inhibition of key enzymes in glutaminolysis (Fig. 2.3.) also has deserved attention by their anticancer potential [302]. Agents targeting GLS have demonstrated to inhibit oncogenic transformation of murine fibroblasts and to impair the growth of human breast carcinoma [197,303]. Several GLS inhibitors are under study in the pre-clinical or clinical setting, namely CB-839 and BPTES (Table 2.1.) [304-307].

Moreover, leukaemia and others types of cancer require high amounts of asparagine, a non-essential amino acid [308]. Thus, L-asparaginase, which catalyses the conversion of L-asparagine to aspartic acid and ammonia, has been approved by the United States Food and Drug Administration for the treatment of acute lymphoblastic leukaemia [30]. L-asparaginase administration deprives cancer cells of circulating asparagine, which leads to cell death [309]. The use of L-asparaginase has already entered in clinical trials in leukaemia, lymphoma and ovarian cancer patients [310-312]. Considering other amino acids, the results of decreasing plasma arginine through systemic administration of pegylated arginine deiminase, which converts circulating arginine into citrulline, has produced encouraging data in clinical trials for various types of tumours (Table 2.1.) [313,314]. Finally, targeting tryptophan catabolism also has been tested in therapy since availability of this amino acid provides advantages to cancer cells by the generation of kynurenine that induces immunosuppression [315]. Indoleamine-2,3-dioxygenase (IDO) is the enzyme that catalyses the conversion of tryptophan to kynurenine and it is highly expressed in several malignancies [315]. IDO inhibitors, namely epacadostat and indoximod, are under clinical trials as anticancer therapy for melanoma and other cancer types (Table 2.1.) [316-319].

Table 2.2. Overview of target metabolic pathways for cancer treatment.

Metabolic pathway	Target	Compound	Stage as anti-cancer therapy	Examples of application	Refs
Glycolysis	GLUTs	Silybin	Phase IV	Colorectal cancer patients	[339]
			Phase II	Prostate cancer patients	[340]
			Preclinical	Human cervical and hepatoma cancer cells	[341]
		Dehydrosilybin	Preclinical	Cervical cancer cell line (HeLa)	[342]
		WBZ117	Preclinical	Breast cancer cell lines	[343]
			Preclinical	Lung cancer cell line (A549)	[344]
		Fasentin	Preclinical	Prostate cancer cell lines and leukaemia cell line	[345]
		Bay876	Preclinical	Ovarian cancer cell lines	[346]
		STF-31	Preclinical	Renal cell carcinoma	[347]
			Preclinical	Breast cancer cell lines	[348]

		Apigenin	Preclinical	Pancreatic cancer cell lines	[349]
			Preclinical	Lung cancer cell lines	[350]
			Preclinical	Adenoid cystic carcinoma cell line	[351]
			Phase II	Colorectal cancer patients	[352]
		Ritonavir	Phase I	Breast cancer patients	[353]
			Phase II	Patients with several types of brain tumours	[354]
	HK	2-Deoxy-D-glucose	Preclinical	Various tumours cell lines	[355]
			Phase I	Lung, breast, pancreatic and gastric cancer patients	[356]
			Phase I/II	Prostate cancer patients	[357]
		methyl jasmonate	Phase III	Benign prostatic hyperplasia patients	[330,358]
			Preclinical	Myeloma cells	[243]
		3-bromopyruvate	Preclinical (discontinued)	Anticancer activity *in vitro* and *in vivo*	[242,330,358]
		Clotrimazole	Preclinical	Melanoma cancer cell line (B16 F10)	[359]
			Preclinical	Cervical cancer cell line (HeLa)	[360]
			Preclinical	Glioblastoma cell lines and animal model	[361]
		Bifonazole	Preclinical	Melanoma cancer cell line (B16 F10)	[359]
		lonidamine	Preclinical	Breast cancer cell lines	[362]
			Phase III	Benign prostatic hyperplasia patients	[247]
			Phase II	Advanced breast cancer patients	[246]
			Phase II	Advanced ovarian cancer patients	[245]
	PFKFB3	3PO	Preclinical	Adenocarcinoma cell lines	[363]

			Preclinical	Several cancer cell lines	[248]
		PFK15	Preclinical	Lung carcinoma cell lines	[353]
		N4A	Preclinical	Cervical cancer cell line (HeLa)	[252]
		YN1	Preclinical	Cervical cancer cell line (HeLa)	[252]
		YZ9	Preclinical	Human adenocarcinoma cell line (T47D)	[352]
	PKM2	TLN-232	Phase II	Recurring metastatic melanoma patients	[354]
		Shikonin	Preclinical	Breast and lung cancer cell lines	[254]
		Alkannin	Preclinical	Breast and lung cancer cell lines	[254]
	LDH	AT-101	Phase I and Phase II	Solid tumours and castrate resistant prostate cancer	[259,260]
		FX11	Preclinical	Prostate cancer cell lines	[365]
			Preclinical	Human lymphoma and pancreatic cancer cell lines	[261]
		Gossypol and derivatives	Preclinical	Melanoma, lung, breast, cervix and leukaemia cell lines	[366]
			Phase II	Glioblastoma patients	[258]
			Phase III	Non-small cell lung cancer patients	[257]
		Galloflavin	Preclinical	Hepatocellular carcinoma cell line (PLC/PRF/5)	[367]
		Oxamate	Preclinical	Hepatocellular carcinoma cell lines	[262]
	MCTs	AZD3965	Phase I/II	Prostate cancer	[368]
			Phase I	Lymphoma patients	[369]
			Preclinical	Small cell lung cancer	[268]
		Atorvastatin	Phase II	Prostate Neoplasms	[370]
			Phase I	Solid tumour and leukaemia	[267]

		Simvastatin	Phase II	Adenocarcinoma of rectum patients	[266]
			Phase II	Colorectal cancer patients	[371]
			Phase II	Breast cancer stage IV	[265]
Fatty acid synthesis	FASN	Cerulenin	Preclinical Studies	Colon Cancer, Melanoma, Breast Cancer, Pancreatic Cancer, Ovarian Cancer, Osteosarcoma, Ocular Cancer cell lines and animal studies	[280,293,372-378]
		C75	Preclinical Studies	Breast Cancer, Prostate Cancer, Lung Cancer, Ovarian Cancer, Endometrial Cancer cell lines and animal studies	[282,290,374,378-382]
		Triclosan	Preclinical Studies	Prostate Cancer, Breast Cancer, Ocular Cancer cell lines and animal studies	[291-293]
		G28UCM	Preclinical Studies	Breast Cancer, Ovarian Cancer and animal studies	[288-290]
		EGCG	Preclinical Studies	Breast Cancer, Liver Cancer, Lung Cancer, Prostate Cancer cell lines and animal studies	[282-287]
		Fasnall	Preclinical Studies	Breast Cancer cell lines and animal study	[281]
		IPI-9119	Preclinical Studies	Prostate Cancer cell lines and animal study	[280]
		Orlistat	Preclinical Studies	Melanoma, Endometrial Cancer, Breast Cancer, Prostate Cancer, Colon Cancer, Retina Cancer, Gastric Cancer, Brain Cancer, Leukaemia, Lymphoma cell lines and animal studies	[293,373,383-398]
		TVB-2640	Phase I/II	Breast Cancer, Astrocytoma, Colon Cancer Patients	[294-296]
	ACLY	BMS-303141	Preclinical Studies	Prostate Cancer Cell lines and animal models	[278]
		SB-204990	Preclinical Studies	Prostate Cancer, Ovarian Cancer, Lung Cancer cell lines, Leukaemia cell line and animal models	[276,277]
		Cucurbitacin B	Preclinical Studies	Prostate Cancer Cell lines and animal models	[275]

	ACC	ND-646	Preclinical Studies	Lung Cancer cell lines and animal models	[274]
		ND-654	Preclinical Studies	Hepatic cancer cell lines and animal studies	[273]
		Soraphen A	Preclinical Studies	Breast Cancer, Bladder Cancer, Hepatic Cancer and Prostate Cancer cell lines and animal studies	[271,272]
		BAY ACC002	Preclinical Studies	Pancreatic cancer cell lines and animal studies	[270]
Fatty acid ß-oxidation	CPT1	Etomoxir	Preclinical Studies	Leukaemia, Hepatic Cancer, Prostate Cancer, Gastric Cancer, Lung Cancer cell lines and Nasopharyngeal carcinoma cell lines and glioma cell lines and animal studies	[301,399-405]
		Perhexiline	Preclinical Studies	Leukaemia, Cervical Cancer and Prostate Cancer cell lines and animal studies	[299-301]
		Ranolazine	Preclinical Studies	Prostate Cancer cell lines and animal study	[301]
Glutamine metabolism	GLS	CB-839	Preclinical	Liposarcoma, Ewing sarcoma and melanoma cell lines	[406]
			Phase I	Breast, lung and renal cancer patients	[305]
			Phase II	Several types of lung cancer	[304]
			Phase I	Acute leukaemia	[407]
		BPTES	Preclinical	Liposarcoma, Ewing sarcoma and melanoma cell lines	[406]
			Preclinical	Breast cancer cells lines	[306]
Amino acid metabolism	Arginine availability	Pegylated arginine deiminase (ADI-PEG20)	Phase I	Castrate resistant prostate cancer	[408]
			Phase II	Metastatic melanoma patients	[409]
			Phase II	Acute myeloid leukaemia patients	[410]
			Phase III	Advanced hepatocellular carcinoma patients	[411]
	Asparagine availability	L-asparaginase	Phase I	Lymphoma and leukaemia patients	[312]

			Phase III	Acute lymphoblastic leukaemia patients	[311]
			Phase II	Advanced ovarian cancer patients	[310]
	IDO	epacadostat	Phase II	Several types of melanoma	[412]
			Phase II	Thymus cancer patients	[413]
			Phase III	Urothelial cancer patients	[318]
			Phase I	Rectal cancer patients	[317]
		Indoximod	Phase II	Prostate carcinoma	[414]
			Phase II/III	Melanoma patients	[415]
			Phase I	Brain tumours patients	[316]
Other metabolic routes	HIF1	PX-478	Phase I	Advanced solid tumours	[330]
		Topotecan	Phase I/II/III	Small cell lung cancers patients	[328,329]
			Phase II	Colorectal cancer patients	[327]
		Irinotecan	Phase I	Lung, breast, colon and ovarian cancer patients	[416]
			Phase II	Esophageal and gastric cancer patients	[326]
		Digoxin	Phase II	Prostate and breast cancer patients	[324,325]
		Ganetespib	Phase III	Non-Small-Cell Lung cancer	[323]
		CRLX101	Phase I/II	Advanced solid tumours	[322]
		BAY87-2243	Phase I	Advanced malignancies	[321]
		Bortezomib	Phase II	Primary peritoneal cavity cancer	[320]

	c-Myc	10058-F4	Preclinical	Leukaemia cell lines, pancreatic adenocarcinoma, hepatocellular carcinoma, neuroblastoma and animal studies	[335-338]
		CPI-0610	Phase I/II	Myeloma, Lymphoma, Leukaemia patients	[331-334]

2.8.4. Other routes and general perspective

The inhibition of transcription factors with a central role in the regulation of glycolytic metabolism and glutaminolysis, as is the case of HIF1 and c-Myc, has also been applied in cancer treatment (Table 2.1.). Concerning HIF1, several inhibitors were been developed, namely PX478, topotecan, irinotecan, digoxin, ganetespib, CRLX101, BAY87-2243 and bortezomib, which have been tested for treatment of several human neoplasms, such as lung, breast, esophageal and colorectal cancer [282,284,283,275-277,378,293,375,376,339]. Regarding c-Myc inhibition, 10058-F4 is under preclinical studies and CPI-0610 is in PhaseI/II trials for myeloma, lymphoma and leukaemia patients [371-374,396-399].

In summary, the existence of cancer cell populations metabolically-dependent on several energy sources can be a weakness of many tumours, which has the potential for therapeutic exploitation. However, the liable behaviour of cancer cells and their continued adaptation and reprogramming to hostile environmental conditions can be a drawback in the success of metabolic inhibitors in cancer treatment. In this way, targeting several metabolic pathways simultaneously or in combination with the classical conventional therapies might be a novel promising anticancer approach.

2.9. Conclusion

The peculiarities of cancer cell metabolism started to be disclosed almost 100 years ago. However, the tumour-associated alterations in metabolism and the concept of metabolic reprogramming were recognized as a hallmark of cancer only in the last decade.

The information gathered within this review supports the theory that the metastatic process triggers several pathways of metabolism, depending on the requirements of cancer cells. These pathways supply tumour cells with the necessary resources that enable them starting the migration and invasion processes. Overall, metabolic reprogramming represents a favourable route to metastasization.

Cancer cells respond to unfavourable and stressful environmental conditions reprogramming their metabolism in multiple ways. The panoply of metabolic rewiring actions displayed by tumour cells allowed that numerous targets with therapeutic potential have

been identified in the last years. On the other hand, tumour cells have been shown to be highly sensitive to deprivation of energy substrates. In this context, several chemicals directly targeting metabolic enzymes or transporters have been tested into the clinical setting and many others are under development or in pre-clinical studies. Targeting cancer metabolism as a therapy has been seen as an encouraging strategy since the metabolic differences within and between tumours are less than the ones observed in comparison with the surrounding cell "landscape". However, the exploitation of tumour metabolism as therapeutic approach has represented a challenge since malignant cells are able to easily readapt metabolism using other pathways and, in this way, can overcome specific nutrient depletion effects. Therefore, substantial efforts have been made on combining compounds that target different metabolic pathways. Moreover, the use of classical chemotherapy agents concomitantly with drugs targeting cancer cells metabolism might be a promising therapeutic approach to overcome some of the difficulties perceived on fighting this disease. Nevertheless, medicinal chemistry research efforts still are needed to continue improving the effectiveness of anti-cancer drugs targeting metabolism in metastatic cancers.

2.10. Funding

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project No. 007491; Project No 029114) and National Funds by FCT-Foundation for Science and Technology (Project UID/Multi/00709/2013). Henrique J Cardoso and Marília I Figueira were recipient of FCT fellowships (SFRH/BD/111351/2015 and SFRH/BD/104671/2014, respectively).

2.11. Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 3

Revisiting prostate cancer metabolism: from metabolites to disease and therapy

This Chapter was submitted :

Cardoso HJ, Carvalho TM, Figueira MI, Vaz CV, Socorro S. Revisiting prostate cancer metabolism: from metabolites to disease and therapy. Submitted to Medicinal Research Reviews. 2019

Abstract

Prostate cancer (PCa), one of most commonly diagnosed cancers worldwide, still presents important unmet clinical needs concerning treatment. In the last years, the metabolic reprogramming and the specificities of tumour cells emerged as an exciting field for cancer therapy. The unique features of PCa cells metabolism, and the activation of specific metabolic pathways, propelled the use of metabolic inhibitors for treatment. The present work will revise the knowledge on PCa metabolism and the metabolic alterations that underlie the development and progression of PCa. A focus will be given to the role of bioenergetic sources such as glucose, lipids and glutamine driving PCa cell survival, growth and proliferation. Moreover, it will be described the action of oncogenes/tumour suppressors, and sex steroid hormones in the metabolic reprogramming of PCa. Finally, the status of PCa treatment based on the inhibition of metabolic pathways will be discussed. Globally, this review will update the landscape of PCa metabolism highlight the critical metabolic alterations that could have clinical and therapeutic interest.

3.1. Introduction

Prostate Cancer (PCa) is a heterogeneous and multifactorial disease, being one of the most commonly diagnosed cancers worldwide [1]. Even so, PCa treatment remains a challenging issue, particularly in the metastatic forms of disease. Androgen deprivation therapy (ADT) is the principal treatment for androgen-sensitive primary and metastatic PCa. However, cancer cells become resistant to treatment and disease progresses to the so-called castrate-resistant PCa (CRPC).

The identification of metabolic reprogramming as a hallmark of cancer [2] resulted in a growing interest in the study of cancer metabolomics. It has been widely accepted that cancer cells are metabolically auto-sufficient maximizing energy consumption and production by several mechanisms and adaptations. Moreover, cancer cells display metabolic specificities that contribute to tumour development and progression. In the last years, the metabolic peculiarities and vulnerabilities of cancer cells have been exploited for the development of new anti-cancer therapies. Research on PCa has followed this trend and efforts have been made to disclose the active and functionally relevant metabolic pathways in prostate tumours [3,4]. Several metabolic routes responsible for energy production have been related to the development and progression of PCa by deregulating crucial processes such as cell proliferation, survival and resistance to therapy [5-7]. Therefore, the full understanding of the metabolic specificities of PCa cells is of utmost importance considering metabolism with clinical and therapeutic interest.

Prostate cells present a unique metabolism mainly characterized by the inactivation of the tricarboxylic acid (TCA) cycle, which sustains to the high production of citrate [8-11], a component of prostatic secretions. In PCa cells, the TCA cycle is reactivated, and other

metabolic pathways also have been shown to be overactivated. This includes glycolysis for faster energy production supporting the high cell proliferation rates, though PCa is much less glycolytic than other types of cancer [12,13]. PCa cells also actively use lipids and glutamine as an energy substrates [14-17]. The metabolic adjustments of prostate cells towards malignancy have been related with the activity of oncogenes or loss of function of tumour suppressor genes, which besides governing the cell survival and death pathways regulate the metabolic reprogramming [18]. Moreover, the development of PCa is nurtured by growth factors and hormones, namely the sex steroids androgens, which also have been proposed to have a roleregulating metabolism [19,20].

The present review describes prostate metabolism in the transition to malignancy, and the role of specific metabolites, such as, glucose, lipids, and glutamine, as driving forces in the oncogenic process and progression of PCa. The influence of oncogenes, tumour suppressors and sex steroid hormones in the metabolic reprograming of PCa also is revised. Finally, this review discusses the therapeutic approaches targeting metabolism that have been envisaged for PCa treatment. Overall, this work aims to draw an updated picture of PCa metabolic signatures highlight the targets that could be exploited for therapeutic intervention.

3.2. Prostate metabolic phenotype towards malignancy

Prostate gland presents a specialized and unique metabolism with the main objective of supporting sperm function and viability. However, malignant transformation, and the establishment of cancer phenotype, dramatically change prostate cells metabolism, entailing a set of modifications and the activation of metabolic routes, which culminates in the promotion of their survival and growth.

Healthy prostate cells, mainly the epithelial cells in the peripheral zone of prostate, are characterized by the active production and secretion of citrate [8-11] (Fig. 3.1.). In fact, citrate is the major component of prostatic fluid, and the high concentrations of this metabolite in the ejaculate are important to maintain sperm viability [10,21,11]. In the majority of cells, the citrate originated from the condensation of oxaloacetate and acetyl-coA is oxidized originating isocitrate that fuels the TCA cycle (Krebs Cycle) [22]. In contrast, in prostate cells citrate is the final product of Krebs Cycle [8-11] (Fig. 3.1.). This occurs in consequence of the high concentrations of zinc found in prostate cells [23-26], which are maintained by the uptake of this ion from the interstitial fluid due to the activity of ZIP1 and ZIP3 transporters [17,27]. Zinc is a potent inhibitor of m-aconitase, the Krebs cycle enzyme responsible for oxidation of citrate [26,23,27] (Fig. 3.1.). Moreover, in epithelial cells the production of citrate is possible due to the high concentrations of glucose and aspartate [28-31]. The glucose metabolized in aerobic glycolysis produce pyruvate, which by the activity of the pyruvate dehydrogenase complexis converted to acetyl-coA that enters the TCA cycle by

combining with oxaloacetate to form citrate. The high levels of aspartate in prostate cells are maintained by the efficient activity of membrane transporters [28,32], and serve as substrate for aspartate aminotransferase producing oxaloacetate for citrate synthesis [29,33]. Therefore, citrate accumulates in the prostate epithelial cells being secreted as a component in the ejaculate (Fig. 3.1.).

Healthy prostate epithelial cells also show reduced activity of oxidative phosphorylation (OXPHOS) in consequence of intense citrate production instead of oxidation [8].

In consequence of this unique metabolism and the existence of a truncated Krebs cycle, non-neoplastic prostate cells are extremely poor energetically, sacrificing ATP production for the production and secretion of citrate (Fig. 3.1.). Contrastingly, PCa cells display low levels of zinc [26,23,27] and the m-aconitase activity is no longer inhibited, thus, the oxidation of citrate is restored and the Krebs cycle progresses [24] (Fig. 3.1.). Nevertheless, citrate can be converted back into acetyl-coA for *de novo* fatty acid (FA) synthesis [34] (Fig. 3.1.), which is an important metabolic route in PCa cells [34,35]. Fatty acid synthesis is upregulated in PCa accompanying the progression of the disease. Characteristically, advanced metastatic stages of PCa become highly glycolytic, and other metabolic routes such as glutaminolysis also are over activated in cancer cells compared with non-neoplastic. Considering OXPHOS, it is reactivated in the transition to malignancy, but the highly glycolytic activity in metastatic cancer contributes to the inhibition of mitochondrial respiration [36].

In summary, prostate cells present deficient production of ATP through the Krebs cycle, with glucose being redirected to the citrate production to sustain sperm cell survival and function. In PCa cells, the Krebs cycle is reactivated and fuelled by the high glycolytic activity. Advanced stages of PCa are characterized by the enhanced metabolic activity and active use of glucose and fatty acids, as well as, glutamine. The importance of glucose, glutamine and fatty acid, and the different metabolic pathways driving the development and progression PCa will be explored in the next topics of this review.

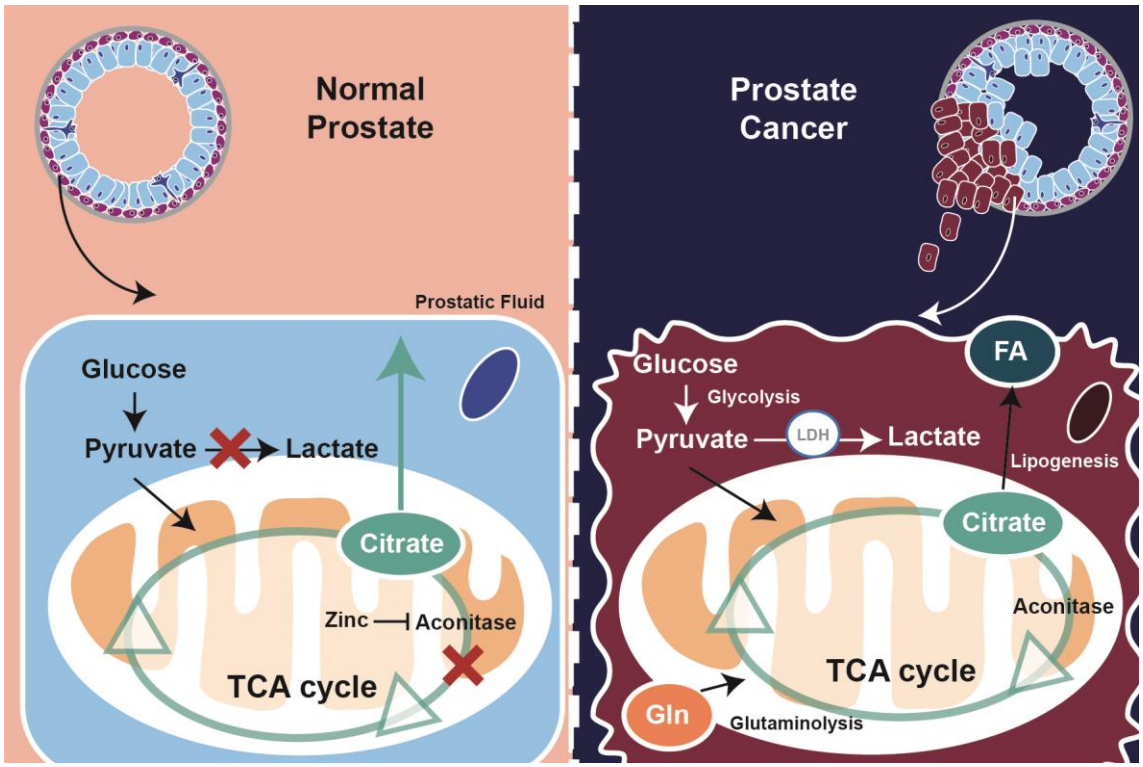

Figure 3.1. General metabolic features of non-neoplastic prostate cells and prostate cancer. The prostate gland produces high amounts of citrate, which is a typical feature of its activity as a secretory organ. This is achieved by the high levels of zinc that inhibit aconitase and, thus, the (tricarboxylic acid) TCA cycle with the subsequent accumulation of citrate. Citrate is then secreted as a component of the ejaculate helping in maintaining sperm viability and function. In contrast, prostate cancer cells are characterized by low levels of zinc, which restores aconitase activity and mitochondrial function, reducing citrate secretion. Advanced stages of PCa display enhanced glycolytic metabolism, and in general, increased expression of the transporters and enzymes involved in glycolysis, as is the case of lactate dehydrogenase (LDH). fatty acids (FA) synthesis and glutamine (Gln) metabolism (glutaminolysis) have been shown to be upregulated during disease initiation and progression.

3.3. Bioenergetics sources and their relevance in prostate cancer

3.3.1. Glucose

To satisfy the high-energy demand, cancer cells present elevated rates of glycolysis, a metabolic adaption known as the Warburg effect [37]. The pioneer studies of Otto Warburg first demonstrated that cancer cells shift the utilization of OXPHOS to aerobic glycolysis to produce ATP [38,37]. Despite less efficient in terms of generating ATP compared with the complete respiratory process, cancer cells preferentially use high rates of glycolysis, as a rapid way of maximizing energy consumption, generating metabolic intermediates for anabolic processes, and increasing biomass growth [39].

However, aerobic glycolysis is not considered a hallmark of primary PCa cases [12,13], being crucial only in advanced prostate tumours [40-42]. In fact, androgen sensitive PCa cells, representing an initial stage of disease, display both active glycolysis and OXPHOS, with the Warburg effect being active only in the metastatic stage [36,42]. For this reason, FTG-PET scans have failed to detect the absorption of radiolabelled glucose in primary PCa [40,43,44].

However, in late stages of disease, high glycolytic activity was related with the stimulation of cell proliferation and migration in PCa cell lines [45]. Also, the intracellular concentrations of glucose have been shown to change the response of PCa cells to different therapeutic drugs [46,47].

Glycolysis is a complex process depending on the activity of several molecular regulators, such as the membrane glucose transporters (GLUTs), and hexokinase (HK) and phosphofructokinase (PFK) enzymes, key targets in this pathway that culminates with the production of pyruvate. GLUT1 overexpression has been detected in PCa cases, with a relevant role enhancing cell proliferation [48-50]. On the other hand, inhibition of GLUT1 enhanced the production of reactive oxygen species and induced apoptosis of PCa cells [51]. Also, the higher expression and activity of glycolytic enzymes HK, pyruvate kinase and PFK underlie PCa progression and the establishment of the glycolytic phenotype [52,41,53-55]. Besides its enzymatic activity in aerobic glycolysis, HK-2 has been shown to promote the viability of PCa cells, and tumour growth in PTEN- and P53-negative xenograft models [56,55]. Another study also showed that the inhibition of HK-2 resulted in a significant reduction of ATP production and cell viability, and augmented caspase-3 activity in PCa cells [13].

In glycolytic cells, pyruvate from glycolysis is converted to lactate by the activity of lactate dehydrogenase (LDH) (Fig. 3.2.). Normally seen as a waste product, lactate has been shown to play a crucial role in cancer cells, including PCa [41,57,58]. Lactate can promote proliferation and the number of colony-forming PCa cells [59]. In an *in vivo* model of PCa, the transgenic adenocarcinoma of mouse prostate (TRAMP), lactate and an acidic tumour microenvironment provided a potential mechanism driving lymph node (86%) and liver (33%) metastases [60]. Similar findings were obtained by knocking-down or inhibiting LDH activity. LDHa knockdown diminished the tumour volume and reduced primary tumour growth, as well as decreased lymph node and visceral metastases in TRAMP mice [60]. Accordingly, the chemical inhibition of LDHa activity *in vitro* decreased cell proliferation, migration, and invasion of castrate resistant PCa (CRPC) cells, and promoted apoptosis [61].

The monocarboxylate transporters (MCTs) responsible for the lactate import/export are central players in the metabolism of cancer microenvironment [62]. MCT1 expression essentially is related with the tumour cells whereas MCT4 seems to be more expressed in the stromal compartment, with their expression being positively correlated in human PCa tissues and associated with poor clinical outcome [59,63,64]. MCTs have been shown to mediate alterations in PCa behaviour. For example, silencing MCT1 abolished the stimulatory effect of lactate on PCa cell survival and tumour growth [59,63], indicating the relevance of lactate as an energy substrate for cancer cells. MCT4 has been indicated as an oncogenic player in PCa with capacity of inhibiting apoptosis and accelerating cell proliferation, as well as, invasion [65].

In the last years, a lactate shuttle system between stroma cells and PCa cells has been proposed (Fig. 3.2.) [66,63]. Fibroblasts, the second most abundant cell type in the cancer microenvironment, have been pointed out as important glucose consumers (high

GLUT1 expression) and active lactate producers [66,59].The lactate produced is then exported to the extracellular space through MCT4 (Fig. 3.2.) [66,59]. Neighbourhood cancer cells in contact with fibroblasts display decreased GLUT1 expression and increased utilization of lactate via MCT1 [66], which indicates the role of lactate as an energy substrate for cancer cells converted back to pyruvate (Fig. 3.2.). The import of lactate also can help cancer cells to regenerate NADH and accelerate glycolysis [57]. The hypothesis of lactate shuttling also is supported by a study showing the high expression of LDH-5 in PCa cells and LDH-1 in fibroblasts [67]. LDH-5 converts pyruvate to lactate whereas LDH-1 enzyme system utilizes the secreted lactate to produce pyruvate (Fig. 3.2.). Therefore, the direct relationship between the increased expression of LDH-5 and MCT1 in PCa cells [67], favours lactate production and simultaneously its uptake, creating a positive feedback loop for lactate use as an energy substrate. This trend has been shown in PCa cells *in vitro*, which gradually become independent of glucose consumption, and start using the lactate imported from the extracellular space to sustain the anabolic pathways and cell growth [63].

3.3.2. Glutamine

Glutamine, although classified as a non-essential amino acid, is the second most common extracellular nutrient, and a key fuel for cancer cell growth, in addition to glucose [16]. Glutamine availability has been shown to maintain higher OXPHOS, being an important substrate for prostate epithelial cells proliferation and growth [17]. In contrast, restriction of glutamine in cell culture medium was associated with diminished viability of metastatic PCa cell line models [68]. Furthermore, glutamine deprivation affected cancer cell motility by changing the actin dynamics, in consequence of altered expression of actin-binding proteins, namely, cofilin and profilin [69].

Indeed, over the years, it has been established that cancer cells growth rely on the reprogramming of other metabolic routes besides glycolysis. The aggressive behaviour of PCa cells has been linked with the anabolic metabolism, fatty acid ß-oxidation, and glutaminolysis (Fig. 3.2.). The mitochondrial enzyme glutaminase transforms glutamine into glutamate, which is converted to α-ketoglutarate, a component of the Krebs cycle [70] (Fig. 3.2.). Overall, cancer cells, including PCa, display enhanced glutamine metabolism, sustained by the increased uptake of glutamine and up-regulated expression of glutaminase [15-17].

The sodium dependent antiporter, the alanine, serine, cysteine, glutamine transporter (ASCT2) is a key molecular player in glutamine homeostasis, being responsible by the glutamine uptake from the extracellular space (Fig. 3.2.) [71-76]. ASCT2 (also named SCL1A5 or L-type amino acid transporter [77,78], is expressed both in healthy and PCa cells [79,80], though its overexpression in tumour samples has been reported [79]. Moreover, ASCT2 expression is significantly increased in patients with recurrent PCa after androgen-deprivation therapy [79]. ASCT2 and enhanced glutamine consumption seem to pave the way for malignant transformation and PCa progression. ASCT2 activity was related with the cell

cycle progression and PCa cell growth *in vitro* [79]. On the other hand, ASCT2 inhibition diminished tumour growth and development of metastasis in PCa xenografts models [79]. Moreover, blocking ASCT2 decreased glutamine uptake, as expected, but also basal oxygen consumption and fatty acid synthesis [79].

Two different subtypes of glutaminase have been identified: a kidney- and a liver-type, respectively, glutaminase 1 and glutaminase 2 [81]. Glutaminase 1 is the isoenzyme predominantly expressed in PCa, and its expression levels were positively correlated with the tumour stage and progression of PCa [82]. *In vitro* studies have been demonstrating the relevance of glutaminase activity promoting PCa cells survival and growth. Glutaminase was related with increased cell viability, growth and invasiveness of PCa cells [82,83]. Also, glutaminase was associated with subclones of metastatic PCa cell lines, which demonstrated an increase of glutamine utilization and higher sensitivity to glutaminase inhibition [82,84]. Blocking glutaminase, besides suppressing cell survival, also was suggested to affect PCa cells metabolism and glycolysis, decreasing ATP production and O_2 consumption, and glutathione production [85].

Finally, the product of glutaminase activity glutamate, also was involved in PCa. Glutamate deprivation or inhibition of glutamate receptor decreased PCa cells' proliferation, migration, and invasion, and augmented the expression levels of pro-apoptotic molecular signals [86].

3.3.3. Lipids

Lipids are a vast class of compounds comprising different types of molecules, namely fatty acids, triglycerides, cholesterol, phospholipids and sphingolipids. In the cell, lipids are crucial structural components of membranes, play a role as second messengers in intracellular signalling, and are an important energy source. In the last years, the capacity of PCa cells in increasing lipids uptake, fatty acids and phospholipids biosynthesis, fatty acid oxidation, and storage of cholesterol, and respective esters, in lipid droplets (Fig. 3.2.), has been described as a relevant mechanism to sustain cell growth and invasiveness.

Lipid (fatty acid, cholesterol, and lipoprotein) uptake by PCa cells was associated with cell growth, cancer progression, and development of bone metastasis [87-89]. However, some inconsistency appears on the role of fatty acids targeting cancer hallmarks, if specific compounds or cell-types are considered. If some articles indicate that fatty acid can have proliferative and growth/invasion promoting action, others report the opposite effect. It is, for example, the case of palmitate that activated apoptosis in PCa PC3 cells, and attenuated C4-2B cells growth [89]. In addition, other fatty acids supplementation protected PC3 cells from the palmitate-induced apoptosis [89]. In contrast, other study described increased migration of PC3 cells and decreased oxidative stress levels (by augmented catalase activity), after palmitate treatment [45].

Also, linoleate, arachidonic acid, and eicosapentaenoic acid have been shown to increase PCa LNCaP cells proliferation [90], whereas a report exists indicating the toxicity of linoleic acid and oleic acid in PC3 cells [91]. Moreover, it was demonstrated that different combinations of fatty acids and the concentration used can change the PCa cells' answer proliferating or dying [92]. This can be the reason for some of the inconsistency of results obtained across the different studies. Nevertheless, the fatty acid transporter CD36 (Fig. 3.2.), that has been characterized in different types of cancer cells [93-95], was associated with an aggressive phenotype of PCa. High CD36 expression positively correlated with lower survival rates and development of metastasis [88]. Studies on the detailed role of CD36 in PCa cells are scarce, but its deletion diminished fatty acid uptake, with impact on cell proliferation and migration, reducing the development of primary prostate tumours, and slowing cancer progression and reducing tumour growth in patient-derived xenografts [88].

The cell incorporated FA can be drive to ß-oxidation or, if not necessary as energy metabolites by proliferating cells, can be stored in lipid droplets (Fig. 3.2.). Fatty acid ß-oxidation was proposed as the dominant bioenergetic pathway in PCa cells [14], which is supported by the fact that primary PCa cases do not rely on intense glycolytic or OXPHOS activity (Fig. 3.1.). The carnitine palmitoyltransferase I (CPT1) catalyses a rate-limiting step in fatty acid ß-oxidation by converting fatty acids to acylcarnitines, which allows their translocation to the mitochondria and energy production (Fig. 3.2.). CPT1A was shown to be overexpressed in PCa compared with benign tissues, especially in high-grade tumours [96]. Moreover, the presence of CPT1A was needed to maintain PCa cells viability and invasion [96]. Lipid catabolism via CPT1 also sustained tumour growth and invasion in xenograft mice models [96,97].

If intracellular fatty acid content exceeds the cell needs, they can be stored in lipid droplets (Fig. 3.2.), which is a process mediated by storage enzymes, such as diglyceride acyltransferase (DGAT). On the opposite direction, the alpha-beta hydrolase domain containing 5 (ABHD5), an activating co-enzyme of adipose triglyceride lipase, promotes the hydrolysis of triacylglycerols generating fatty acids (Fig. 3.2.) [98]. In this way, the inhibition of DGTA1 stopped the formation of lipid droplets in PCa cells, whereas blocking ABHD5 promoted the accumulation of these organelles [99]. Moreover, loss of ABHD5 was also related with the aggressiveness of PCa, with authors suggesting its role as metabolic tumour suppressor gene [100].

Indeed, the metabolism of lipid droplets has been demonstrating to have a fundamental role in cancer cells' survival and growth [101-103]. Accumulation of lipid droplets is a common feature in human PCa [99,102,104] and was associated with the development of high-grade tumours and metastases [105]. Moreover, droplets' storage protects lipids from harmful peroxidation, and has been associated with resistance to therapy [101,106]. However, this relationship is unknown in the case of PCa, and further research is needed to clarify the role of lipid droplets in prostatic cancer.

Fatty acids synthesis is not a relevant process in non-neoplastic cells, being almost inexistent. In contrast, cancer cells, including PCa cells, typically display activated fatty acids *de novo* synthesis. The over-activation of this metabolic route results from the augmented expression of several enzymes such as, ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN), and stearoyl-CoA desaturase (SCD). At an initial step, ACLY converts citrate to acetyl-CoA, a crucial precursor of fatty acid synthesis (Fig. 3.2.). Next, ACC catalyses the conversion of acetyl-CoA in malonyl-CoA (Fig. 3.2.). Both ACLY and ACC were shown to be active in PCa cells, being crucial intermediates in fatty acid production [107,108].

FASN is the enzyme responsible for the synthesis of long-chain fatty acids from malonyl-Coa (Fig. 3.2.). It is overexpressed in prostate malignancies, being associated with the early development and progression of PCa, but also with more aggressive phenotypes, namely the appearance of CRPC bone metastasis [109-115]. Based on these findings, FASN has been suggested as an oncogene [116,112]. Studies in mice have demonstrated that the transgenic expression of FASN potentiated the onset of prostate intraepithelial neoplasia, but not the development of invasive tumours [112]. *In vitro* approaches have shown that FASN overexpression increased PCa cells proliferation and growth [112,117], whereas its inhibition blocked proliferation, induced caspase-dependent apoptosis, and increased ROS production [117].

Other enzymes involved in lipid handling downstream FASN also have been linked with the carcinogenic process. It is the case of SCD that elongates and desaturates palmitic acid. However, SCD expression and function in PCa still is controversial. SCD1 was considered a central regulator of PCa growth and viability [118]. However, Moore et al. [119] showed that SCD expression is lost in prostate carcinoma compared with normal prostate epithelium. In contrast, more recent data reported the association of SCD increased expression levels with malignant transformation and PCa progression [120-122].

Cholesterol metabolism is crucial for structural cell functions, being one of the major components of cell membranes. In normal conditions, a balance exists between cholesterol synthesis, uptake and storage. However, extracellular high cholesterol levels can disturb cholesterol homeostasis, which has been shown contributing to carcinogenesis. Several epidemiological studies have linked hypercholesterolemia with a higher risk of developing PCa, acceleration of the progression to CRPC, and appearance of bone metastasis after ADT [123-125]. This relationship was demonstrated in xenograft models, with diet-induced hypercholesterolemia promoting PCa metastasis [126,127]. Moreover, cholesterol rich-environment and cholesterol metabolites were also related with increased PCa cell proliferation, migration, viability and resistance to docetaxel [128-131], which highlights the importance of characterizing PCa cells dependency on these substrates and their cell and molecular effects over cell fate and metabolism.

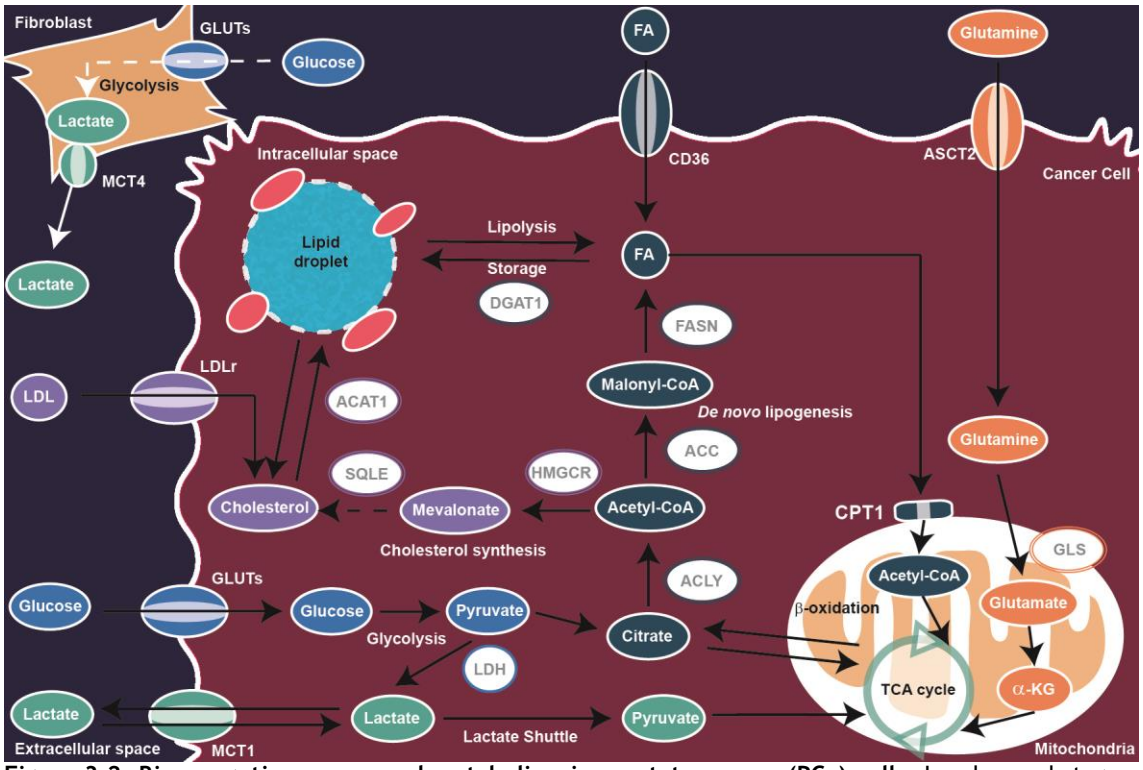

Figure 3.2. Bioenergetic sources and metabolism in prostate cancer (PCa) cells. In advanced stages, PCa cells display increased glucose uptake and high rate of glycolysis and lactate production by the activity of lactate dehydrogenase (LDH). Lactate is exported to the extracellular space through monocarboxilate transporters (MCTs), namely MCT1. PCa cells also collaborate with fibroblasts in a lactate shuttle. High glycolytic fibroblasts actively produce lactate that is exported by MCT4 an uptake by PCa cells, being converted back to pyruvate and used for fuelling tricarboxylic acid (TCA) cycle. PCa cells also use amino acids and glutamine uptake occurs through the membrane amino acids transporter ASCT2. Glutamine is converted to glutamate by glutaminase (GLS). Glutamate in turn is transformed into α-ketoglutarate (α-KG) that enters the tricarboxylic acid (TCA) cycle. Fatty acids (FA) uptake from the extracellular space is mediated by the CD36 transporter. FA ß-oxidation starts with conjugation with carnitine by carnitine palmitoyltransferase 1 (CPT1), which allows the transport to the mitochondria generating acetyl-CoA, that enters the TCA cycle producing citrate. In turn, citrate can be used for the de novo FA synthesis, a metabolic pathway known to be activated in PCa cells and dependent on the activity of adenosine triphosphate citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and FA synthase (FASN). If not being used by the proliferating cells FA can be stored in lipid droplets by the activity of storage enzymes, such as diglyceride acyltransferase (DGAT1). On the contrary, lipid droplets can undergo lipolysis with the conversion of monoacylglycerols to FA. Cholesterol biosynthesis from acetyl-CoA and mevalonate, is mediated by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and squalene monooxygenase (SQLE) enzymes. The generated cholesterol can suffer esterification by acetyl-CoA acetyltransferase1 (ACAT1), being stored in lipid droplets. Internalization of LDL-cholesterol complexed with the low density lipoprotein receptor (LDLr) also was described in PCa cells.

Cholesterol is internalized by binding to the low-density lipoprotein receptors (LDLr). High LDL-cholesterol availability and the LDLr have been implicated in breast cancer cell growth, in both cell lines and a mouse model of hyperlipidaemia [132,133]. The impact of LDL availability and LDLr in PCa is much less known, but it was shown that PCa cells can uptake cholesterol in a process mediated by LDLr [134]. Moreover, upon inhibiting cholesterol biosynthesis with statins, PCa cells seem to develop a compensatory mechanism increasing the expression of LDLr [135].

Also, *de novo* cholesterol synthesis (via squalene epoxidase, SQLE), rather than transcellular uptake (via LDLr) or esterification (via SOAT1) [136] (Fig. 3.2.), was shown to be important for PCa progression, and linked to lethal forms of disease. The expression and

activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR, Fig, 3.2.), a crucial enzyme in the mevalonate pathway for sterol biosynthesis, was related with PCa aggressiveness and CRPC resistance to the anti-androgen enzalutamide [137]. The mevalonate pathway including the HMGCR1 and 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) were shown to be upregulated in prostate stromal cells with stimulating effects on PCa cells growth [138]. This suggests that cholesterol synthesised by stromal cells can be supplied to the PCa cells, promoting their proliferative activity.

Cholesterol esterification is another event that has been shown to potentiate the development and growth of PCa metastatic lesions [104]. An aberrant accumulation of esterified cholesterol in lipid droplets was detected in high-grand PCa and metastases, while the depletion of cholesteryl ester induced the opposed scenario, significantly reducing cancer cell proliferation and invasion, and suppressing tumour growth [105]. Acetyl-CoA acetyltransferase1 (ACAT1) is the enzyme responsible for converting intracellular free cholesterol into cholesteryl esters for storage in lipid droplets (Fig. 3.2.), and its expression and activity were described in PCa [139]. Also, the inhibition of cholesterol esterification by blocking ACAT1 was shown to supress the development of PCa, decreasing cell migration both *in vitro* and *in vivo* and impairing cancer invasion [104,105].

3.4. Oncogenes and tumour suppressor genes driving the metabolic reprogramming in prostate cancer

Oncogenes and tumour suppressor genes' signalling networks are widely known to target the classical hallmarks of cancer, namely, the self-sustained cell proliferation and resistance to cell death [140]. After the Hanahan and Weinberg [141] proposal of the metabolic reprogramming as a new cancer hallmark in 2011, numerous reports have been showing that activation of oncogenes and/or loss of tumour suppressor genes can coordinate the metabolic adaptations of cancer cells. Several molecular players with established or putative roles as oncogenes/tumour suppressors, or activators of oncogenic signalling pathways, have been described as metabolic regulators in PCa, shaping the reprogramming of cell metabolism. Based on the available literature this list includes, for example, the AMP-activated protein kinase (AMPK), hypoxia-inducible factor 1 (HIF-1), phosphatase and tensin homolog (PTEN), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), P53, c-Myc, and sterol regulatory element-binding protein (SREBP).

AMPK is the central energy sensor activated by the increased levels of AMP and ADP (decreased ATP), namely, in consequence of glucose deprivation or hypoxia [142]. Thus, AMPK activity contributes increasing ATP levels by activating cell catabolism and/or suppressing the biosynthetic pathways. AMPK actions also seem to be cancer-related, and in the case of PCa cells, they were shown to regulate glucose and lipid metabolism. AMPK regulation of glucose metabolism was crucial for maintaining PCa cell survival and avoiding apoptosis under glucose

deprivation [143]. This article also showed that AMPK actions were dependent on c-Jun N-terminal kinase (JNK), with Considering the relationship of AMPK with glycolysis, it was also shown that the calcium/calmodulin-dependent protein kinase kinase 2 can phosphorylate and activate AMPK increasing the expression of GLUT12 and its presence at plasma membrane, which resulted in enhanced glucose uptake and cell growth [144]. Also, *de novo* lipogenesis was identified as a relevant metabolic pathway downstream AMPK activation. AMPK activity suppressed this pathway decreasing the concentration of malonyl CoA (Fig. 3.2.) [145,146]. Increased phosphorylation of AMPK was shown to inactivate ACC, blocking malonyl CoA, and thus, reducing fatty acid synthesis [99]. Moreover, the concomitant inhibition of lipid synthesis and AMPK resulted in excess of oxidative stress, PCa cell death and inhibition of tumour growth [117]. Overall, AMPK actions regulating metabolism were translated in physiological effects, decreasing cell proliferation and the malignant behaviour of PCa cells, and inhibiting tumour growth [147,145].

Hypoxia is a common event in the tumour microenvironment that induces the expression of HIFs transcription factors, namely, HIF-1. HIF-1 modulates several transcriptional changes affecting the invasive and migratory behaviour of PCa cells, and is one of the most recognized drivers of the glycolytic phenotype in different types of cancer cells [148-150]. In PCa cells, HIF-1 regulates the expression of aconitase and LDH [151], contributing to the reactivation of the TCA cycle and promoting the establishment of the glycolytic phenotype (Fig. 3.1.). Metabolic reprogramming of both stromal and PCa cells becoming independent of glucose consumption and developing a dependence on lactate as an energy substrate (Fig. 3.2.) also is regulated by HIF-1 [63].

The tumour suppressor PTEN, a negative regulator of the PI3K/AKT pathway, is usually deleted in PCa cases and linked with the development of CRPC [152,153]. In this way, increased AKT activity, in the consequence of PTEN loss, has been detected in PCa, with several reports showing its association with a poor clinical outcome [154-156]. Indeed, PTEN deletion and AKT activity have been shown to play a determinant role in promoting cell survival, but its role as regulators of PCa metabolism also has been reported. A very recent study showed that PTEN deletion had a broad role reprogramming metabolism of PCa cells, increasing glycolysis and glutaminolysis metabolites, as well as, fatty acid synthesis and ß-oxidation [157]. PTEN also was related with the re-programming of mitochondrial respiratory in PCa cells, which displayed a substantial shift towards succinate-supported respiration compared to benign cells. This metabolic shift was shown to depend on PTEN, as its loss was associated with increased respiration and accumulation of succinate [158]. Naguib et al. [159] also reported the dependency of mitochondria to consume instead of producing ATP in PTEN null PCa cells relative to PTEN wild-type. Moreover, PTEN null cells showed higher vulnerability to the inhibition of mitochondria complex I displaying decreased cell viability and proliferation.

In turn, AKT activation was related with the accumulation of aerobic glycolysis metabolites [160], indicating a higher glycolytic flux in PCa cells. Accordingly, the inhibition

of AKT reduced aerobic glycolysis in PCa cells [161]. Besides the regulation of glucose use, AKT also has been connected with glutamine metabolism in prostate carcinoma. The inhibition of AKT decreased glutamine and glutamate levels PCa *in vitro* models, which would indicates a higher activity of glutaminolysis in the absence of AKT signalling [162]. However, increased glutamine levels were found in a PCa xenograft model upon inhibition of AKT [162]. Therefore, further studies are needed to clarify the role of AKT in the control of glutamine handling in PCa.

PTEN loss and PI3K/AKT activation in advanced and metastatic PCa were also associated with the accumulation of cholesteryl esters in lipid droplets, which had a positive effect on cell proliferation [105]. Moreover, the absence of PTEN and AKT activation resulted in FASN overexpression and increased activity [163,105,164], indicating that the fatty acid *de novo* synthesis also was enhanced in response to the activation of this oncogenic pathway. Some of the effects of PTEN loss on lipid metabolism were related with the SREBP transcription factor (discussion below). Finally, AKT localized in lipid rafts also was shown to stimulate cholesterol synthesis [165,166,105] and cholesteryl esters accumulation in lipid droplets [163,105].

c-Myc is one of the oncogenes commonly overexpressed in PCa, being extremely important for cancer progression, and also promoting the alteration of cancer cells metabolic phenotype [167-169]. c-Myc overexpression has been related with glutamine and lipid metabolism in PCa, though its role in glycolysis also is reported. c-Myc has been shown to increase the expression of ASCT2 glutamine transporter [170]. Also, glutaminase expression was increased by ten times in consequence of c-Myc action repressing the expression of regulatory microRNAs miR-23a and miR-23b [81]. Moreover, c-Myc was shown to activate glutaminolysis in PCa cells by increasing glutaminase activity [171]. Interestingly, it was also demonstrated that c-Myc stimulation of glutaminolysis influenced the glycolytic response of PCa cells. Augmented glutaminase activity was shown to repress the expression of thioredoxin-interacting protein, a potent negative regulator of glucose uptake, which resulted in stimulation of glycolysis [171]. Overall, c-Myc has been proposed as a strong stimulator of glutaminolysis. c-Myc overexpression was also associated with the deregulation of lipid metabolism in PCa cells, being reported the increased glycerolipid metabolism and fatty acid and cholesterol synthesis [160,172]. However, the molecular partners of c-Myc actions in regulating lipid metabolism in PCa cells remain to be fully characterized.

SREBPs are transcription factors that regulate the expression of genes involved in fatty acid and cholesterol biosynthesis. The transcriptional activity of SREBP requires the cleavage-activating protein (SCAP) that allows the subsequent cleavage and release of the SREBP [173]. SREBP and SCAP have been shown to regulate cholesterol homeostasis in PCa cells. SCAP overexpression and SREBP stimulated the expression of HMGCR in PCa cells [174,175] and, thus, the cholesterol synthesis pathway (Fig. 3.2.). Increased cholesterol biosynthesis triggered by SREBP-2, induced proliferation, invasion and migration of PCa cells, and was shown to be mediated by the transcriptional activation of c-Myc [172]. Moreover,

augmented expression of SCAP increased the expression levels of LDLr, which was related with the increased intracellular cholesterol levels [174]. SREBP function also increased the expression of FASN and SCD1 in PCa cells [176,177,122,175]. Moreover, SREBP transcriptional activity promoted the accumulation of lipid droplets in PCa cells [177], though the exact mechanism involved needs to be disclosed. Nevertheless, the response was functionally relevant as promoted PCa cell growth, migration and invasion [177].

Deletion of PTEN (with the reactivation of MAPK pathway) was a condition known to increased the SREBP expression with the activation of the lipogenic program by augmenting FASN and HMGCR expression [178]. The metabolic reprogramming induced by SREBP has shown to have impact on PCa cell growth, migration and invasion. Interestingly, SREBP inhibition has been used with positive results decreasing cell growth and inducing apoptosis of PCa, if in combination with docetaxel [177,175,179,178]. The most frequently mutated tumour suppressor gene in human cancers, the p53, [180] also was reported to play an important role in the regulation of metabolic pathways in cancer cells [181,182]. p53 enhanced the HK-2 mRNA stability through the inhibition of miR143 biogenesis, which increased aerobic glycolysis rates in xenograft mouse models of PCa [55]. Moreover, the activation of p53 by tenovin-1 in combination with metformin reduced the glucose consumption and lactate production, reducing the glycolysis capacity in PCa cells [183].The liaison of p53 with metabolic rewiring in PCa cells is relatively scarce, but a report exists associating this tumour suppressor with the regulation of lipid metabolism. Obese mice with increased p53 levels showed reduced levels of SREBP-1, whereas the deletion of p53 restored both SREBP-1 and FASN levels [184].

3.5. The role of sex steroids in regulating prostate cancer metabolism

Androgens and the androgen receptor (AR) are essential for the normal prostate cell growth and differentiation, as well as for maintaining prostate physiology in the adult [185]. However, the androgens/AR actions also play a crucial role in promoting prostate carcinogenesis [186].

The AR is a ligand-dependent transcription factor that upon activation by androgens, translocate to the nucleus and bind to the androgen response elements in the promoter region of target genes, controlling the expression of cell signaling and proliferation regulators [187]. In the last years, several studies have described the role of androgens as metabolic regulators in PCa, controlling the expression levels of genes involved in lipid and cholesterol metabolism, glycolysis and glutaminolysis [188-191]. Nevertheless, lipid metabolism is the best characterized metabolic pathway under the influence of androgens. A study from Swinnen et al. [192] first described the relationship between androgens and the control of lipid metabolism. Thereafter, several articles have been detailing the androgens actions in

regulating the expression of several molecular targets involved in the fatty acid and cholesterol biosynthesis, lipid uptake, lipolysis, and lipid storage.

The most well known AR target gene is FASN (Fig. 3.2.). Androgens stimulated the expression and activity of FASN, whereas antiandrogen treatment inverted this effect [193,188]. The same findings were reported for ACC (Fig. 3.2.). Androgen deprivation resulted in a marked reduction of ACC expression with testosterone supplementation reverting this scenario [188]. The AR effect regulating FASN expression depend on the presence of SREBPs transcription factors, with androgens increased the nuclear levels of active SREBP and promoting the translocation of SCAP from its site of synthesis in the endoplasmic reticulum to the site of proteolytical activation in the Golgi [194,195]. The importance of androgens in controlling FASN cell levels also was demonstrated by a study investigating the degradation of AR by the ASC-J9. This compound promoted the degradation of AR concomitantly with suppressed expression of FASN and SREBP-1, pointing out for an AR→SREBP-1→FASN signaling pathway [163]. Interestingly, it was demonstrated that androgens stimulation of lipogenesis depends on glycolysis as the main carbon source supplier, more exactly on the up-regulation of PFKFB2 (6-phosphofructo-2- kinase/fructose-2,6-bisphosphatase, allosterically control of PFK-1) and increased activity of HK-2, dependent on PKA signalling [196].

Cholesterol synthesis also was regulated by androgens. Expression of enzymes involved in cholesterol synthesis like HMG-CoA synthase, HMG-CoA reductase, and farnesyl diphosphate synthase were upregulated by androgens both *in vitro* and *in vivo* [197,188,198,139,199]. Moreover, free cholesterol available from increased biosynthesis or uptake, is likely a precursor for intratumoral *de novo* androgen synthesis. Increased serum cholesterol levels in PTEN-null transgenic mouse model fed ad libitum with high fat/high cholesterol diet were associated with elevated intraprostatic DHEA, androstenedione and testosterone levels [200,201].

Lipid uptake also was recently described as a target of androgenic regulation. Treatment with androgens increased cellular uptake of medium and long chain fatty acids, cholesterol, and low-density lipoproteins [87,202]. Moreover, androgen exposure or deprivation were shown to affect mRNA and protein expression of multiple lipid transporters in PCa cell lines and tumour xenografts [87]. Androgens are also involved in modulation of lipid content in cancer cells, by regulating lipolysis of adipose tissue, as well as, recruiting the intern reserves from intracellular lipid droplets [203-205]. However, how androgens exert all these effects is not entirely understood, and there are regulators of lipid metabolism that remain to be identified as androgen target genes. Moreover, androgens treatment increased lipid accumulation in lipid droplets, more exactly triglycerides and cholesterol esters [192].

The last decade has witnessed the identification of androgens as regulators of glycolytic metabolism in PCa. The synthetic androgen R1881 and the main prostatic androgen 5α-dihydrotestosterone stimulated glucose uptake and lactate production in PCa cells [189,188] by altering the expression levels of several targets in the glycolytic pathway. The increased expression of GLUT1, GLUT3, HK, PFK and MCT4 was reported [189,188]. Also,

testosterone was responsible for the increased expression of m-aconitase, inducing citrate oxidation [206], and, thus, activating the Krebs cycle and modulating the prostate cells to the cancer phenotype (Fig. 3.2.).

AR signaling driving metabolic alterations also was show to have a role in glutamine metabolism. Androgens/AR promoted glutamine metabolism by increasing the expression of glutamine transporter ASCT2 [170,207]. However, the interplay between androgens actions and glutaminolysis in PCa cells deserves further research.

Androgens are widely recognized as the main drivers of PCa, but other steroid hormones, namely, estrogens have been shown to influence prostate physiology. However, the estrogens' functions in PCa cells has remained a subject of some controversy. These steroid hormones have been used in PCa treatment for years, and disregarded because of the associated side-effects, but their role suppressing tumour growth has been widely reported [208,19]. Our research group demonstrated the protective actions of estrogens suppressing PCa cells proliferation and inducing apoptosis in both cancer cell lines and rat prostate [209]. Nevertheless, other studies defended a causative effect of estrogens in PCa, promoting tumour progression [210,211]. This duality of estrogenic effects in PCa cells is supported by the opposite roles of estrogen receptors (ER) subtypes. The ERα that has been indicated as oncogenic, while the ERβ has pro-apoptotic and anti-proliferative actions counteracting PCa cells growth [212,213]. Both ERα and ERβ have been implicated in the metabolic reprogramming in breast cancer [214], but little information exists on the activity of estrogens as regulators of prostate metabolism. However, ERα increased cell proliferation, promoting the neoplastic growth of PCa cells, by increasing the expression of GLUT1 and the sensitivity to glucose availability [215]. Moreover, proliferation of PCa cells with ERα knocked-down were less affected by glucose withdrawal than control cells [215]. Considering lipid metabolism, a prospective study showed that transdermal estrogen therapy decreased total cholesterol and LDL cholesterol, and increased HDL serum levels [216], but the action of these hormones affecting prostate lipid metabolism is unknown. The exploitation of the ER actions modulating PCa metabolism is a relevant research focus for the next upcoming years, which would allow the development of new treatment approaches.

3.6. Emerging therapeutic approaches targeting prostate cancer metabolism

There are several metabolic alterations trigging the development and progression of PCa that have been identified as new promising anti-cancer therapy targets. Table 3.1. summarizes the information on the metabolic targets that are actually being used for PCa treatment in pre-clinical and clinical studies.

Although glycolysis is not the main energy source used by primary PCa, blockage of this pathway showed usefulness for treatment. Overall, inhibition of glycolysis decreased tumour volumes and metastasis, delayed the onset of CRPC, increased the expression of pro-

apoptotic factors, and maintained PSA levels low. Moreover, suppression of glycolytic activity in PCa cells had synergic effects with other anticancer drugs [12,13]. The metabolic targets of glycolytic pathway that have been used for treatment of PCa are GLUTs, HK, PFK, pyruvate kinase isozymes M2 (PKM2), LDH and MCTs. Concerning GLUTs, the membrane proteins responsible for glucose uptake, three inhibitors have been tested, namely, silybin, apigenin and ritonavir [217-252]. However, only silybin and ritonavir reached the stage of clinical trials, showing good efficacy decreasing prostate tumour growth and progression to metastasis [253-257]. The inhibition of HK, the first step of glycolysis, also has been used as strategy for PCa treatment by using methyl jasmonate, 3-bromopyruvate, 2-Deoxy-D-glucose and ionidamine [258-268]. From this list, only the last two compounds have already been investigated in clinical trials [269-272]. The most promising compound might be methyl jasmonate since it was more studied and the only one that demonstrated the ability to inhibit the expression of the anti-apoptotic protein Bcl-2 inducting cell death of radioresistant human PCa cell line [260]. Sodium orthovanadate has been used as inhibitor of PFK in preclinical models of PCa, whereas shikonin has been exploited as an inhibitor of PKM2 [273-276]. Regarding LDH, the enzyme that converts pyruvate to lactate (Fig. 3.1.), there are two inhibitors that are being tested at clinical stages: AT-101 and FX11 [277-286]. In preclinical models, the use of AT-101 and FX11 inhibitors in combination with other therapies, namely ADT or surgical castration, improved disease outcomes [277,278]. In the end of glycolytic flux, the inhibition of MCTs, which will attenuate the export of lactate reducing the acidification of tumour microenvironment, also has been explored as an anti-PCa therapy. Two inhibitors of this class of transporters, namely, atorvastatin and simvastatin, showed effectiveness inhibiting tumour formation and growth, re-growing of tumours after castration, and reducing PSA levels [287-311]. Both statins are currently in test in clinical trials for PCa treatment [312,313]. However the specificity of these compounds for MCTs is very low [314-326].

As discussed above, the augmented proliferation of cancer cells requires the generation of new membrane phospholipids, and PCa displays enhanced lipid synthesis, thus targeting this anabolic pathway might be a crucial approach for PCa therapy. FASN, ACLY, ACC and SCD are the enzymes more explored in this context. Several inhibitors namely, cerulenin, C75, triclosan, EGCG, IPI-9119 and orlistat have been used for inhibition of FASN [327-352]. Overall, these compounds showed tumour growth and angiogenesis inhibitory properties, decreasing serum PSA levels, promoting apoptosis, and increasing the efficacy of radiotherapy and enzalutamide. However, from the extensive list, only EGCG reached the phase of clinical trials, which can be due to the weak solubility of the previously mentioned compounds [353-360]. Concerning the other enzymes of lipid biosynthetic pathway (Fig. 3.2.), ACLY, ACC and SCD, isolated compounds have been tested as inhibitors, namely, cucurbitacin B, soraphen A and SCD, respectively [361-364]. However, none of these molecules reached clinical phases. On other hand, it has been shown that fatty acid ß-oxidation has an important role as an energy source in PCa cells [14]. Therefore, inhibition of fatty acid ß-oxidation has

aroused as a promising therapeutic approach for PCA. Concerning the mitochondrial translocator CPT1, a limiting step in fatty acid β-oxidation, also there are known inhibitors tested in PCa treatment. It is the case of etomoxir, perhexiline and ranolazine that decreased cancer growth and metastasis, and improved the outcomes of enzalutamide treatment [365-370]. However, only ranolazine has been used in clinical stages, while others never passed through the preclinical stages due to their side-effects such as neurotoxicity, hepatotoxicity, risk of cardiovascular disease and heart failure [371]. ACAT (Fig. 3.2.), the enzyme linked with the formation of lipid droplets, is another target for PCa therapy through the utilization of two inhibitors as available inhibitors, avasimibe and beauvericin [372-374]. Lastly, in the fatty acid processing molecular players, the inhibition of DGAT (Fig. 3.2.) with xanthohumol using preclinical and *in vivo* models seemed to have benefits against the progression of PCa [375-380].

Regarding the cholesterol synthesis pathway, HMGCR is the most common target for prostate anti-cancer therapy. The inhibition of this enzyme was achieved through the use of statins compounds namely, atorvastatin, lovastatin, pravastatin, fluvastatin, rosuvastatin and simvastatin [287,295,293,381,382,292,383,290,289,291,288,384,294,296,385-390,297,391-395,301,396,303,299,397,298,309,308,305,302,398,311]. All of them have been tested in clinical trials with PCa patients [399,314-323,400-404,324-326].

Since cancer cells also depend on glutamine for proliferation and survival, the inhibition of key modulators of glutaminolysis also has earned consideration by their anticancer potential. The two key regulators of glutamine metabolism, glutaminase and ASCT2, have been targeted for PCa treatment. CB-839 and BPTES are the compounds used as glutaminase inhibitors, while in the case of ASCT2 the inhibitor is the l-γ-glutamyl-p-nitroanilide [405-407]. However, none of these inhibitors has reached clinical trials.

The inhibition of other metabolic modulators with a central role in the regulation of glycolysis metabolism, lipid metabolism and glutaminolysis, as is the case of HIF1, AKT, AMPK, PTEN and SREBP, also has been applied in cancer treatment. Regarding HIF1, several inhibitors were developed, namely chetomin, chrysin and YC-1 [408-414]. AZD5363, Afuresertib, Ipatasertib and MK-2206 are the inhibitors clinically tested for the inhibition of AKT towards PCa therapy [415-432]. AICAR and salicylate are the molecules tested for the inhibition of AMPK as a possible treatment for PCa in preclinical models [433,350,434-437].

One of the most common recognized events leading to PCa development is the loss of function of the PTEN tumour suppressor [152,153]. Thus, several efforts have been focused in the "recuperation of function" of this tumour suppressor. The stimulation of PTEN reached the clinical trials by using rituximab, rosiglitazone, sodium selenite, sunitinib, trastuzumab and pertuzumab [438-485]. Sodium selenite was the compound more tested in preclinical studies, and demonstrated to induce apoptosis through several pathways such as, TRAIL receptor, p53 and generation of oxygen reactive species [446-454]. Moreover, sodium selenite has the ability to inhibit the expression of vascular endothelial growth factor, which is

important for the metastization process [451]. Finally, fatostatin A, which is an inhibitor of SREBP, has been tested in preclinical and *in vivo* models for the treatment of PCa [486,487].

The dependency of PCa cells on several metabolic pathways endows metabolism the potential as a therapeutic target. However, PCa continuously reprogram metabolism in consequence of accumulation of new mutations and environmental pressure, which can hamper or strongly limit the success of metabolic inhibitors. Then, the combination of several metabolic pathways or metabolic regulators simultaneously might be a more effective anticancer approach.

Table 3.1. Overview of target metabolic pathways for prostate cancer treatment

Metabolic pathway	Target	Compound	Stage as anti-cancer therapy	Refs
Glycolysis	GLUTs	Silybin	Preclinical	[217-228]
			In vivo models	[229-233,226]
			Clinical Studies	[253-255]
		Apigenin	Preclinical	[234-248]
			In vivo models	[249,237,250,243-246,251]
		Ritonavir	Preclinical	[252]
			In vivo models	[252]
			Clinical Studies	[256,257]
	HK	methyl jasmonate	Preclinical	[258-261]
			In vivo models	[262,263]
		3-bromopyruvate	Preclinical	[264,265]
		2-Deoxy-D-glucose	Clinical Studies	[269,270]
		Lonidamine	Preclinical	[266]
			In vivo models	[267,266,268]
			Clinical Studies	[271,272]
	PFK	Sodium Orthovanadate	Preclinical	[273]
	PKM2	Shikonin	Preclinical	[274-276]
	LDH	AT-101	Preclinical	[277]
			In vivo models	[278]
			Clinical Studies	[279-285]
		FX11	Preclinical	[286]
	MCTs	Atorvastatin	Preclinical	[287-295]

			In vivo models	[288,290,296]
			Clinical Studies	[314-323]
		Simvastatin	Preclinical	[297-310]
			In vivo models	[300,311,303]
			Clinical Studies	[324-326,323]
Fatty acid synthesis	FASN	Cerulenin	Preclinical	[327]
		C75	Preclinical	[327]
			In vivo models	[328]
		Triclosan	Preclinical	[329]
		EGCG	Preclinical	[330-343]
			In vivo models	[344-348,341]
			Clinical Studies	[353-360]
		IPI-9119	Preclinical	[349]
			In vivo models	[349]
		Orlistat	Preclinical	[350-352]
	ACLY	Cucurbitacin B	Preclinical	[361]
			In vivo models	[361]
	ACC	Soraphen A	Preclinical	[362,363]
	SCD	SCD inhibitors	Preclinical	[364]
Fatty acid ß-oxidation	CPT1	Etomoxir	Preclinical	[365-369]
			In vivo models	[368]
		Perhexiline	Preclinical	[369]
		Ranolazine	Preclinical	[369]

			In vivo models	[370]
			Clinical Studies	[371]
	ACAT	Avasimibe	Preclinical	[372]
			In vivo models	[372]
		Beauvericin	Preclinical	[373,374]
	DGAT	Xanthohumol	Preclinical	[375-379]
			In vivo models	[380]
Glutamine metabolism	GLS	CB-839	Preclinical	[488]
			In vivo models	[488]
		BPTES	Preclinical	[406]
	ASCT2	l-γ-glutamyl-p-nitroanilide	Preclinical	[407]
			Preclinical	[407]
Cholesterol Synthesis	HMGCR	Atorvastatin	Preclinical	[287,295,293,381,382,292,383,290,289,291,288,384,294]
			In vivo models	[290,296,288]
			Clinical Studies	[399,314-323]
		Lovastatin	Preclinical	[385-388]
			In vivo models	[389]
			Clinical Studies	[400,401]
		Pravastatin	Clinical Studies	[323]
		Fluvastatin	Clinical Studies	[402]
		Rosuvastatin	Preclinical	[390]
			In vivo models	[390]

			Clinical Studies	[403,323]
		Simvastatin	Preclinical	[297,391-395,301,396,303,299,397,298,309,308,305,302,398]
			In vivo models	[395,311,303]
			Clinical Studies	[404,324-326,323]
Other metabolic routes	HIF1	Chetomin	Preclinical	[408,409]
		Chrysin	Preclinical	[410-412]
			In vivo models	[410]
		YC-1	Preclinical	[413,414]
	AKT	AZD5363	Preclinical	[415-418]
			In vivo models	[415-417,419,418]
			Clinical Studies	[420-423]
		Afuresertib	Clinical Studies	[424,425]
		Ipatasertib	Clinical Studies	[426-429]
		MK-2206	Clinical Studies	[430-432]
	AMPK	AICAR	Preclinical	[433,350,434-436]
		Salicylate	Preclinical	[437]
	PTEN	Rituximab	Clinical Studies	[438-440]

		Rosiglitazone	Preclinical	[441-443]
			Clinical Studies	[444,445]
		Sodium Selenite	Preclinical	[446-454]
			In vivo models	[455,456]
			Clinical Studies	[457,458]
		Sunitinib	Preclinical	[459-463]
			In vivo models	[459,464,463]
			Clinical Studies	[465-475]
		Trastuzumab	Preclinical	[476]
			In vivo models	[477-480]
			Clinical Studies	[481,482]
		Pertuzumab	Clinical Studies	[483-485]
	SREBP	Fatostatin A	Preclinical	[486,487]
			In vivo models	[486,487]

ACAT - Sterol O-acyltransferase; ACC - Acetyl-CoA carboxylase; ACLY - ATP citrate lyase; AKT - Protein kinase B; AMPK - AMP-activated protein kinase; CPT1A - Carnitine palmitoyltransferase IA; DGAT - Diglyceride acyltransferase; FASN – Fatty acid synthase; GLS – glutaminase; Gluts – glucose transporters; HIF1- hypoxia inducible factor 1; HK – hexokinase; HMGCR - 3-hydroxy-3-methylglutaryl-CoA reductase; LDH – lactate dehydrogenase; MCTs – monocarboxylate transporters; PFK –phosphofructokinase; PFKM2 – phosphofructokinase M2; PTEN - Phosphatase and tensin homolog; SCD - Stearoyl-CoA desaturase; SREBP - Sterol regulatory element-binding protein

3.7. Conclusions

The landscape of cancer cell metabolism has been intensively investigated over the last years mainly with the rationale of: i) disclosing the metabolic alterations driving carcinogenesis and tumour growth and ii) identifying the vulnerabilities of cancer cells that can be used with treatment purposes. Prostate cells have peculiar and unique metabolic features privileging the use of less efficient metabolic routes to the production of citrate, but a shift occurs in the metabolism PCa cells. Citrate secretion decelerates and the use of glycolysis, lipid metabolism and glutaminolysis is empowered. Moreover, the metabolic reprograming of PCa cells displays several specificities compared to other types of cancer.

This review revisited the metabolic alterations accompanying the development and progression of PCa, and the cancer cells' dependency on specific metabolites. PCa cells, mainly in advanced stages of disease, displayed high glycolytic activity, increased lipid uptake and oxidation, and enhanced use of glutamine. Overall, all these features were largely stimulated by the cell fate "decision makers" namely, the over-activation of oncogenes or the loss of function of tumour suppressors. Also, the hormonal milieu, and specifically the actions of androgens/AR, create a suitable environment to shape PCa cells metabolism, favouring the utilization of energy to sustain increased cell proliferation rates, as well as cell migration and invasion. However, a detailed understanding of the molecular mechanisms underlying the metabolic responses of PCa cells in different stages of disease or under specific environmental conditions still is far from being completed. Future research is need in this field to fully draw the picture of the metabolic PCa cell.

Nevertheless, and following the trend observed in other cancer types, several drugs targeting the metabolic peculiarities of PCa cells have been tested in pre-clinical and clinical studies. Therapies suspending the use or production of metabolites essential for PCa cell survival showed to be promising strategies to regulate tumours growth. However, metabolic therapy also has shown some limitations, namely by, the acquisition of mechanisms of resistance, the weak specificity of compounds, associated-toxicity or adverse side-effects.

Moreover, the "rich-soup" of metabolites and regulators present in PCa cells and tumour microenvironment represents a complexity that can hamper treatment effects when specific metabolic enzymes/ transporters and metabolic regulators are targeted. PCa cells, and cancer cells in general, have a recognized plasticity in adapting the metabolic pathways dependently on the nutrient availability. This means that, blocking a metabolic route can be efficient suppressing tumour growth only transiently, until cancer cells reprogram the machinery to start using another pathway. A strategy to overcome this constrain could encompass the identification of synergic effects combining compounds that target different metabolic pathways. Alternatively, the use of metabolic inhibitors together with suppressors of oncogene activity or with the classical ADT may represent interesting approaches for management and treatment of PCa. The use of drugs targeting metabolic pathways also might

be an auspicious therapeutic approach for CRPC, a resistant and lethal stage of disease with unmet clinical needs concerning treatment.

3.8. Funding

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project No. 007491; Project No 029114) and National Funds by FCT-Foundation for Science and Technology (Project UID/Multi/00709/2013). Henrique J Cardoso and Marília I Figueira were recipient of FCT fellowships (SFRH/BD/111351/2015 and SFRH/BD/104671/2014, respectively).

3.9. Conflict of interest

The authors declare that they have no conflict of interest.

3.10. References

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Chapter 4

Aim and Outline of the thesis

Aim and outline of the thesis

The development and progression of PCa are driven by numerous factors and complex cellular and molecular mechanisms. However, the initial progression of PCa mainly depends on androgens, which enables the use of ADT reducing androgens production and the AR-mediated effects.

In the past few years, the reprogramming of energy metabolism was added to the list of cancer hallmarks. This has been highly related with rewiring of aerobic glycolysis, indicated as the pathway mostly explored by cancer cells to sustain their growth. In the case of PCa, and unlike other types of neoplasms, initial tumour growth does not rely on aerobic glycolysis. In this context, the hypothesis that PCa cells differ from other cancer cells, predominantly utilizing fatty acids and glutamine as energy substrates, has been gaining consistency. Deregulated lipid metabolism, namely, hypercholesterolemia, augmented lipid and glutamine uptake and increased *de novo* fatty acid synthesis, have been associated with PCa development and progression. Nevertheless, the inter-relationship among energy substrates and the contribution of the different metabolic routes to PCa cells survival and growth remains unknown. Indeed, only more advanced stages of the disease have been shown to be highly glycolytic, namely, those characterized by the loss of androgen responsiveness, the so-called CRPC. Also, it has been shown that androgens have a stimulatory effect on multiple metabolic pathways, both in benign and malignant prostate cells. Moreover, androgens were shown to fuel the progression to the highly glycolytic more aggressive stages of PCa, also driving important alterations in lipid metabolism. Notwithstanding, the role of androgens controlling PCa metabolism needs to be more detailed. Although new therapies for CRPC have emerged recently, the therapeutic options for this stage of disease are restricted and display limited duration of clinical and survival benefits. Presently, there are drugs targeting metabolism that have been envisaged for the treatment of PCa and CRPC. However, there are important knowledge gaps in the understanding of PCa cells metabolism that should be fulfilled to improve its efficacy and to avoid resistance and bypassing metabolic pathways. For example, how the tumour metabolic microenvironment influences the response of PCa cells to therapy is a subject clearly underexplored.

To shed light on some of these questions, the present thesis aims to:

1. Establish the PCa cells dependency on the different metabolic pathways and the relationship between each route;

2. Determine the relevance of glutaminolysis for PCa cells survival and growth and the effect of androgens in the regulation of glutamine metabolism;

3. Study the role of androgens in regulating lipid metabolism and the influence of these hormones and LDL-cholesterol in modulating PCa cells fate;

4. Investigate the effect of glucose availability on PCa cells response to therapy: the case of imatinib in two cell line models of CRPC.

After a brief description of the prostate gland anatomy and physiology and characterization of the cellular and molecular basis of PCa (**Chapter 1**), cancer cell metabolism (**Chapter 2**) and PCa specific metabolic features were revised **(Chapter 3).** Chapter 4 establishes the main objectives of the thesis, and **Chapters 5-9** were organized as follows:

Chapter 5 describes the dependency and capacity of PCa cells using glucose, glutamine and fatty acid metabolism. Also, it determines the dependency of PCa cells on glycolysis and their utilization of glycolysis and aerobic glycolysis in conditions of glucose deprivation or hyperglycaemia.

Chapter 6 explores the relevance of glutaminolysis for PCa cells survival and growth and the effect of androgens in the regulation of glutamine metabolism. It characterizes the expression of target regulators of glutaminolysis in PCa cell line models and describes the effect of 5α-dihydrotestosterone (DHT) in regulating glutamine metabolism in PCa cells and rat dorsolateral prostate. Moreover, this chapter addresses whether inhibition of glutaminase would sensitize PCa cells to anti-androgens. The effect of inhibiting glutaminase on PCa cells viability, apoptosis, and migration is described. Also, it evaluates the utilization of other metabolic pathways, like glycolytic metabolism after blockage of glutaminolysis.

Chapter 7 establishes the effect of androgens (DHT) in the regulation of lipid metabolism, and the influence of these hormones and LDL-cholesterol in modulating PCa cells viability, proliferation and migration. Moreover, it characterizes the expression of target regulators of lipid metabolism and lipid content in PCa cells under different LDL-cholesterol availabilities with or without DHT.

Chapter 8 investigates the response of CRPC cells to the tyrosine kinase inhibitor imatinib under hypoglycaemic and hyperglycaemic conditions, evaluating cell viability, apoptosis, glucose consumption, lactate production and expression and activity of glycolytic regulators.

Finally, **Chapter 9** contains an integrative view of the results presented in the thesis and discusses the potential impact of the obtained findings in the context of PCa prevention and treatment.

Chapter 5

Metabolic features of human androgen-sensitive and castrate-resistant prostate cancer cells

Abstract

Cancer cells have the ability of reprogramming metabolism to sustain biomass synthesis and cell growth. Glucose, glutamine and fatty acids are crucial fuels for prostate cancer (PCa) cells, and their differential utilization has been proposed accompanying the transition of PCa from the androgen-sensitive stage to the castrate-resistant PCa (CRPC). However, the full understanding of PCa metabolism still needs clarification. Conditions of different glucose availability (high/low) have been shown to switch on specific metabolic pathways in several cell types, but the effect of hyperglycaemia driving alterations on PCa cell metabolism remains unexplored. The present study characterises the glycolytic phenotype of androgen-sensitive (LNCaP) and CRPC (PC3 and DU145) cells by the robust analysis of extracellular acidification using the Seahorse methodology. Also, it is investigated the PCa cells dependency on glucose, glutamine and fatty acids, and their capacity of using these fuels. In addition, it is assessed the impact of glucose availability (glucose depletion vs. hyperglycaemia) on PCa cells' use of glycolysis and lipid metabolism. The results obtained demonstrated that CRPC cells have higher metabolic rates being more glycolytic than the androgen-sensitive cells, especially PC3 cells, which also showed a higher capacity to oxidize glutamine. LNCaP cells displayed a higher capacity for using fatty acids as mitochondrial fuels. Hyperglycaemia decreased glycolytic activity in PCa cells and promoted lipid metabolism. Overall, these findings characterized PCa cells metabolism, showing a differential dependency and capacity of fuel use between androgen-sensitive and CRPC cells. Also, it was shown that hyperglycaemia induced a metabolic shift in PCa cells from glycolysis to lipid metabolism.

5.1. Introduction

The capacity of metabolic reprogramming is a known feature of tumour cells that is recognized since 2011 as a hallmark of cancer [1]. This flexibility on using different metabolic routes provides cancer cells with the necessary energy for maintenance of biomass synthesis and sustainment of cell growth [2,3]. The peculiar metabolic features of cancer cells, known since the pioneer studies of Otto Warburg, are characterized by the use of glucose for lactate production, acidifying the tumor microenvironment and promoting cancer cells growth and dissemination [4,5]. Moreover, due to these specificities, cancer cells metabolism has been gaining increasing interest in therapy [6]. The initial stages of prostate cancer (PCa) are less glycolytic than other cancer types. However, an increase in the glycolytic phenotype accompanies the transition of disease from the androgen-sensitive stage to the castrate-resistant prostate cancer (CRPC) [7,8], which represents an aggressive and lethal form of disease [9,10]. Moreover, PCa cells also have been shown to use glutaminolysis and fatty acid metabolism [11,12]. Nevertheless, there are important knowledge gaps in the understanding

of PCa metabolism, and their clarification can provide new insights for the development of new therapies targeting metabolic pathways.

On the other hand, the reduced blood flow and nutrient deprivation act as engines, forcing the cancer cells to adapt their metabolism to the tumoral microenvironment and allowing them to survive in hostile conditions [13,14]. In this way, nutrient availability is responsible for the reactivation of specific metabolic pathways and the acquisition of more aggressive disease phenotypes [13]. In addition, nutrient availability (e.g. hyperglycaemia) have been shown to influence cancer cells' response to therapy [15,16] In the case of PCa, glucose deprivation have been related with aggressive phenotypes and reactivation of several survival pathways [17,18]. Also, reports exist indicating that the aggressive behaviour of PCa cells depends on fatty acids supplementation and lipid *de novo* synthesis [19,20]. Nevertheless, PCa cell's use of glycolysis and lipid metabolism in hyperglycaemia remains unexplored.

The present study characterises the glycolytic phenotype of androgen-sensitive and CRPC cells using a robust methodology for metabolism analysis. The PCa cells dependency on glycolysis, glutaminolysis and lipid metabolism, and their capacity to oxidize the corresponding fuels also is investigated. In addition, it is assessed the impact of glucose availability (glucose depletion vs. hyperglycaemia) on glycolysis and lipid metabolism.

5.2. Materials and methods

5.2.1. Chemicals

All chemicals and culture media unless otherwise stated were purchased from Sigma–Aldrich (St Louis, MO, USA).

5.2.2. Cell lines and treatments

Human PCa cell lines (LNCaP, DU145, and PC3) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). LNCaP cells, originated from a PCa lymph node metastasis, express the androgen receptor and are an androgen-sensitive model. DU145 and PC3 cell lines are nonresponsive to androgens and known models of metastatic (advanced) stages of human PCa. DU145 and PC3 cell lines have origin in brain and bone metastasis of undifferentiated grade IV prostate adenocarcinomas [21,22].

LNCaP, DU145, and PC3 cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/ streptomycin at 37 ºC in an air incubator equilibrated with 5 % CO_2. In all experiments, cells were maintained up to 60% confluence and then culture medium was replaced by glucose-free medium or medium supplemented with 30 mM glucose (hyperglycaemia) for 24 h.

5.2.3. Glycolytic Rate and Mito Fuel Flex Assays

PCa cells dependency/capacity on glucose, glutamine and fatty acids, and cell glycolytic response were evaluated by measurement of the extracellular acidification (proton efflux rate, PER) using the Seahorse XF-96 analyser (Seahorse Bioscience, Agilent, Santa Clara, CA, USA). LNCaP (15000 or 35000 cells/well), DU145 (5000 or 9000 cells/well), and PC3 (5000 or 8000 cells/well) cells were seeded in 96-well plates (Seahorse Bioscience) and maintained for 24 h before the Seahorse XF Glycolytic Rate and Mito Fuel Flex Assays, In parallel, an XFe96 sensor cartridge for each cell plate was placed in a 96-well calibration plate containing 200 µL/well calibration buffer and left to hydrate overnight at 37°C. The sensor cartridge and the calibration plate were loaded into the XFe96 Extracellular Flux Analyzer for calibration, and then, the utility plate, containing the calibration solution, was replaced with the cell culture plate and the assay started. Cell culture medium was replaced by low-buffered serum-free medium without phenol red with 2 mM glutamine, 10 mM glucose, 1 mM pyruvate, and 5.0 mM HEPES (Glycolytic Rate Assay) or 2 mM glutamine, 10 mM glucose, 1 mM pyruvate (Mito Fuel Flex) at pH 7.4, and cells were maintained at 37°C for 1 h in the absence of CO_2. Next, for Glycolytic Rate Assay, mitochondrial inhibitors rotenone plus antimycin A (0.5 µM each) and glycolysis inhibitor 2-deoxy-D-glucose (2-DG, 50 µM) were injected via ports A and B, respectively, and PER was measured (Fig. 5.1.A). For, Mito Fuel Flex different combinations of glutaminase inhibitor BPTES (5 µM), carnitine palmitoyltransferase 1A (CPT1A) inhibitor Etomoxir (10 µM), and mitochondrial pyruvate carrier inhibitor UK5099 (4 µM) were injected in Ports A and B, and oxygen consumption rate was measured (Fig. 5.1.C). Data were analysed using the XFe96 Extracellular Flux Analyzer software (Seahorse XF Glycolytic Rate Assay or Seahorse XF Mito Fuel Flex Test). All results were normalized by the sulforhodamine B (SRB) assay. Briefly, cells were fixed with 50 µl of 60% trichloroacetic acid (TCA), overnight at 4 ºC. After removing the fixing solution and washing with distilled water, fixed cellular proteins were stained with the SRB solution (0.05 % w/v in 1% acetic acid) for 1 h at 37 °C. Unbound dye was removed with 1% acetic acid, and cell bound dye solubilized with 10 mM Tris base solution, pH 10. Colour intensity was measured at 540 nm using the Cytation 3 microplate reader, from Biotek (Winooski, VT, USA).

5.2.3. Western blot (WB) analysis

PCa cells were homogenized by pippeting in the appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail and 10 % PMSF , kept on ice for 20 min with occasional mixing, and then centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA).

Total proteins were resolved by SDS-PAGE on 7.5, 10 or 12,5% gels and electro-transferred to PVDF membraned (Bio-Rad). Membranes were incubated overnight at 4 °C with rabbit anti-

CD36 (1:400, ab64014; Abcam, Cambridge, UK), rabbit anti-acetyl-CoA carboxylase (ACC) (1:1000, no.3662; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-fatty acid synthase (FASN, 1:1000, no.3180; Cell Signaling Technology), mouse anti-CPT1A (1:1000, ab128568; Abcam), Then, membranes were washed and incubated 1 h at room temperature with the anti-rabbit IgG, HRP-linked (1:20000, no.7074; Cell Signaling Technology) or anti-mouse-IgGκ HRP-linked (1:20000, sc-516102, Santa Cruz Biotechnology, Heidelberg, Germany) secondary antibodies. Protein expression was normalized using a mouse anti-ß-actin (1:1000, A5441) antibody. Membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and immunoreactive proteins were visualized with the ChemiDoc™ MP System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab software (Bio-Rad) and normalized by division with the respective ß-actin band density.

5.2.4. Statistical analysis

Statistical significance of differences between experimental groups was evaluated by unpaired t-test with Welch's correction or one-way ANOVA followed by Tukey post-hoc test, using GraphPad Prism v6.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean (S.E.M).

5.3. Results and Discussion

The present study investigated the LNCaP, DU145, and PC3 PCa cells dependency on different metabolic pathways, as well as the utilization of glycolysis and aerobic glycolysis in glucose deprivation or hyperglycaemic conditions.

First, we characterized the glycolytic profile of different PCa cells using the Seahorse XF Glycolytic Rate Assay (Fig. 5.1.A and 1B). Pyruvate from glycolysis can be converted to lactate in the cytoplasm, or, alternatively, to CO_2 and water in the mitochondria. Both pathways contribute to the extrusion of protons to the extracellular medium (Fig. 5.1.A). Basal PER represents the contribution of both pathways to acidification at the initial moment. Basal PER was significantly higher in CRPC cells relative to LNCaP (3.03 ± 0.18 and 1.31 ± 0.07 fold-change, respectively, Fig. 5.1.B). These results follow the knowledge that DU145 and PC3 cells have higher metabolic rates than LNCaP cells [21,23,24].

Discounting the contribution from CO_2-dependent effect on acidification from Basal PER, give as the contribution from glycolysis (Glyco PER) (Fig. 5.1.A). Also, Glyco PER was higher in DU145 and PC3 cells compared to LNCaP (3.67 ± 0.23 and 1.61 ± 0.09 fold-change, respectively, Fig. 5.1.B), which indicates that CRPC display higher glycolytic metabolism. Glyco PER measurement is highly correlated with the extracellular lactate production rate. In fact, our research group has shown the augmented production of lactate in PC3 cells relative to LNCaP cells and non-neoplastic prostate cells PNT1A, which was sustained by the increased activity of lactate dehydrogenase (LDH) and enhanced expression of monocarboxylate transporter 4 (MCT4) [8]. Note worthy, these *in vitro* outcomes are in accordance with

patients' clinical data. It has been described that CRPC patients present a higher glycolytic activity with LDH activity being used to predict progression-free survival [25-27].

The Compensatory Glycolysis indicates the maximum cells' capacity to use glycolysis to meet energy demands in response to the inhibition of mitochondria by addition of rotenone plus antimycin A (Fig. 5.1.A). Both DU145 and PC3 cells presented a higher capacity to use

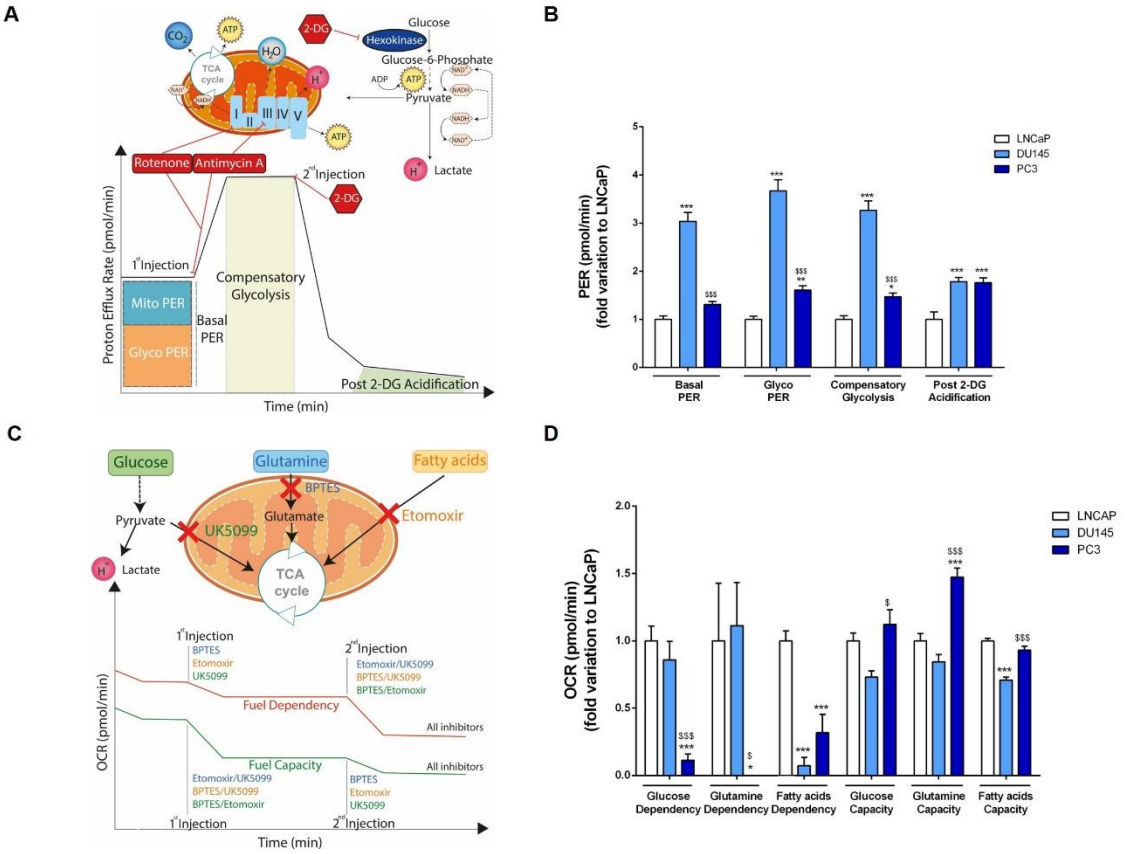

Figure 5.1. Glycolytic profile of LNCaP, DU145 and PC3 cells, and dependency and capacity of utilization of glucose, glutamine and fatty acids. (A) Scheme illustrating the experimental strategy for analysis of glycolytic metabolism. Proton efflux rate (PER) was obtained by the Seahorse XF96 Glycolytic Rate Assay. Basal PER represents the initial total PER. The use of mitochondrial inhibitors, rotenone and antimycin A (0.5 µM each), enabled calculating the mitochondrial-associated acidification (Mito PER). The Glycolytic PER (Glyco PER) resulted from subtracting Mito PER from Basal PER results. Inhibition of mitochondria drives cells compensatory changes to use glycolysis to meet energy demands (Compensatory Glycolysis). Secondly, glycolysis was inhibited by using the glucose analogue 2-deoxy-D-glucose (2-DG, 50 mM, Post-2-DG acidification). **(B)** Basal PER, Glyco PER, Compensatory Glycolysis and Post 2-DG Acidification in LNCaP, DU145 and PC3 cells. **(C)** Schematic panel illustrating the experimental strategy to evaluate cells' dependency on specific fuels, and their capacity to oxidize glucose, glutamine and fatty acids. The rate of oxidation of each fuel was determined by measuring mitochondrial respiration, i.e. oxygen consumption rate (OCR) using the Seahorse XF96 Mito Fuel Flex Test Kit. Glucose, Glutamine, and Fatty acids Dependency is the measurement of cancer cells reliance on each of these pathways to maintain baseline respiration upon inhibition of mitochondrial oxidation with UK5099, BPTES or Etomoxir, respectively. Glucose, Glutamine, Fatty acids Capacity is the ability of a cancer cell's mitochondria to oxidize each of these fuels when the other fuel pathways are inhibited, i.e upon inhibition of BPTES/Etomoxir, UK5099/Etomoxir or UK5099/BPTES, respectively. **(D)** LNCaP, DU145, PC3 cells dependency and capacity to oxidize glucose, glutamine, and fatty acids. All experimental results were normalized to cell mass determined by the SRB assay. Results are expressed as fold-variation relative to LNCaP cells. Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$, *** $p<0.001$ when compared with LNCaP, $ $p<0.05$; $$$ $p<0.001$ when compared with DU145.

glycolysis in these conditions compared to LNCaP cells (3.27 ± 0.19 and 1.47 ± 0.08 fold-change, respectively, Fig. 5.1.B). CRPC cells have a lower activity of mitochondrial respiratory chain complexes III and IV naturally, whereas LNCaP cells were described as having a more oxidative phenotype [28]. These findings support our results, with the CRPC cells more easily compensating inhibition of mitochondrial activity using glycolysis.

It is note worthy, that DU145 cells, which are originated from brain metastasis of prostate adenocarcinomas, displayed significantly higher Basal PER, Glyco PER and Compensatory Glycolysis when compared with PC3 (Fig. 5.1.B). Indeed, brain metastasis of breast and lung cancers are characteristically described as being highly glycolytic [29]. Moreover, it was shown that DU145 tumour xenografts had enhanced capacity of converting pyruvate into lactate relative to those originated from PC3 cells, also displaying a greater sensitivity to inhibition of LDH [30].

Injection of the glucose analogue 2-DG inhibits glycolysis through its competitive binding to hexokinase, the first enzyme in the glycolytic pathway. Post-2-DG acidification indicates the sources of extracellular acidification that are not attributed to glycolysis or mitochondrial phosphorylation (Fig. 5.1.A). CRPC cells showed a significantly higher post-2-DG acidification relative to LNCaP cells (1.79 ± 0.09 and 1.76 ± 0.10 fold-change, respectively, Fig. 5.1.B), which indicates that these cells are more able to use alternative energy sources that contribute to acidification.

Next, the dependency of androgen-sensitive and CRPC cells on specific fuels, and their capacity to oxidize glucose, glutamine and fatty acids in the mitochondria were determined using the Seahorse XFp Mito Fuel Flex Test. Dependency indicates that the cells' mitochondria are unable to compensate for the blocked pathway by oxidizing other fuels to maintain the baseline respiration (Fig. 5.1.C). Whereas, capacity is the ability of a cancer cell's mitochondria to oxidize a fuel of interest when other alternative fuels pathways are inhibited (Fig. 5.1.C). PC3 cells dependency on glucose was significantly lower relative to LNCaP and DU145 cells (0.11 ± 0.05 fold-change relative to LNCaP, Fig. 5.1.D). However, PC3 cells were the cancer line with the highest capacity for oxidation of glucose (1.05 ± 0.10 vs 0.73 ± 0.05 fold-change in DU145 compared to LNCaP, Fig. 5.1.D). The inhibition of pyruvate entry into the mitochondria by UK5099 has been tested in several studies, which showed the reprogramming of aggressive cancer cells metabolism to use aerobic glycolysis and glutamine uptake [31-33]. Compared with LNCaP and DU145 cells, PC3 cells do not rely on glutamine (Fig. 5.1D). However, PC3 cells displayed a higher capacity to oxidize glutamine relative to LNCaP and DU145 cells (1.47 ± 0.07 fold-change relative to LNCaP, Fig. 5.1.D).

Dependency on fatty acids was significantly lower in CRPC cells (DU145 and PC3) relative to the androgen-sensitive LNCaP cells (0.03 ± 0.02 and 0.32 ± 0.14 fold change compared to LNCaP cells, respectively, Fig. 5.1.D). These findings are sustained by the fact that fatty acid ß-oxidation is extremely regulated by androgens and androgen receptor in the androgen-sensitive stages of PCa [53, 54]. Moreover, treatment with etomoxir, a specific inhibitor of CPT1A, showed to be effective in suppressing viability and proliferative activity of

androgen-sensitive PCa cells [34,35]. DU145 cells also presented lower fatty acids capacity relatively to LNCaP and PC3 cells (0.71 ± 0.02 fold changed compared to LNCaP, Fig 1.D).

Altogether, the results obtained showed a predominant capacity of PC3 cells to use the glucose and glutamine as fuels to sustain the mitochondrial respiration when the other metabolic pathways were inhibited. Moreover, LNCaP presented more dependency on fatty acids and a higher capacity to oxidize this fuel relative to CRPC cells (Fig. 5.1.D).

It has been shown that glucose availability and the glycolytic environment can change the metabolic machinery, as well as influence the PCa cells response to therapy [15,16]. Therefore, we decided to investigate the glycolytic metabolism of PCa cells maintained in glucose deprivation or hyperglycaemic conditions for 24 h. LNCaP and PC3 cells in hyperglycaemic conditions presented lower levels of Basal PER relative to glucose deprivation conditions (52.44 ± 3.15 vs. 61.08 ± 2.78 pmol/min in LNCaP and 61.15 ± 3.24 vs 79.65 ± 3.97 pmol/min in PC3, Fig. 5.2.A). However, GlycoPER measurement was only diminished by high glucose availability in PC3 cells (49.23 ± 2.75 vs. 72.01 ± 3.48 pmol/min in glucose deprivation conditions, Fig. 5.2.B).

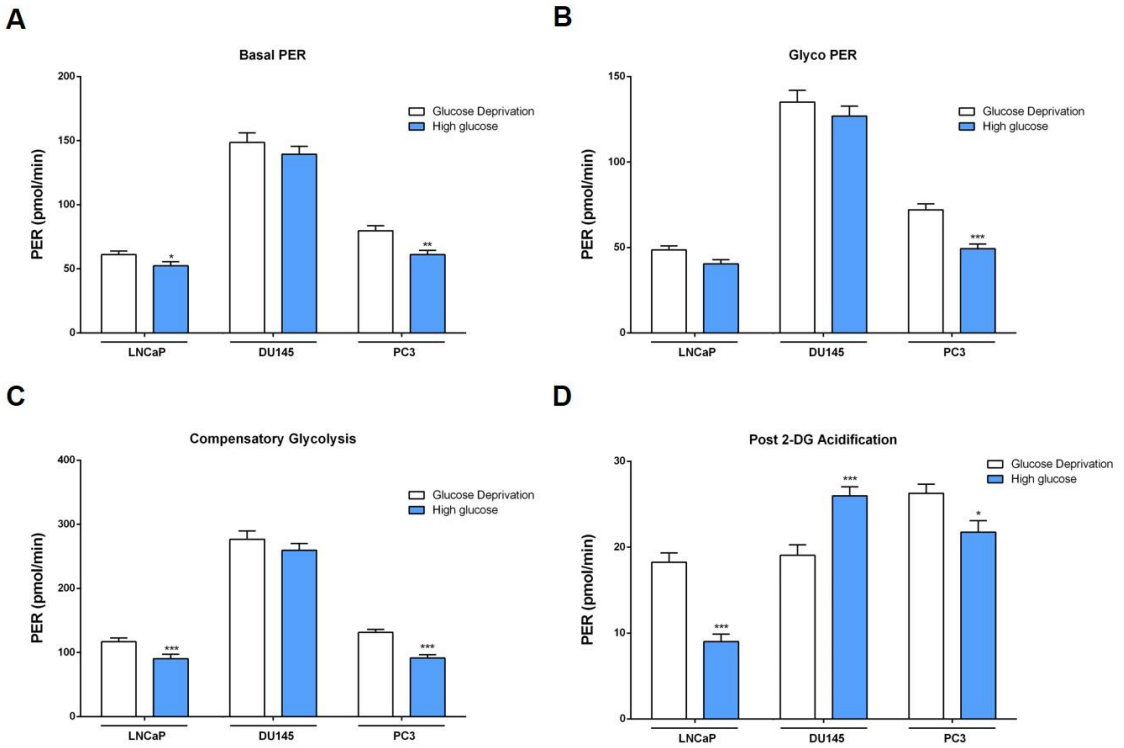

Figure 5.2. Effect of glucose deprivation and hyperglycaemia (30 mM glucose) on Basal Proton Efflux Rate (PER), Glycolytic PER, Compensatory Glycolysis, and Post 2-DG Acidification in LNCaP, DU145, PC3 cells. PER was obtained by the Seahorse XF96 Glycolytic Rate Assay. **(A)** Basal PER represents the initial total PER. The use of mitochondrial inhibitors, rotenone and antimycin A (0.5 μM each), enabled calculating the mitochondrial-associated acidification. **(B)** The Glycolytic PER (Glyco PER) resulted from subtracting mitochondrial acidification from Basal PER results. **(C)** Inhibition of mitochondria drives cells compensatory changes to use glycolysis to meet energy demands (Compensatory Glycolysis). **(D)** Secondly, glycolysis was inhibited by using the glucose analogue 2-deoxy-D-glucose (2-DG, 50 mM, Post-2-DG acidification). All experimental results were normalized to cell mass determined by the SRB assay. Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$, *** $p<0.001$ when compared with glucose deprivation.

Following the addition of mitochondrial inhibitors, the compensatory glycolysis in LNCaP and PC3 cells also was lower in hyperglycaemia relatively to glucose deprivation (90.38 ± 6.77 vs 116.7 ± 5.84 pmol/min in LNCaP, and 91.33 ± 5.30 vs 127.6 ± 4.59 pmol/min in PC3, Fig. 5.2.C). Furthermore, post-2-DG acidification (Fig. 5.1.A), that indicates the usage of others energy sources besides glycolysis or mitochondrial phosphorylation, was significantly decreased by hyperglycaemia in LNCaP and PC3 cells (9.019 ± 0.86 vs. 18.24 ± 1.09 pmol/min

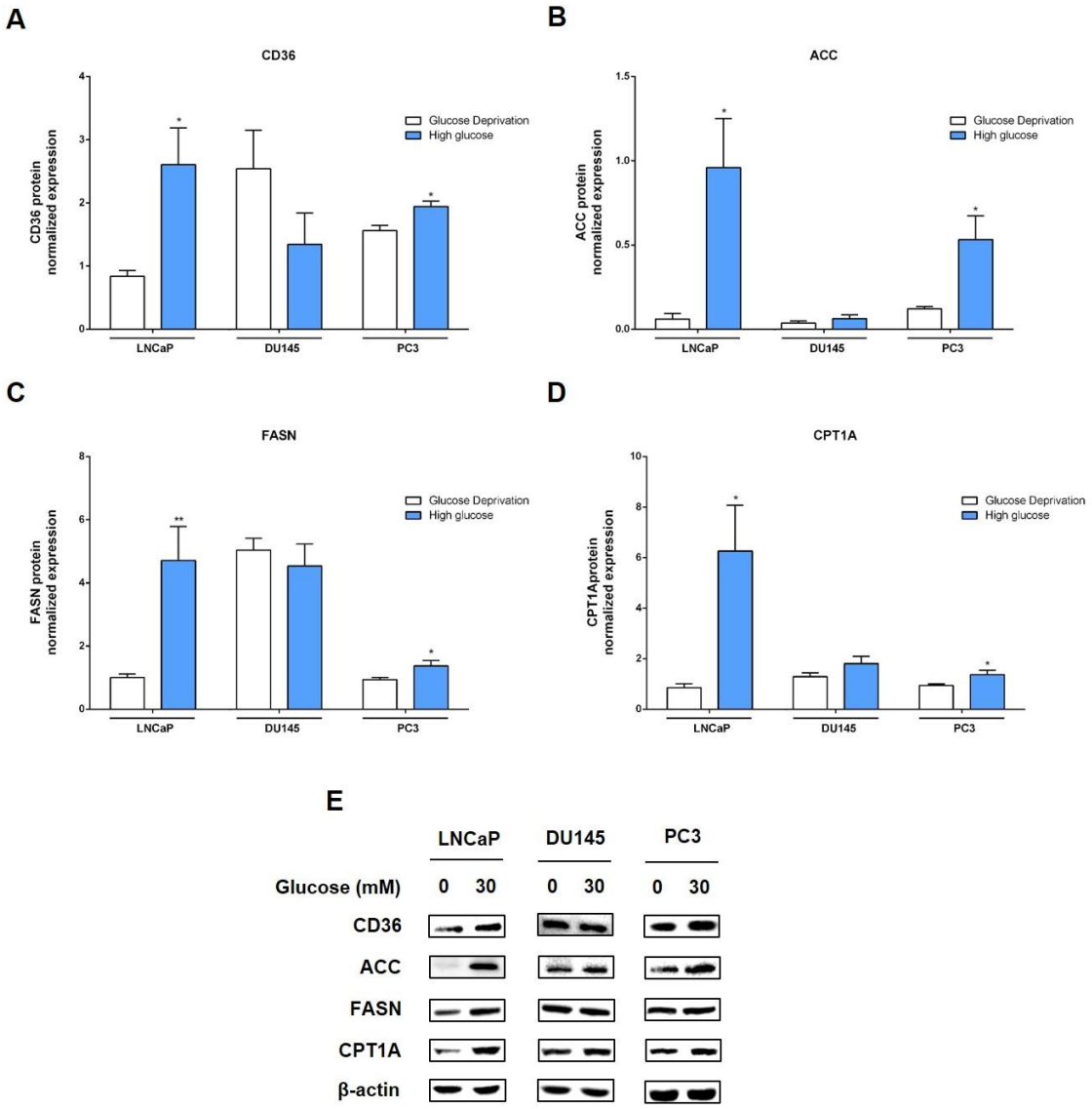

Figure 5.3. Effect of glucose deprivation and hyperglycaemia (30 mM glucose) on the expression of target regulators of lipid metabolism in LNCaP, DU145, PC3 cells. Protein expression of CD36 **(A),** ACC **(B),** FASN **(C)** and CPT1A **(D)** in LNCaP, DU145, PC3 cells analysed by WB after normalization with β-actin. Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$ when compared with glucose deprivation. Representative immunoblots are shown in panel E.

and 21.74 ± 1.351 vs. 26.26 ± 1.062 pmol/min , respectively, Fig. 5.2.D), whereas was augmented in DU145 cells (19.04 ± 1.24 vs 25.98 ± 1.05, Fig. 5.2.D). These results showed that DU145 cells present some plasticity to face alterations on glucose availability, which is in accordance with other findings describing the resistance of DU145 cells to radiation in glucose deprivation conditions [36].

The available literature also shows that glucose deprivation, as well as hypoxia and hypoxia-inducible factor-1, activate glycolysis and upregulate the expression of glucose transporter 1 (GLUT1), which was a mechanism of resistance to cell death [37-39]. Overall, others and our results have confirmed the dynamics of the Warburg Effect in PCa cells to answer to cell's energy necessities [40].

Finally taking into account that glucose metabolism is one of the principal sources to lipid metabolism, we decided to evaluate the expression of target regulators of lipid metabolism in hyperglycaemic and glucose deprivation conditions (Fig. 5.3.). The expression of the fatty acid transporter, CD36, was significantly increased in LNCaP and PC3 cells in hyperglycaemia relative to glucose deprivation ($p=0.024$ and $p=0.018$, respectively Fig. 5.3.A). ACC, the enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA is a key element in the biosynthesis and oxidation of fatty acids. Hyperglycaemia induced ACC expression relative to glucose deprivation, both in LNCaP and PC3 cells ($p=0.015$ and $p=0.027$, respectively, Fig. 3B). In fact, the majority of acetyl-CoA used for lipid synthesis is generated from glucose by the conversion of pyruvate to citrate [41,42]. Similarly, the expression of FASN, a crucial enzyme in fatty acid synthesis, was increased in LNCaP and PC3 cells in high glucose availability ($p=0.007$ and $p=0.045$, respectively Fig. 5.3.C). Knowledge of the mechanisms that underpin the hyperglycaemia actions influencing lipid metabolism is very limited. However, it was reported that glucose could activate the sterol regulatory element-binding proteins (SREBPs) [43], transcription factors known to regulate the expression of genes involved in lipid and cholesterol metabolism, including FASN [44-47]. Also, the expression of CPT1A, a rate-limiting component in fatty acid oxidation by catalysing the carnitine-dependent transport of fatty acids across the inner mitochondrial membrane, was shaped by the glycolytic environment. Hyperglycaemia induced CPT1A expression relative to glucose deprivation, both in LNCaP and PC3 cells ($p=0.017$ and $p=0.047$, respectively, Fig. 5.3.D). No effect was seen on the expression of fatty acid metabolism regulators, CD36, ACC, FASN, CPT1A in DU145 cells, in response to different glucose concentrations (Fig. 5.3.). In LNCaP and PC3 cells, our results showed that high glucose availability diminished the glycolytic activity and augmented lipid metabolism, indicating a -shift of PCa metabolism dependently on glucose availability.

In summary, this is the first study fully characterizing the metabolism of PCa cells by the Seahorse analysis and showing a differential dependency and capacity of PCa cells. In addition, hyperglycaemia conditions induced a metabolic shift turning off glycolytic pathways and activating lipid metabolism. Overall, these findings contribute to the deep understanding of PCa metabolism and are of utmost importance for development of therapeutic options targeting metabolic pathways.

5.4. Acknowledgments

None.

5.5. Funding

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Projects No. 007491 and 029114) and National Funds by FCT-Foundation for Science and Technology (Project UID/Multi/00709/2013). Cardoso HJ and Figueira MI were recipient of FCT fellowships, respectively, SFRH/BD/111351/2015 and SFRH/BD/104671/2014.

5.6. Conflict of Interest

The authors declare that they have no conflicts of interest.

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Chapter 6

Glutaminolysis is a metabolic route essential for survival and growth of prostate cancer cells, and a target of 5α-dihydrotestosterone regulation

This Chapter was submitted for publication:

Cardoso HJ, Figueira MI, Carvalho TM, Vaz CV, Madureira PA, Oliveira PJ, Sardão VA , Socorro S. Glutaminolysis is a metabolic route essential for survival and growth of prostate cancer cells, and a target of 5α-dihydrotestosterone regulation. Submitted to Cancer Letters.

Abstract

Resistance to androgen-deprivation therapies and the progression of disease to the so-called castrate-resistant prostate cancer (CRPC) remain a challenge in prostate cancer (PCa) management and treatment. Among other alterations, the establishment of CRPC is associated with a set of metabolic alterations driven by androgens. The present study aimed to characterize the importance of glutaminolysis in controlling the survival and growth of androgen-sensitive (LNCaP) and CRPC (DU145 and PC3) cells, and investigate the role of androgens regulating this metabolic route. Moreover, the consequence of inhibiting glutaminase on the PCa cells response to anti-androgens, and their capability to use other metabolic pathways, were evaluated. Western Blot (WB) analysis demonstrated the lower expression of the glutamine transporter, ASCT2 in DU145 and PC3 cells relative to LNCaP, whereas glutaminase was increased. 5α-dihydrotestosterone (DHT) up-regulated the expression of ASCT2 and glutaminase both in LNCaP cells (10 nM) and rat prostate (500 μg/kg/day). Glutaminase inhibition (BPTES, 10 μM) diminished PCa cell viability, migration and c-Myc oncogene expression, while increasing the expression of the cell cycle inhibitor p21 and augmenting caspase-3 activity. Moreover, co-treatment with BPTES and the anti-androgen bicalutamide had a synergic effect suppressing LNCaP cells viability. Biochemical assays, WB analysis and the extracellular flux analyses demonstrated that inhibiting glutaminolysis had a differential impact on glycolysis and lipid metabolism in androgen-sensitive and CRPC cells. Overall, the present findings support glutaminolysis as a central metabolic route controlling PCa cell fate and highlight for the benefit of co-targeting androgen receptor and glutamine metabolism in PCa treatment.

Keywords: 5α-dihydrotestosterone, bicalutamide, BPTES, prostate cancer, glutamine, glutaminolysis.

6.1. Introduction

Prostate cancer (PCa) is one of the most diagnosed cancers among men, although treatment and monitoring remain a clinical and research challenge. The progression of PCa is initially dependent on the stimulatory action of androgens, which validates the use of therapies reducing androgens biosynthesis or antagonizing the androgen receptor (AR) [1,2]. However, the majority of PCa cases becomes resistant to androgen-deprivation therapy (ADT), progressing to the so-called castrate-resistant PCa (CRPC), on average after 38 months [3]. Thus, this stage of the disease is characterized by the proliferation of PCa cells independently of androgen depletion and, despite the important advances in the last years, effective treatment options for CRPC are still needed [4,5]. Preclinical and clinical research aimed at developing strategies for better management of PCa and slowing the progression to CRPC, have involved investigating the efficacy of ADT in combination with other drugs, namely docetaxel or cabazitaxel [6]. However, the benefits for patients are modest, increasing the

overall survival by a few months only, while the development of resistance also occurs [6]. The identification of new therapeutic targets in connection with the androgens and AR actions remains a fundamental aspect to improve management and treatment of PCa.

Metabolic reprogramming favouring cancer development and progression is one of the hallmarks of cancer that has been intensively exploited with therapeutic interest in the last years [7-9]. In the case of PCa, progression to CRPC has been associated with a set of metabolic alterations that can be driven by androgens [10-12]. These steroid hormones have been characterized as master regulators of several metabolic pathways in PCa, such as aerobic glycolysis, glutamine metabolism, pyruvate import, oxidative phosphorylation, fatty acid ß-oxidation, and *de novo* lipid synthesis [13,14,12]. Indeed, the androgens' role stimulating glucose and lipid usage and metabolism has been detailed, but their action modulating other routes, namely, glutaminolysis is less known.

Glutamine is one of the principal energy sources, supplying nitrogen for purine and pyrimidines biosynthesis, being also a carbon substrate for anabolic processes in cancer [15,16]. Glutamine uptake is mediated by the alanine, serine, cysteine transporter 2 (ASCT2) (also called SLC1A5 or L-type amino acid transporter) whose expression has been shown to be significantly increased in several types of cancer, including PCa [17]. In the intracellular space, glutamine is converted to glutamate by the activity of the mitochondrial enzyme glutaminase [18]. In turn, glutamate originates α-ketoglutarate, an intermediate metabolite that fuels the tricarboxylic acid (TCA) cycle. Moreover, glutaminase activity and the α-ketoglutarate have been shown to influence other metabolic pathways, such as glycolysis [19].

Glutaminase has been shown to be highly expressed in PCa and to correlate positively with tumour stage and disease progression [20,19]. Also, there are indications that glutaminolysis is a relevant metabolic pathway in PCa, and that inhibiting glutaminase can suppress PCa cell proliferation [21-26]. However, the broad actions of androgens regulating this metabolic route and the effect of glutaminase inhibition in PCa cells in the presence (or absence) of anti-androgens still need attention. Also, it is entirely unknown whether the inhibition of glutaminolysis by blocking glutaminase activity shapes PCa cells towards higher glucose and lipid utilization.

Our main objective was to characterize the importance of glutaminolysis in controlling cell fate in androgen-sensitive and CRPC cells, and investigate the androgenic regulation of this metabolic route. We determined not only whether inhibition of glutaminase sensitizes PCa cells to anti-androgens, but also ascertained how PCa cells respond using other metabolic pathways, such as glycolysis, after blockage of glutaminolysis.

6.2. Materials and methods

6.2.1. Chemicals

All chemicals, culture media, and antibodies unless otherwise stated were purchased from Sigma–Aldrich (St Louis, MO, USA). All solutions were prepared with ultra-pure water. BPTES was dissolved in DMSO and 5α-dihydrotestosterone (DHT) and bicalutamide in ethanol.

6.2.2. Cell lines and treatments

Human prostate cancer adenocarcinoma cell lines (LNCaP, DU145, and PC3) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). LNCaP cells have origin in PCa lymph node metastasis, express the AR and are used as an androgen-sensitive model. DU145 and PC3 cell lines are originated from brain and bone metastasis of undifferentiated grade IV prostate adenocarcinomas, and mimic the castrate-resistant stage of PCa [27,28].

LNCaP, DU145, and PC3 cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/ streptomycin at 37 ºC in an atmosphere equilibrated with 5 % CO_2. At 60% confluence, culture medium was replaced by phenol red-free RPMI1640 medium containing 5% charcoal-stripped FBS. Next, cells were maintained for additional 24 h and then, exposed to DHT (10 nM) or vehicle for 12, 24, 48 h. Alternatively, cells were exposed to different concentrations of glutaminase inhibitor, BPTES (1, 2, 5, 10, 25, and 50 μM), alone or in the presence of the AR inhibitor bicalutamide (10, 20, and 40 μM).

6.2.3. Animals and hormone treatment

Adult male Wistar (*Rattus norvegicus*) rats, approximately aged 3 months old (250–300 g) were maintained in an animal room at constant temperature and humidity, under a 12 h light-dark cycle, with standard food pellets and water available *ad libitum*. Animal handling and all experimental procedures complied with the guidelines established by the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the National and European Union rules for the care and handling of laboratory animals (Directive 2010/63/EU).

A total of 16 rats were orchidectomised (ORCHX) under anaesthesia (Clorketam 1000, Vétoquinol, Lure, France). Five days after surgery, animals were randomly divided into two groups receiving daily intraperitoneal injections of DHT (500 μg/kg/day) (ORCHX + DHT) or vehicle alone (physiologic serum/ethanol 30% alone; ORCHX + Vehicle) for 5 days. Another group of animals that did not undergone surgery was treated daily with vehicle alone (Intact group, n = 8). After treatment, animals were euthanized by cervical dislocation under anesthesia (100 mg ketamine/8 mg xilazine per Kg), and prostates removed and immediately frozen in liquid nitrogen for protein extraction.

6.2.4. Protein extraction

Human prostate cells and rat prostate tissues were homogenized in the appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail and PMSF (0,1 mM), kept on ice for 20 min with occasional mixing, and then centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer´s instructions.

6.2.5. Western blot (WB) analysis

Twenty-five microgram of protein extracts were resolved on a 12.5% acryl-bisacrylamide gel by SDS–PAGE. Proteins were electro-transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA), which were incubated with rabbit anti-ASCT2 (1:1000, V501; Cell Signalling Technology, Danvers, MA, USA), rabbit anti-glutaminase (1:1000, ab93434, Abcam, Cambridge, UK), rabbit anti-Glucose transporter type1 (GLUT1, 1:1000,CBL242, Millipore), rabbit anti-GLUT2 (1:1000, sc-9117, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-GLUT3 (1:1000, sc-30107, Santa Cruz Biotechnology), rabbit anti-Phosphofructokinase 1 (PFK1, 1:1000, sc-67028, Santa Cruz Biotechnology), rabbit anti-lactate dehydrogenase (LDH, 1:10,000, Ab52488, Abcam,), rabbit anti-monocarboxylate transporter 4 (MCT4, 1:1000, sc-50329,Santa Cruz Biotechnology), rabbit anti-Fatty Acid Synthase (FASN, 1:1000, no.3180; Cell Signaling Technology), mouse anti-Carnitine Palmitoyltransferase 1A (CPT1A, 1:1000, ab128568; Abcam), rabbit anti-p21 (1:500, sc-397, Santa Cruz Biotechnology) and rabbit anti-phospho-c-Myc (1:1000, no.13748; Cell Signaling Technology) primary antibodies. After that, membranes were washed and incubated with either anti-rabbit IgG HRP-linked (1:20000, sc-2004, Santa Cruz Biotechnology) or anti-mouse-IgGκ HRP-linked (1:20000, sc-516102, Santa Cruz Biotechnology) secondary antibodies for 1 h at room temperature. Protein expression was normalized using an anti-α-tubulin monoclonal antibody (1:10000, T9026) or mouse anti-β-actin (1:1000, A5441) antibodies. Membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and immunoreactive proteins were visualized using the ChemiDoc™ MP System (Bio-Rad). Band densities were obtained according to standard methods using Image Lab software (Bio-Rad) and normalized by the respective housekeeping band density.

6.2.6. Fluorescence immunocytochemistry

LNCaP cells were fixed with 4% PFA for 10 min and permeabilized with 1% Triton X-100 for 5 min at room temperature. Non-specific staining was blocked by incubation with PBS containing 0.1% (w/v) Tween-20 (PBS-T) and 20% FBS for 1 h. After washing, cells were incubated with the rabbit anti-ASCT2 (1:100, V501; Cell Signalling Technology) primary antibody for 1 h at room temperature. Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen,

Darmstadt, Germany) was used as secondary antibody. Alternatively, cells were incubated with rabbit anti-Calnexin (1:50, sc-11397, Santa Cruz Biotechnology) primary antibody for 1 h at room temperature and Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen) was used as secondary antibody. Specificity of staining was assessed by omission of the primary antibodies. Cell nuclei were stained with Hoechst 33342 (5 mg/mL, Invitrogen) for 10 min. Lamellae were washed with PBS-T and mounted with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using a Zeiss LSM710 laser scanning confocal microscope (Carl Zeiss, Gottingen, Germany). The threshold for the co-localization of ASCT2-calnexin was defined through the omission of anti-Calnexin or anti-ASCT2 antibodies. The degree of co-localization was measured by the Pearson's Correlation Coefficient obtained in the Zeiss LSM710 laser scanning confocal microscope.

6.2.7. Cell viability assay

LNCaP (25.000 cells/well), DU145 (8000 cells/well) and PC3 (8000 cells/well) cells were grown in 96-well plates, and cell viability was determined by the colorimetric MTT assay. After BPTES or bicalutamide treatments, MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), at a final concentration of 0.5 mg/ml, was added to the cell culture medium and reaction occurred in the dark at 37 ºC for 4 h. After that, MTT solution was carefully removed, and the formed formazan crystals were solubilized with 100 µL DMSO. The absorbance of the resultant purple coloured solution was measured at 570 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The absorbance value is directly proportional to the number of viable cells in each experimental group.

6.2.8. Migration assay

Cell migration assay was performed using 8 µM pore size inserts (35224, SPL, Life Sciences, Naechon-Myeon Pocheon, South Korea). Briefly, LNCaP (3.0 x10^5 cells/transwell), DU145 (10^5 cells/transwell) and PC3 (1.5 x10^5 cells/transwell) cells were placed into the upper chambers with serum-free media in the presence or absence of BPTES. The lower chambers contained media supplemented with 20% FBS. After 24 h, cells on the lower surface of the transwell were fixed and stained with haematoxylin. Cells were then counted in 10, randomly selected, ×40 magnification fields per transwell.

6.2.9. Caspase-3-like activity

The caspase-3-like activity was determined spectrophotometrically at 405 nm by detecting the presence of the yellow product p-nitro-aniline (pNA), upon cleavage of caspase-3 substrate (Ac-DEVD-pNA). In brief, 10 µl of total protein extract was incubated overnight at 37 °C with reaction buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT)

and 200 µM of Ac-DEVD-pNA. The amount of generated pNA was calculated by extrapolation with a standard curve with known amounts of pNA. All measurements were normalized to the total amount (µg) of protein in each sample.

6.2.10. Quantification of glutamine, glucose and lactate

The concentration of glutamine (NZYTech, Lisboa, Portugal), glucose and lactate (Spinreact, Girona, Spain) in the cell culture medium of DHT- or BPTES-treated cells was determined by spectrophotometric analysis using commercial kits according to the manufacturer's instructions. The glutamine and glucose consumption, as well as lactate production, were calculated relatively to the initial concentration of these metabolites at 0 h of treatment. All measurements were normalized to the total amount (µg) of protein in each sample.

6.2.11. Glycolytic rate assay

The cell glycolytic response upon BPTEs treatment was evaluated by measurement of the extracellular acidification (proton efflux rate) using the Seahorse XF-96 analyser (Seahorse Bioscience, Agilent, Santa Clara, CA, USA). LNCaP (15000 cells/ well), DU145 (5000 cells/ well), PC3 (5000 cells/ well) cells were seeded in 96-well plates (Seahorse Bioscience) and maintained at 37º C, 5% CO_2 for 24 h. Cells were then exposed to 10 µM BPTES for 24 h. In parallel, an XFe96 sensor cartridge for each cell plate was placed in a 96-well calibration plate containing 200 µL/well calibration buffer and left to hydrate overnight at 37°C. The sensor cartridge and the calibration plate were loaded into the XF^e96 Extracellular Flux Analyzer for calibration, and then, the utility plate, containing the calibration solution was replaced with the study plate, and the assay started. After 24 h of treatment with BPTES, cell culture medium was replaced by 180 µL/well of low-buffered serum-free medium without phenol red containing 2 mM glutamine, 10 mM glucose, 1 mM pyruvate, and 5.0 mM HEPES at pH 7.4, and cells maintained at 37°C for 1 h in the absence of CO_2. Mitochondrial inhibitors rotenone plus antimycin A (0.5 µM each) and glycolysis inhibitor 2-deoxy-D-glucose (2-DG, 50 mM) were injected via ports A and B, respectively.

Data were analysed using the Agilent Seahorse Glycolytic Rate Assay Report Generator, and the raw data were used to calculate the initial proton efflux rate, mitochondrial- and glycolysis-associated acidification, and the acidification rate upon mitochondria and glycolysis inhibition were calculated. All results were normalized by the sulforhodamine B (SRB) assay [29]. Briefly, cells were fixed with 50 µl of 60% trichloroacetic acid (TCA), overnight at 4 ºC. After removing the fixing solution and washing with distilled water, fixed cellular proteins were stained with the SRB solution (0.05 % w/v in 1% acetic acid) for 1 h at 37 °C. Unbound dye was removed with 1% acetic acid, and cell bound dye solubilized with 10 mM Tris base solution, pH 10. Colour intensity was measured at 540 nm using the Cytation 3 microplate reader, from Biotek (Winooski, VT, USA).

6.2.12. Alanine aminotransferase (ALT) enzymatic activity

The enzymatic activity of ALT in BPTES-treated cells was determined using a commercial kit (Spinreact) according to the manufacturers' instructions. ALT catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate forming pyruvate and glutamate. The pyruvate produced is reduced to lactate by LDH together with the oxidation of NADH to NAD^+. The rate of decrease in NADH concentration, measured spectrophotometrically, is proportional to the catalytic concentration of ALT present in the sample. The enzymatic activity was calculated by measuring samples variation in the absorbance (340 nm). The activities achieved were calculated as U/μg of protein.

6.2.13. Statistical analysis

Statistical significance of differences among experimental groups were evaluated by unpaired T-test with Welch's correction or one-way ANOVA followed by Tukey post-hoc test, using GraphPad Prism v6.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean (S.E.M).

6.3. Results

6.3.1. ASCT2 and glutaminase are differentially expressed in androgen-sensitive compared to CRPC cells

The protein expression of glutamine metabolism regulators ASCT2 and glutaminase (Fig. 6.1.A) in PCa cell line models was evaluated by WB analysis. The expression of the glutamine transporter, ASCT2, was significantly lower in CRPC cell lines (DU145 and PC3) relatively to the androgen-sensitive LNCaP cell line (0.57 ± 0.07 and 0.61 ± 0.02 fold variation, respectively, Fig. 6.1.B). On the other hand, glutaminase expression (Fig. 6.1.A), was significantly higher in DU145 and PC3 cells relatively to LNCaP (3.66 ± 0.26 and 2.686 ± 0.22, fold variation, respectively, Fig. 6.1.B). Amongst all the PCa cell lines, DU145 cells displayed the highest expression of glutaminase (Fig. 6.1.B).

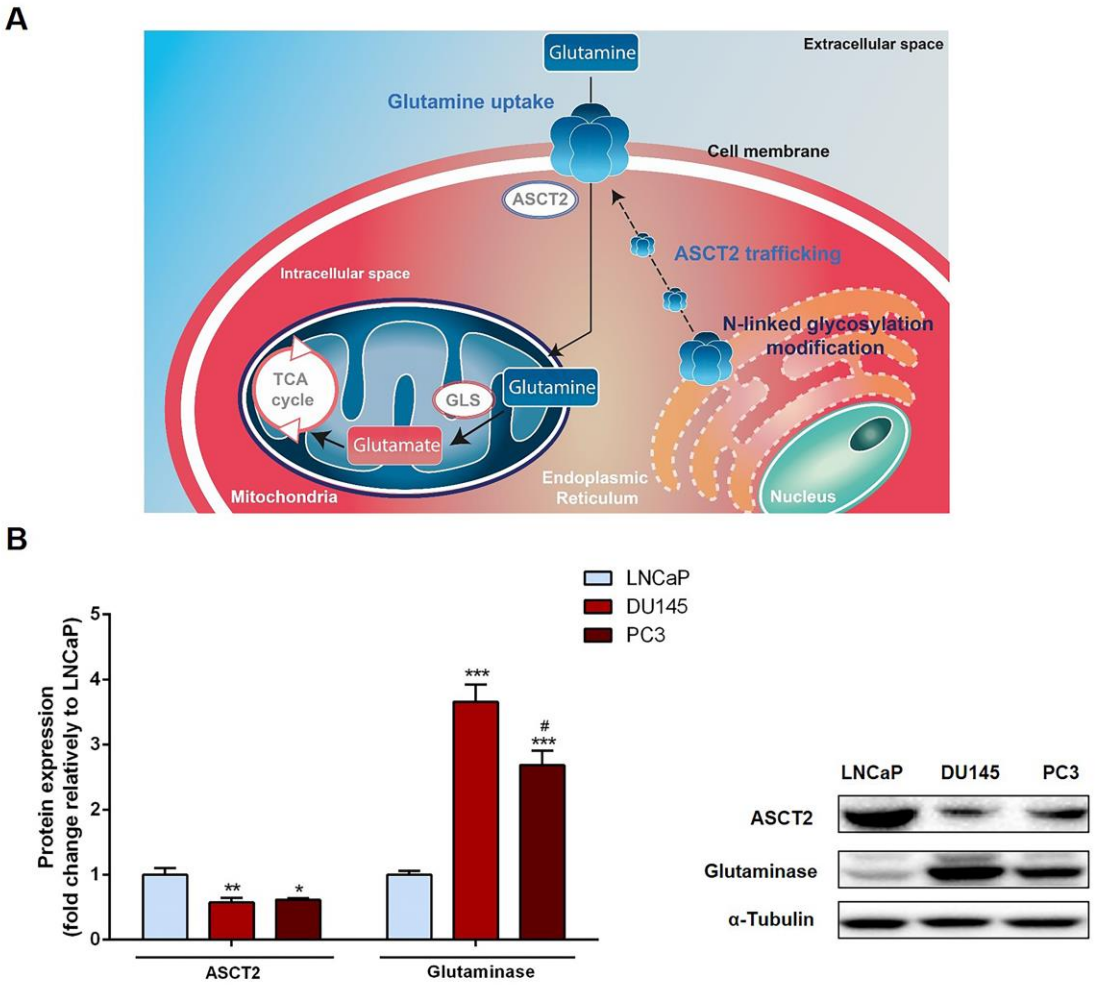

Figure 6.1. ASCT2 and glutaminase in androgen-sensitive (LNCaP), and CRPC (DU145 and PC3) cell line models. (A) Schematic representation of glutamine metabolism. Glutamine uptake from the extracellular space occurs through the activity of the ASCT2 transporter, whose translocation to the cell membrane via endoplasmic reticulum seems to depend on post-translational modifications, namely, N-linked glycosylation. At the mitochondria, glutaminase (GLS) converts glutamine into glutamate that enters the tricarboxylic acid (TCA) cycle generating ATP. **(B)** ASCT2 and glutaminase protein expression determined by WB analysis after normalization with α-tubulin. Error bars indicate mean ± S.E.M (n= 5) * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared to LNCaP cells and # $p<0.05$; when compared to DU145 cells. Representative immunoblots are shown in the right panel.

6.3.2. DHT stimulated glutamine metabolism in LNCaP cells and rat prostate in vivo

Androgens have been shown to play a role as metabolic regulators fuelling PCa cells growth and proliferation [12,10,30]. In this work, we evaluated the effect of DHT (10 nM) in regulating glutamine consumption (Fig. 6.2.A), and ASCT2 (Fig. 6.2.B and Fig. 6.2.D) and glutaminase (Fig. 6.2.C and Fig. 6.2.D) expression in PCa cells. DHT-treatment significantly augmented glutamine consumption in the androgen-sensitive LNCaP cells for 24 h of treatment (1.36 ± 0.15 fold variation vs. control, Fig. 6.2.A). Moreover, DHT significantly increased the protein expression levels of ASCT2 and glutaminase in these cells upon 24 h and 48 h of exposure (ASCT2, 1.68 ± 0.27 and 1.98 ± 0.33 fold variation vs. control, respectively, Fig. 6.2.B; glutaminase, 1.20 ± 0.06 and 1.26 ± 0.12 fold variation vs. control, respectively,

Fig. 6.2.C). Overall, no effect was seen on the expression of ASCT2 (Fig. 6.2.B) and glutaminase (Fig. 6.2.C) in CRPC cells, DU145 and PC3, in response to DHT.

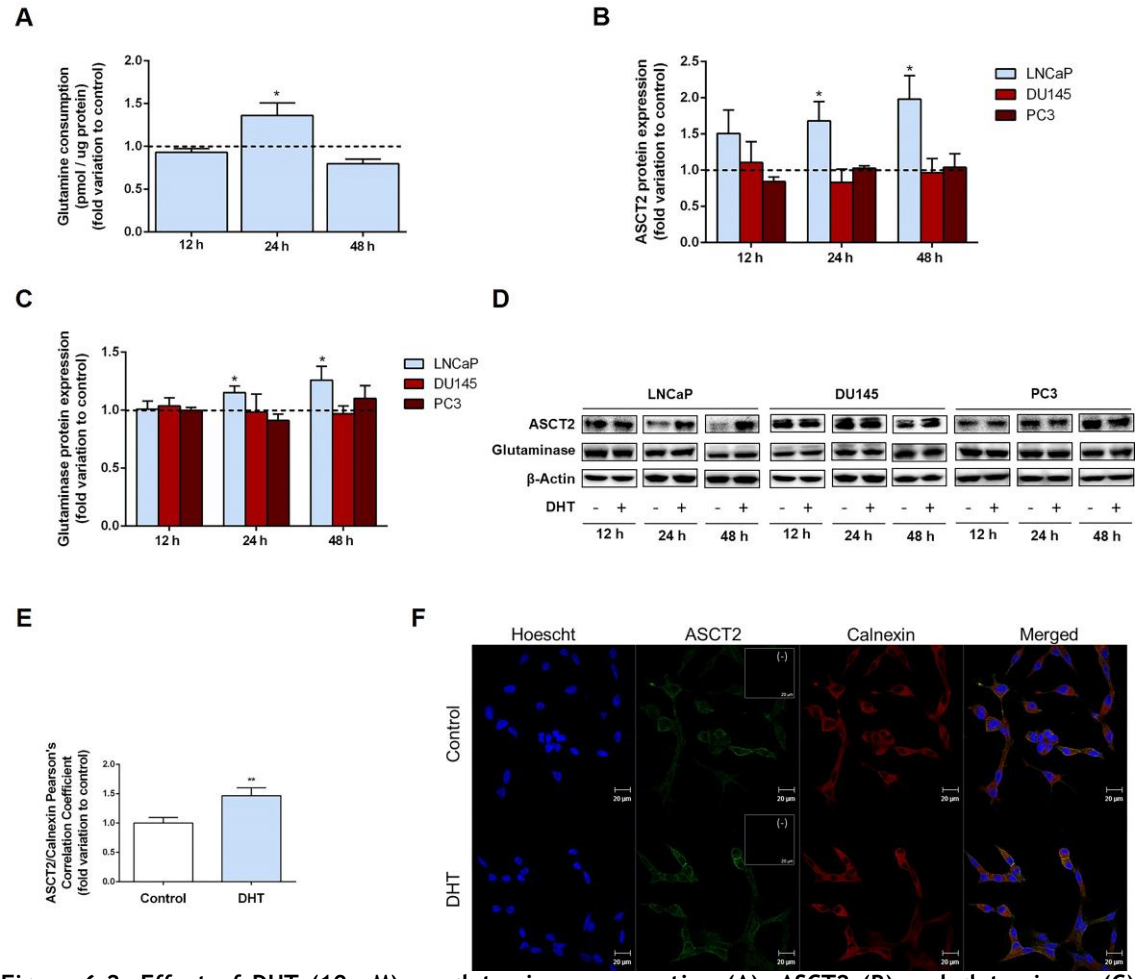

Figure 6.2. Effect of DHT (10 nM) on glutamine consumption (A), ASCT2 (B) and glutaminase (C) expression, and ASCT2 subcellular localization (E, F) in androgen-sensitive (LNCaP) and CRPC (DU145 and PC3) cells treated for 12, 24, or 48 h. (A) Glutamine consumption was determined spectrophotometrically using a commercial kit. **(B,C)** Protein expression was determined by WB after normalization with ß-actin. All results are expressed as fold-variation relative to the control untreated group (0 nM DHT, dashed line). **(D)** Representative immunoblots. **(E)** ASCT2 co-localization with the endoplasmic reticulum marker calnexin assessed by immunofluorescence and calculated using the Pearson's Correlation Coefficient. **(F)** Representative confocal microscopy images showing co-localization of ASCT2 (green) with calnexin (red). Nuclei are stained blue with Hoechst 33342. Negative controls for ASCT2, obtained by omission of the primary antibody, are provided as insert panels (-).Error bars indicate mean ± S.E.M (n= 5) * $p<0.05$, ** $p<0.01$ when compared to control.

Recently, it was shown that N-linked glycosylation is an important post-translational modification required for ASCT2 trafficking from the endoplasmic reticulum to the cell membrane (Fig. 6.1.A) [31]. Also, it has been shown that androgens can upregulate global protein glycosylation in PCa cells [32], and alter the distribution of several molecular targets across cell compartments [33-35]. Therefore, we investigated whether DHT-stimulation would affect ASCT2 subcellular localization. For this purpose, ASCT2 co-localization with calnexin was analysed by confocal microscopy (Fig. 6.2.E and Fig. 6.2.F). Calnexin is a membrane protein of the endoplasmic reticulum that functions as a molecular chaperone responsible for glycoprotein folding and quality control [36]. DHT-treatment increased the co-localization of ASCT2 with the endoplasmic reticulum protein calnexin by approximately 50% (1.47 ± 0.14

fold variation vs. control, Fig. 6.2.E), determined by immunofluorescence staining (Fig. 6.2.F) and analysed by the Pearson's correlation coefficient (Fig. 6.2.E).

We also investigated whether the increase of expression levels of glutamine regulators, ASCT2 and glutaminase induced by DHT found in LNCaP cells (Fig. 6.2.) could be recapitulated *in vivo*. For this purpose, orchidectomised rats were treated with DHT (ORCHX + DHT, 500 µg/kg/day) for 5 days. Both ASCT2 and glutaminase expression were significantly augmented in the ORCHX + DHT group when compared to the ORCHX + Vehicle and/or intact groups (Fig. 6.3.). ASCT2 and glutaminase expression changed by 2.06 ± 0.24 and 1.61 ± 0.11 fold variation relative to intact animals, respectively, Fig. 6.3.). No effect was perceived on the protein expression of ASCT2 and glutaminase upon castration only (Fig. 6.3.).

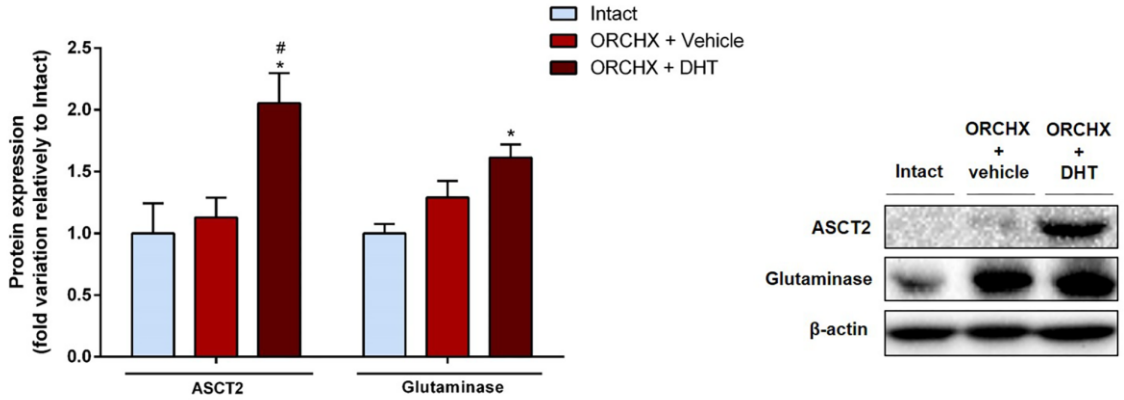

Figure 6.3. Effect of DHT (500µg/kg/day) on ASCT2 and glutaminase protein expression levels in rat dorsolateral prostate. Three months old ORCHX animals received intraperitoneal injections of vehicle (ORCHX + vehicle) or DHT (ORCHX + DHT) for 5 consecutive days. The intact group includes animals that did not undergone surgery and receiving vehicle only. Protein expression was determined by WB after normalization with ß-actin. All results are expressed as fold-variation relative to the control intact group. Error bars indicate mean ± S.E.M (n= 5) * $p<0.05$ when compared to intact group. # $p<0.05$; when compared to ORCHX + vehicle group. Representative immunoblots are shown in the right panel.

6.3.3. Inhibition of glutamine metabolism decreased PCa cells viability and migration whereas increasing caspase-3-like activity

Viability of LNCaP, DU145 and PC3 cells was determined by the MTT assay after treatment with BPTES, a specific glutaminase inhibitor, which was used to inhibit glutamine metabolism [37]. All BPTES concentrations tested (1-50 µM) significantly decreased the viability of DU145 and PC3 cells (Fig. 6.4.A), though not always on a concentration-dependent manner. BPTES also decreased the viability of LNCaP cells. However, the effect was seen only at the highest (25 µM and 50 µM) concentrations (78 ± 3 % and 73 ± 2 % cell viability compared to the control, respectively, Fig. 6.4.A). Moreover, 1 µM and 2 µM BPTES increased LNCaP cells viability (141 ± 9 % and 127 ± 8 % compared to the control, Fig. 6.4.A).

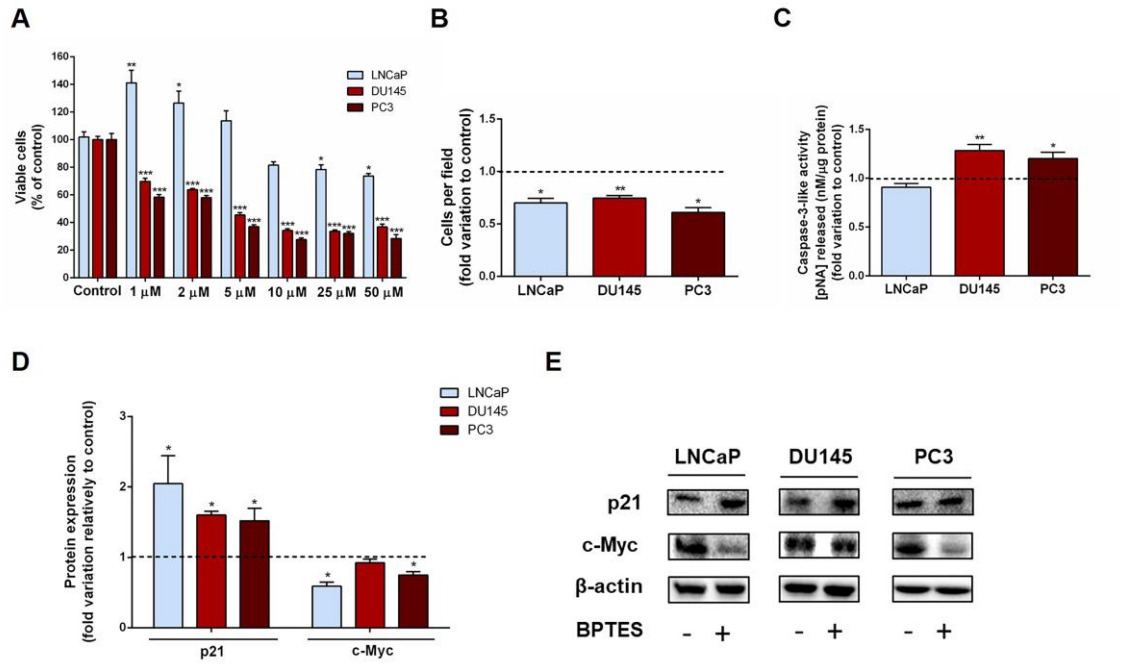

Figure 6.4. Effect of BPTES (1-50 μM) on cell viability (A), migration (B), caspase-3-like activity (C), and p21 and c-Myc protein expression (D, E) in androgen-sensitive (LNCaP), and CRPC (DU145 and PC3) cells treated for 24 h. (A) LNCaP, DU145 and PC3 cells viability was determined by the MTT assay (% of control). **(B)** PCa cells migration was determined by a trans-wells assay in uncoated chambers. The upper chamber contained serum free medium and cells in the presence or absence of BPTES (10 μM). FBS complete medium in the lower chamber was used as chemoattractant. Data are expressed as the mean number of migrating cells per 20x magnification field (10 fields were assessed for each experimental condition). **(C)** Caspase-3-like activity in 10 μM BPTES-treated cells was measured spectrophotometrically based on the release of pNA chromophore. **(D)** Protein expression upon BPTES-treatment (10 μM) was determined by WB analysis after normalization with β-actin. **(E)** Representative immunoblots. Results are expressed as fold-variation relative to the control untreated group (0 nM BPTES, dashed line). Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared to the control.

BPTES effects on migration and apoptosis of PCa cells were assessed with the single concentration of 10 μM, also following previous studies in other cancer cell types [23,38]. BPTES supplementation decreased the migration capacity of LNCaP, DU145 and PC3 cells in trans-wells assays (0.70 ± 0.04, 0.75 ± 0.03 and 0.61 ± 0.05 fold variation vs. control, respectively, Fig. 6.4.B).

The activity-like of the executioner caspase-3, as an end-point indicator of apoptosis, was measured in the cell lines after treatment with BPTES. Caspase-3-like activity was significantly increased in CRPC cell lines (1.28 ± 0.06 and 1.20 ± 0.06 fold variation vs. control, respectively, Fig. 6.4.C), whereas no differences were detected in LNCaP cells (Fig. 6.4.C).

WB analysis showed that the alterations observed in PCa cell fate after BPTES treatment were accompanied by the altered expression of the cell cycle inhibitor p21 and the oncogene c-Myc (Fig. 6.4.D and Fig. 6.4.E). p21 expression was increased in response to BPTES treatment in all PCa cells (2.05 ± 0.40, 1.60 ± 0.06 and 1.52 ± 0.18 fold variation vs. control, respectively, Fig. 6.4.D). On the other hand, BPTES significantly decreased c-Myc

expression in LNCaP and PC3 cells (0.59 ± 0.06 and 0.75 ± 0.05 fold variation vs. control, respectively, Fig. 6.4.D).

6.3.4. BPTES increased the sensitivity of LNCaP cells to the anti-androgen bicalutamide

Next, we investigated whether BPTES would influence the response of PCa cells to standard anti-androgen therapy. For this purpose, LNCaP, DU145, and PC3 cells were treated with

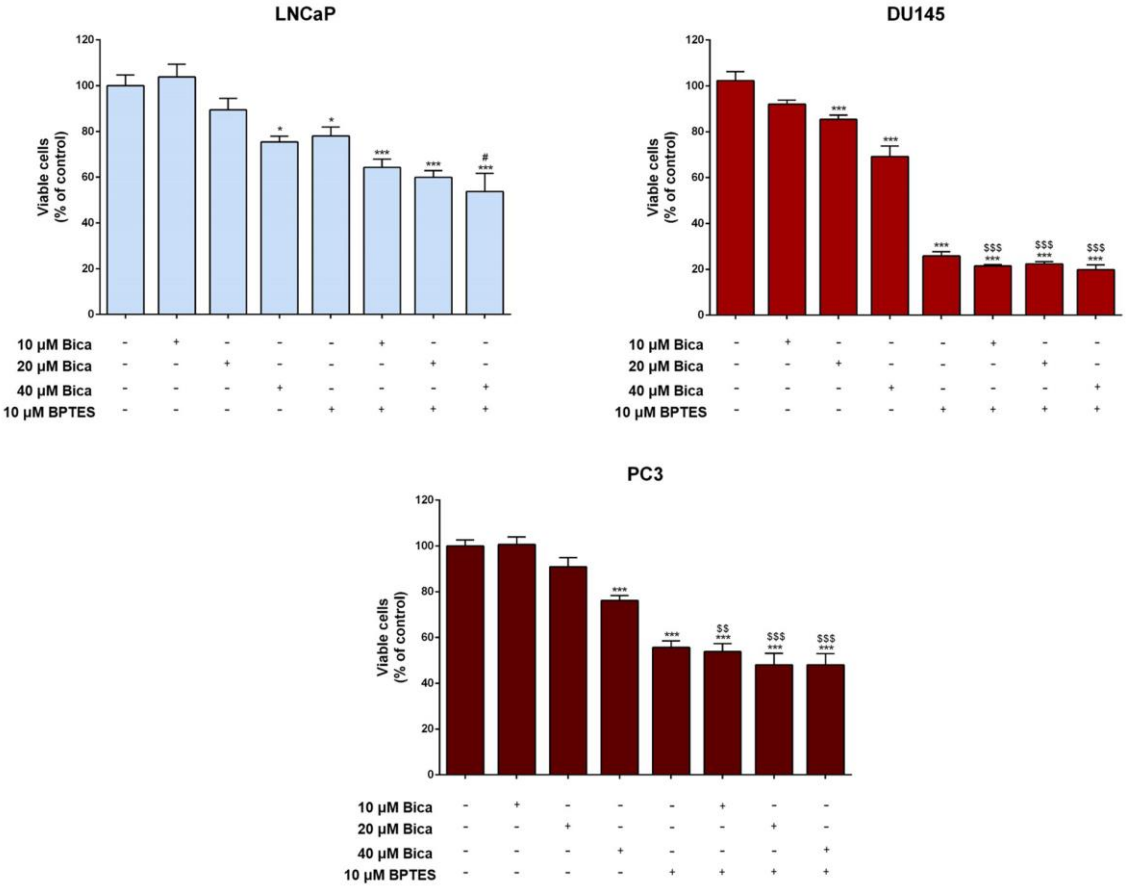

Figure 6.5. Viability of androgen-sensitive (LNCaP), and CRCP (DU145 and PC3) cells after BPTES-treatment (10 μM) together with bicalutamide (Bica, 10-40, μM) for 24 h. Cell viability was determined by MTT assay. Results are expressed as % of control. Error bars indicate mean ± S.E.M (n= 5). * p<0.05, *** p<0.001 when compared with the control. $$ p<0.01, $$$ p<0.001 when compared with bicalutamide (40 μM); # p<0.05 when compared with BPTES (10 μM).

BPTES (10 μM) and a concentration range of bicalutamide (10-40 μM). 40 μM bicalutamide significantly decreased the viability of LNCaP, DU145, PC3 cells (75 ± 3%, 69 ± 4% and 76 ± 2% of control, respectively, Fig. 6.5.). Also, 20 μM bicalutamide was effective in reducing the viability of DU145 cells (85 ± 2% of control, Fig. 6.5.). Furthermore, 10 μM BPTES plus 40 μM bicalutamide had a synergistic effect suppressing the viability of the androgen-sensitive LNCaP cells (54 ± 8% of control, Fig. 6.5.).

6.3.5. Glycolytic metabolism of PCa cells was affected by inhibition of glutaminase

Cancer cells display astonishing metabolic plasticity, reprogramming metabolic routes according to the environmental conditions and nutrients availability [8,39]. Also, the glycolytic metabolism of PCa cells has been shown to be modulated by the increased expression of glutaminase [19]. With this rationale, we investigated if glutaminase inhibition resulting from BPTES treatment would disturb the glycolytic metabolism of PCa cells (Fig. 6.6. and Fig. 6.7.).

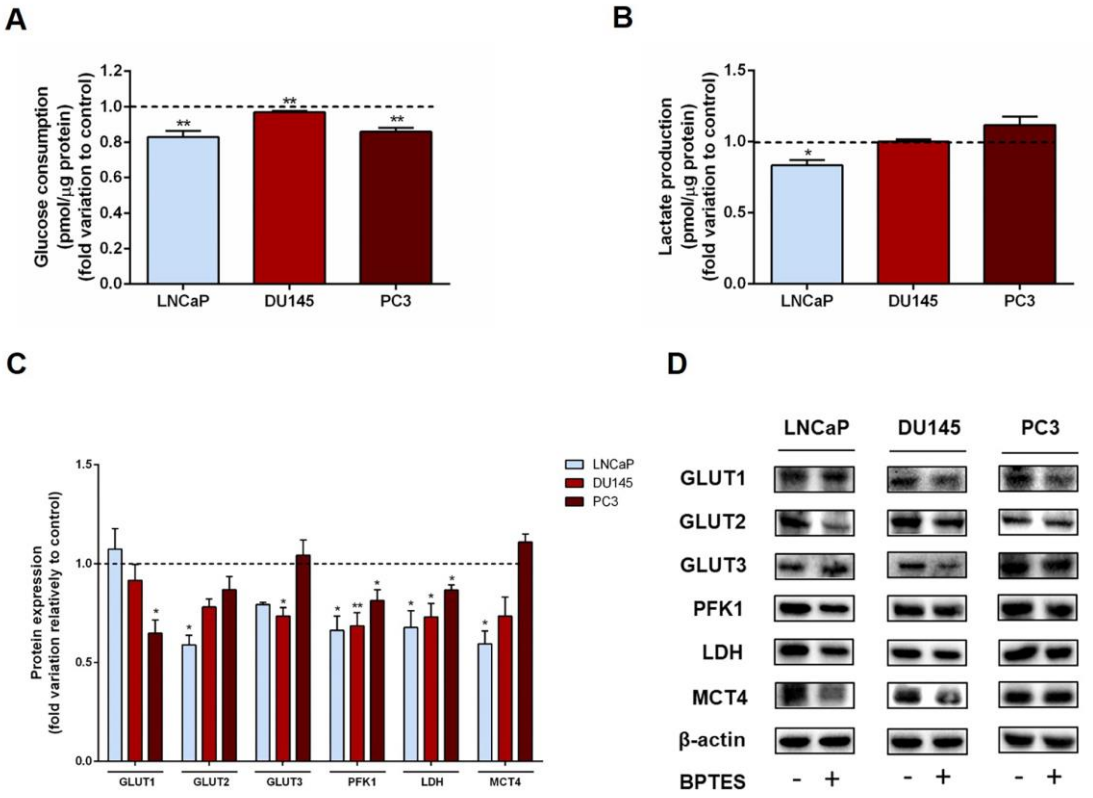

Figure 6.6. Glucose consumption (A), lactate production (B) and GLUT1 (C), GLUT2 (C), GLUT3 (C), PFK1 (C), LDH (C) and MCT4 (C) protein levels in androgen-sensitive (LNCaP), and CRPC (DU145 and PC3) cells treated with BPTES (10 µM). (A,B) Glucose consumption and lactate production were determined spectrophotometrically using commercial Kits. **(C)** Protein expression was determined by WB after normalization with ß-actin. d Representative immunoblots. Results are expressed as fold-variation relative to the control untreated group (0 nM BPTES, dashed line). Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$, when compared with control.

Glucose consumption and lactate production were determined spectrophotometrically, and the protein levels of key transporters and enzymes involved in glucose metabolism were evaluated by WB analysis (Fig. 6.6.). BPTES treatment decreased glucose consumption in all PCa cells (0.83 ± 0.04, 0.97 ± 0.01 and 0.86 ± 0.02 fold variation vs. control in LNCaP, DU145 and PC3, respectively, Fig. 6.6.A). This was accompanied by the decreased production of lactate in LNCaP cells (0.83 ± 0.04 fold variation vs. control, Fig. 6B), whereas no significant differences were observed in the other cell lines (Fig. 6.6.B).

Glucose consumption is first mediated by the uptake of glucose through the activity of GLUTs, namely, GLUT1, GLUT2 and GLUT3, which have been identified in PCa cells [40,11].

BPTES treatment differently affected GLUTs expression levels in PCa cell line models (Fig. 6.6.C and Fig. 6.6.D). GLUT1 expression was significantly decreased with BPTES treatment in PC3 cells (0.65 ± 0.07 fold variation vs. control, Fig. 6.6.C). Diminished expression of GLUT2 upon BPTES-treatment was observed in LNCaP cells (0.59 ± 0.05 fold variation vs. control, Fig. 6C). Concerning GLUT3, BPTES exposure diminished its expression only in DU145 cells (0.73 ± 0.04 fold variation vs. control, Fig. 6.6.C).

Considered one of rate-limiting steps in glycolysis, the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate by PFK1 is a crucial step in the glycolytic flux. BPTES-treatment significantly decreased PFK1 expression in all PCa cells (0.66 ± 0.07, 0.69 ± 0.07 and 0.81 ± 0.05 fold variation vs. control in PC3, DU145 and LNCaP cells, respectively, Fig. 6.6.C). Also, a decreased expression of LDH, the enzyme that converts the end-product of glycolysis pyruvate into lactate, was found in all PCa cell lines in response to BPTES (0.68 ± 0.08, 0.73 ± 0.07 and 0.87 ± 0.03 fold variation vs. control in PC3, DU145 and LNCaP cells, respectively, Fig. 6.6.C). The lactate produced is exported to the extracellular space by the activity of specific members of the MCTs family, namely MCT4 that has been associated with PCa progression and poor prognosis [41,42,11]. MCT4 expression was significantly decreased upon BPTES treatment in LNCaP cells (0.59 ± 0.07 fold variation vs. control, Fig 6.6.C).

The Seahorse XF Glycolytic Rate Assay was used to investigate the effect of BPTES in terms of the glycolytic metabolism of PCa cells (Fig. 6.7.). The contribution of glycolysis for extracellular acidification before and after mitochondrial inhibition was evaluated (Fig. 6.7.A). Pyruvate from glycolysis can be converted to lactate in the cytosol, or, alternatively, to CO_2 and water in the mitochondria. Both pathways result in the acidification of extracellular media by the extrusion of protons or by the reaction of CO_2 with water forming carbonic acid, which also produces protons (Fig. 6.7.A). Basal proton efflux rate (PER) represents the contribution of both pathways to the acidification of cell culture medium under normal culture conditions (Fig. 6.7.B). Basal PER was significantly decreased by BPTES treatment in LNCaP and PC3 cells (0.60 ± 0.04 and 0.83 ± 0.05 fold variation vs. control, respectively, Fig. 6.7.C) whereas remaining unaltered in DU145 (Fig. 6.7.C). Subtracting the contribution from the effect of CO_2-dependent acidification (mitochondrial-associated acidification) gives the contribution from glycolysis (Glyco PER, Fig. 6.7.B). BPTES-treatment decreased Glyco PER only in LNCaP cells (0.64 ± 0.05 fold variation vs. control, Fig. 6.7.C), which was correlated with the observed reduction of lactate extrusion rate (Fig. 6.6.B). The Compensatory Glycolysis (Fig. 6.7.B) indicates the maximum cell capability to use glycolysis after mitochondrial inhibition. Adding rotenone and antimycin A, inhibitors of mitochondrial electron transport chain Complex I and III, respectively, upon BPTES-treatment showed a significant diminution of Compensatory Glycolysis in all PCa cells (0.70 ± 0.04, 0.83 ± 0.04 and

0.71 ± 0.04 fold variation vs. control in DU145, LNCaP and PC3 cell, respectively, Fig. 6.7.C).

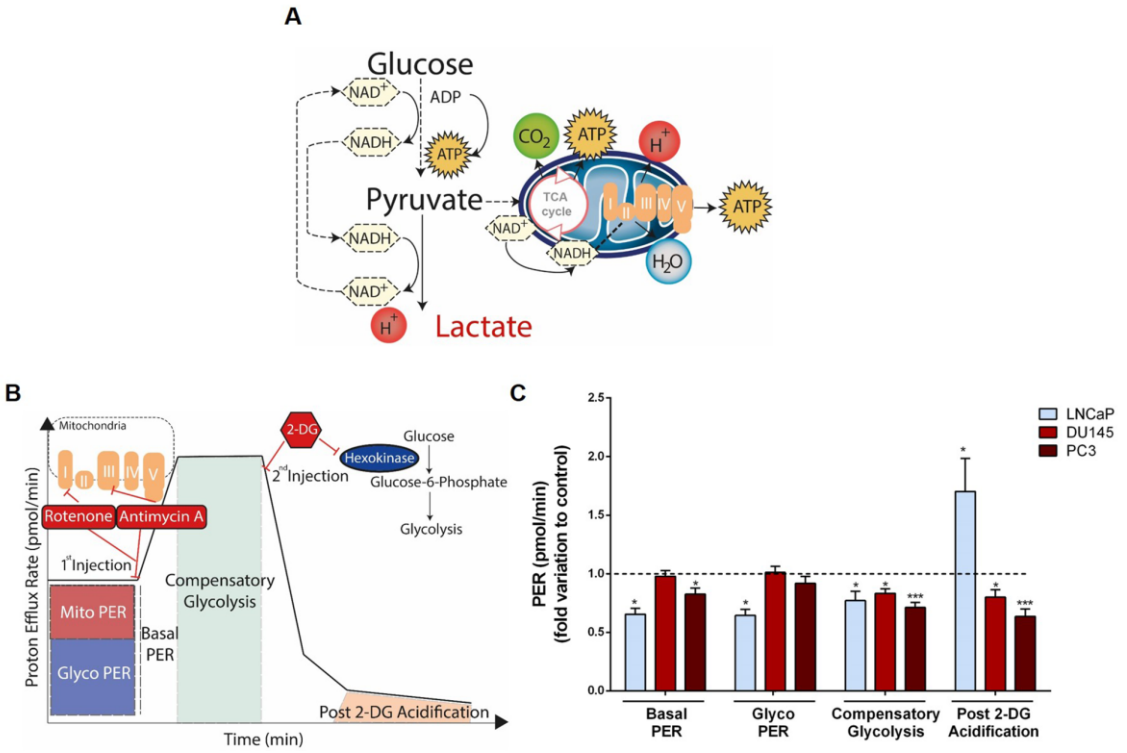

Figure 6.7. Effect of BPTES (10 μM) on glycolysis and extracellular acidification in androgen-sensitive (LNCaP), and CRPC (DU145 and PC3) cells. (A) Glycolysis and mitochondrial respiration as cell energy sources. Glucose is transformed into lactate during glycolysis, with the resulting protons from NADH conversion to NAD+ being extruded into the extracellular space. Mitochondrial activity (TCA cycle and oxidative phosphorylation) produces CO2 and water, and generates H+, which contributes to acidify the surrounding environment. **(B)** Schematic panel illustrating the experimental strategy. Proton efflux rate (PER) was obtained by the Seahorse XF96 Glycolytic Rate Assay. Basal PER represents the initial total PER. The use of rotenone and antimycin A (0.5 μM), inhibitors of mitochondrial complexes I and III, respectively, enabled calculating the mitochondrial-associated acidification (Mito PER). The Glycolytic PER (Glyco PER) resulted from subtracting Mito PER from Basal PER results. Inhibition of mitochondria drives cells compensatory changes to use glycolysis to meet energy demands (Compensatory Glycolysis). Secondly, glycolysis was inhibited by using the glucose analogue 2-deoxy-D-glucose (2-DG, 50 mM, Post-2-DG acidification). **(C)** Basal PER, Glycolytic PER, Compensatory Glycolysis and Post 2-DG Acidification in LNCaP, DU145 and PC3 BPTES-treated cells, Each experimental result was normalized to cell mass determined by the SRB assay. Results are expressed as fold-variation relative to the control untreated group (0 nM BPTES, dashed line). Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$, *** $p<0.001$ when compared with control.

The Post-2-DG acidification (Fig. 6.7.B), obtained after inhibition of glycolysis by 2-DG injection, induced a differential response in LNCaP and CRPC cells. BPTES-treatment significantly increased Post-2-DG acidification in LNCaP cells (1.70 ± 0.28 fold variation vs. control, Fig. 6.7.C), whereas the opposite effect was observed in DU145 and PC3 cells (0.80 ± 0.06 and 0.63 ± 0.06 fold variation vs. control, respectively, Fig. 6.7.C).

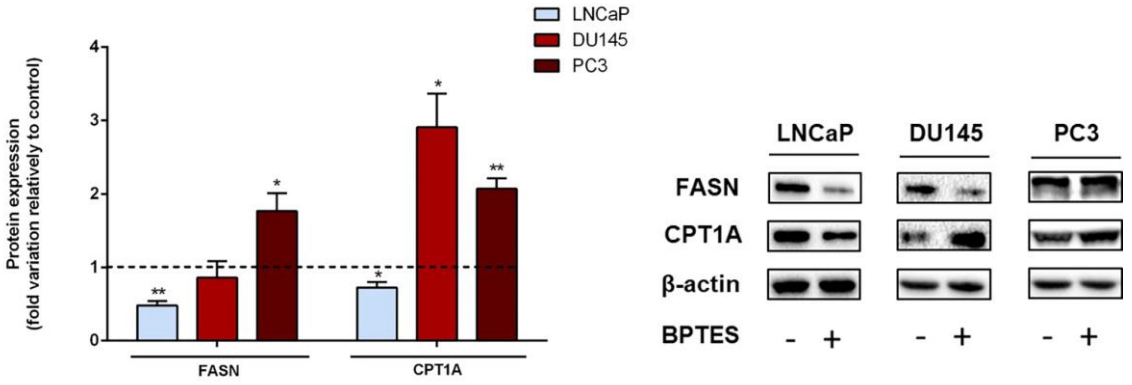

Figure 6.8. Effect of BPTES (10 μM) on enzymatic activity of ALT in androgen-sensitive (LNCaP), and CRPC (DU145 and PC3) cells. All results are expressed as fold-variation relative to the untreated control group (0 nM BPTES, dashed line). Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$ when compared with control.

Ultimately, extracellular acidification can occur through glucose- and glutamine-independent pathways, namely by the activity of aminotransferase enzymes. It is the case of ALT that catalyzes the reversible conversion of alanine into glutamate and pyruvate [43]. By measuring ALT activity, we found that BPTES-treatment significantly increased enzyme activity in LNCaP cells (2.60 ± 0.63 fold variation vs. control, Fig. 6.8.), whereas a diminution was observed in PC3 cells (0.44 ± 0.05 fold variation vs. control, Fig. 6.8.).

Glutamine and glutamate are the main suppliers of the anaplerotic reaction that produces citrate, which in turn, is the principal source for new lipid synthesis during cancer cell growth [44,45]. For this reason, the relationship between glutaminolysis and fatty acid metabolism has been reported [46-48]. Therefore, we evaluated the expression of FASN, a crucial enzyme in fatty acid synthesis, and CPT1A, a rate-limiting component in the carnitine-dependent transport of fatty acids across the inner mitochondrial membrane, upon glutaminase inhibition (Fig. 6.9.). Androgen-sensitive and CPRC cells displayed a different response to BPTES (Fig. 6.9.). FASN expression was significantly decreased in BPTES-treated LNCaP cells (0.48 ± 0.06 fold variation vs. control, Fig. 6.9.), but increased in PC3 cells upon BPTES treatment (1.77 ± 0.24 fold variation vs. control, Fig. 6.9.). Similarly, CPT1A expression was decreased in LNCaP cells (0.73 ± 0.077 fold variation vs. control, Fig. 6.9.) whereas increased in DU145 and PC3 (2.9 ± 0.46 and 2.10 ± 0.14 fold variation vs. control, respectively, Fig. 6.8.) upon exposure to BPTES.

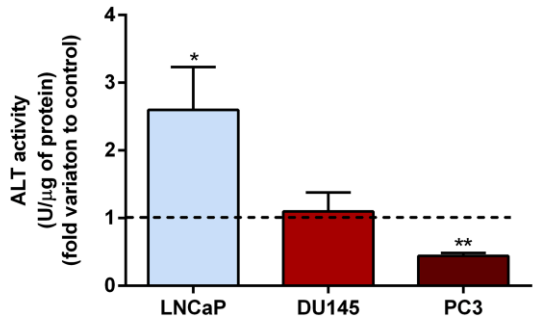

Figure 6.9. Effect of BPTES (10 µM) on the expression of FASN and CPT1A in androgen-sensitive (LNCaP), and CRPC (DU145 and PC3) cells. Protein expression was determined by WB after normalization with ß-actin. All results are expressed as fold-variation relative to the control untreated group (0 nM BPTES, dashed line). Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$ when compared with control. Representative immunoblots are shown in right panel.

6.4. Discussion

This study showed the importance of glutaminolysis in maintaining survival and migration ability of androgen-sensitive and CRPC cells, as well as the relevance of inhibiting this metabolic pathway for PCa cells response to the anti-androgen bicalutamide. Also, it demonstrated that glutaminase inhibition could have an impact on other metabolic pathways, namely, in the glycolytic metabolism.

We started by characterizing the expression of key regulators of the glutamine metabolism, the glutamine transporter ASCT2 and glutaminase (Fig. 6.1.A), in different cell lines models of PCa, namely, LNCaP (androgen-sensitive) and CRPC DU145 and PC3 cells. CRPC cells displayed a lower basal expression of ASCT2 compared with LNCaP (Fig. 6.1.B and Fig. 6.1.C). ASCT2 expression has been shown to be increased in PCa samples compared to the non-cancer tissues [49]. On the other hand, patients undergoing anti-androgen therapy displayed a significant decrease of prostate ASCT2 protein expression relative to untreated individuals [49], which supports the expression pattern we observed in androgen-sensitive and CRPC cell lines models.

In contrast, glutaminase expression was significantly higher in the more aggressive CRPC cells relative to LNCaP cells (Fig. 6.1.B and Fig. 6.1.C). Data in patients have shown the overexpression of glutaminase in PCa tissues compared with benign prostate hyperplasia or non-cancer tissues, which positively correlated with Gleason scores [20,19], also linking increased glutaminase expression with the aggressiveness of the disease. The higher expression of glutaminase in PCa cells relative to the normal prostatic epithelial cell line RWPE-1 also was previously reported [20]. Nevertheless, the present study is the first showing the differential glutaminase expression between castration-resistant and androgen-sensitive PCa cell line models, which is in accordance with patients' findings.

Androgens are widely recognized as the principal hormones responsible for maintaining prostate cells proliferation and survival [50,51]. These sex hormones also have been pointed out as important regulators of prostate cell metabolism, including the modulation of lipid metabolism and glycolysis [51,52,12,10,30]. However, the action androgens in the regulation of glutamine metabolism is much less known. Herein, we

analyzed the effect of DHT in regulating glutamine consumption, ASCT2 and glutaminase expression, and ASCT2 sub-cellular localization (Fig. 6.2). Our results showed that DHT-treatment enhanced glutamine consumption in LNCaP cells (Fig. 6.2.A), which was supported by the increased expression of both ASCT2 (Fig. 6.2.B and Fig. 6.2.D) and glutaminase (Fig. 6.2.C and Fig. 6.2.D). ASCT2 is the major molecular player mediating glutamine uptake, and, thus, its increased expression levels clearly support the enhanced glucose consumption observed. Indeed, other studies have shown the upregulation of ASCT2 expression in LNCaP and VCaP cells in response to androgens, [53,54], which was also accompanied by the increased uptake of glutamine [53].

The human amino acid transporter ASCT2 contains two N-glycosylation sites (N163 and N212) that, although not altering the activity of the protein, have been shown to increase its stability, and are responsible for the intracellular trafficking from the endoplasmic reticulum to theplasma membrane (Fig. 6.1.A) [31]. Aberrant glycosylation has been identified as a typical event in PCa and demonstrated to be a target of regulation by androgens [32,55-57]. These steroid hormones are also capable of altering the subcellular localization of several molecular targets [33-35]. Therefore, we sought to investigate whether exposure to DHT affects the intracellular distribution of ASCT2. Indeed, DHT-treatment significantly increased the co-localization of ASCT2 with the endoplasmic reticulum chaperone calnexin (Fig. 6.2.E and 6.2.F), which coordinates N-linked protein glycosylation, glycoprotein folding and quality control [36,58,59]. The endoplasmic reticulum is the major organelle involved in protein glycosylation and the central player in the distribution of proteins to the cell membrane through the endoplasmic reticulum - Golgi apparatus pathway, thus, the obtained findings support the role of androgens driving the traffic of ASCT2 from the endoplasmic reticulum to the plasma membrane. Future studies are needed to clarify the role of androgens regulating glycosylation and intracellular traffic of ASCT2, but herein we first demonstrated the co-localization of ASCT2 with the endoplasmic reticulum. Moreover, increased co-localization of ASCT2 with calnexin was concomitant with the augmented glutamine consumption in LNCaP cells (Fig. 6.2.A), supporting the increased density of the transporter molecules at the cell membrane.

Intracellular glutamine can be converted to glutamate through the activity of glutaminase (Fig. 6.1.A). Also in line with the observed augment in glucose uptake, DHT-treatment increased the expression of glutaminase in LNCaP cells (Fig. 6.2.C and Fig. 6.2.D). This observation is first in this PCa cell type and follows a previous study showing the action of androgens regulating glutaminase expression in other androgen-sensitive cells, the VCaP [53]. The obtained findings indicate that LNCaP cells are actively using glutamine as an energy substrate, which implicates glutaminolysis as a potential therapeutic target, which was further investigated by us.

Strikingly, stimulation of glutaminolysis by androgens in LNCaP cells *in vitro* (Fig. 6.2.) were also mimicked *in vivo* (Fig. 6.3.). ASCT2 and glutaminase expression was upregulated in the prostate of castrated rats receiving DHT supplementation (Fig. 6.3.), which supports a

direct stimulation of glutaminolysis by DHT in prostatic tissues *in vivo*. Altogether, these findings broaden the role of androgens as central regulators of glutamine metabolism in PCa cells both *in vitro* and *in vivo*.

Our next aim was to analyze the survival and metabolic response of PCa cells upon inhibition of glutaminolysis. BPTES, a recognized inhibitor of glutaminase [37], was used, and PCa cells fate was evaluated. BPTES exposure decreased the viability of all PCa cell lines studied (Fig. 6.4.A). However, CRPC and androgen-sensitive cells displayed a differential response to BPTES concentrations range. Low concentrations of glutaminase inhibitor (1-10 μM) effectively suppressed the viability of DU145 and PC3 cells, whereas LNCaP showed to be resistant to these BPTES concentrations. Only the highest (25 and 50 μM) concentrations of BPTES effectively diminished the viability of LNCaP cells (Fig. 6.4.A). Interestingly, this differential response to BPTES was correlated with the distinct glutaminase basal expression levels in PCa cell line models, which were higher in CRPC (DU145 and PC3) cells relative to LNCaP (Fig. 6.1.B). Altogether the obtained results also indicate that CRPC cells are more dependent on the utilization of glutamine as an energy source to support their growth than LNCaP. The inhibition of glutaminolysis by using enzyme inhibitors, such as BPTES or CB-839, or silencing glutaminase expression also has been shown to decrease the proliferation of several cancer cell types (lung, myeloma and breast) including the PC3 prostate cancer cells [19,60,26,21]. Moreover, the present findings follow a previous report showing that the cancer cell lines that more actively use glutamine, are the most sensitive to the inhibition of this metabolic pathway [61].

Glutaminase inhibition with BPTES also induced apoptosis of CRPC cells, DU145 and PC3 (Fig. 6.4.C), evidenced by the augmented caspase-3-like activity, a recognized end-point of apoptosis at the convergence of both intrinsic and extrinsic pathways [62,63]. The inhibition of glutaminase had no effect over LNCaP (Fig. 6.4.C) caspase-3-like activity, which is in line with the results of cell viability (Fig. 6.4.A). Nevertheless, the BTPES effects modulating PCa cells behavior were underpinned by the altered expression of target regulators of cell cycle and oncogenic pathways in all PCa cell lines (Fig. 6.4.D). The expression of the potent cyclin-dependent kinase inhibitor (CKI) p21, a cell cycle inhibitor functioning as tumor suppressor and an activator of apoptosis by cleavage of precursor caspase-3 [64,65], was significantly increased in LNCaP, DU145 and PC3 cells (Fig. 6.4.D). There are no other reports on the inhibition of glutaminase by compound 968 in PCa cells, but in ovarian cancer cells compound 968 also increased p21 expression, concomitantly with the reduced levels of CDK4 and cyclin D [66].

c-Myc is one of the oncogenes widely known to be overexpressed in PCa, being important for cancer progression and establishment of the CRPC phenotype [67-69]. Moreover, c-Myc has been shown to be involved in the metabolic reprogramming of PCa, specifically promoting the expression of glutaminase [70,18]. Noteworthy, c-Myc expression decreased in LNCaP and PC3 cells (Fig. 6.4.D) after treatment with BPTES. The mechanisms in the interplay of c-Myc expression upon glutaminase inhibition in PCa cells need to be clarified

in the future. However, BPTES inhibition in renal cell carcinoma stopped renal cancer progression driven by c-Myc [71].

Besides the effects on cell viability and caspase-3-like activity, this study showed that the inhibition of glutaminase also has the potential of decreasing the migration capability of PCa cells (Fig. 6.4.B), effects that were transversal to all cell lines. These findings follow similar reports in oral, lung and breast cancer cells, where glutaminase activity was shown to be related to the promotion of cancer cell migration [72-74].

Overall, our data revealed the effectiveness of the glutaminase inhibitor BPTES in decreasing viability, inducing apoptosis and suppressing the migration of CRPC cells, whereas showing only moderate effects in the hormone-sensitive LNCaP cells.

ADT remains the mainstream therapy for hormone-sensitive primary and metastatic PCa [75,6]. However, tumours that initially respond to therapy, inevitably acquire treatment resistance and progress to more aggressive stages, the castration resistance form of the disease [75,6]. It has been shown that metabolic reprogramming concurs to the establishment of the androgen independent phenotype and treatment resistance, which includes changes on glucose, lipid and glutamine metabolism [76,30,77]. The metabolic peculiarities of PCa cells and potential therapeutic advantage of such have been gaining increasing interest [8]. In fact, supporting *in vitro* and *in vivo* evidence exist showing that the combination of ADT with, for example, blockage of lipid or cholesterol metabolism, had a positive effect suppressing cancer cells growth and overcoming resistance to ADT [76,78,79]. With this rationale, we hypothesized that inhibiting glutaminolysis (BPTES treatment) would sensitize PCa cells to the effect of the anti-androgen bicalutamide. In accordance with our hypothesis, the concomitant administration of BPTES and bicalutamide showed a synergistic effect diminishing the viability of the androgen-sensitive LNCaP cells (Fig. 6.5.A). It is well established that anti-androgens treatment has associated adverse side-effects such as gynecomastia, breast pain, fatigue, and decreased libido [80,81]. The present findings suggest that the possible use of bicalutamide, or other anti-androgens, together with BPTES would allow using lower doses of the anti-androgen minimizing its adverse effects. Also, they open interesting perspectives for the exploitation of these results in future preclinical and clinical studies.

Cancer cell metabolism is a complex mixture of diverse and intricate processes that depend on a variety of nutrients fuelling the TCA cycle. Previous reports have shown that a relationship exists between glucose and glutamine handling, and that glutamine availability can change glucose uptake and metabolism [82,19]. Glutamine is converted into glutamate by the activity of glutaminase (Fig. 6.1.A) and subsequently to α-ketoglutarate. It has been shown that α-ketoglutarate itself could directly regulate glucose uptake [83,84]. Also, a metabolic crosstalk exists between glucose and glutamine, and their intermediate metabolites. Pyruvate, the end product of glycolysis, can also be obtained from glutamine by the activity of ALT, which catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate forming pyruvate and glutamate [85].

Herein, we analysed how inhibition of glutaminolysis re-shapes the glycolytic metabolism of PCa cells (Fig. 6.6 and Fig. 6.7). Suppression of glutaminase by BPTES treatment significantly reduced glucose consumption in all PCa cells (Fig. 6.6.A). Similar effects were described in PC3 cells upon siRNA silencing of glutaminase or BPTES administration, being observed a decrease in glucose uptake [19].

Glucose consumption starts with the uptake from the extracellular medium ensured by the activity of GLUTs family members, namely GLUT1, GLUT2 and GLUT3 in PCa cells [86,87]. BPTES-treatment decreased glucose consumption in all PCa cell line models, which was supported by the diminished expression of GLUTs. However, a differential response was observed concerning specific GLUTs family members. Treatment with BPTES decreased GLUT1, GLUT2 or GLUT3, respectively, in PC3, LNCaP, and DU145 cells (Fig. 6.6.C). The distinct utilization of GLUTs among PCa cell lines was also found in another study [88].

A crucial step in the glycolytic process is the irreversible conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, a reaction catalysed by PFK1. Diminished expression of PFK1 (Fig. 6.6.C) was found in BPTES-treated PCa cells, which indicates that besides the reduced glucose consumption, the rate of glycolytic flux is lower upon glutaminase inhibition. The possible correlation between PFK1 activity and glutamine metabolism in PCa cells is unknown for the moment. However, in Hela cells both glucose and glutamine were required for cell cycle progression, which was also related with the decreased activity of APC/C-Cdh1 ubiquitin ligase leading to the accumulation of glutaminase and glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3 [89].

The last step in the glycolytic metabolism is the conversion of pyruvate to lactate through the activity of LDH, which is followed by the export of lactate to the extracellular space. In BPTES-treated LNCaP cells, the reduced glucose consumption observed was accompanied by decreased export of lactate (Fig. 6.6.B), supported by the decreased expression of LDH and MCT4 (Fig. 6.6.C). Information in PCa is very limited or non-existent, but the inhibition of glutaminase by Compound 968 in ovarian cancer cells also showed to decrease lactate export [90]. Similar findings were also found in colorectal cancer cells, with glutaminase inhibition suppressing glucose uptake and lactate export [91].

The results obtained with the Seahorse XF Glycolytic Rate Assay (Fig. 6.7.) also confirmed the effect of BPTES altering the glycolytic metabolism of PCa cells. BPTES-treatment decreased the basal efflux of protons (Basal PER) in LNCaP and PC3 cells (Fig. 6.7.B and Fig. 6.7.C), which in the case of LNCaP cells seems to result from the direct contribution from glycolysis (Glyco PER) (Fig. 6.7.B and 6.7.C). The Glyco PER parameter is directly correlated with the export of lactate, and strictly follows the decreased lactate production observed in LNCaP cells after BPTES treatment (Fig. 6.6.B). Interestingly, when oxidative phosphorylation was inhibited none of the PCa cell lines had the capability to compensate this phenomenon (Fig. 6.7.C) by using glycolysis (Compensatory Glycolysis, Fig. 6.7.B).

These results demonstrated the relevance of glutaminase activity influencing the glycolytic pathway. Also, they pointed glutaminase as a metabolic checkpoint in the interplay of glutamine and glucose metabolism, with impact on cell survival and growth, and migration. When inhibiting glutaminolysis and oxidative phosphorylation, the addition of 2-DG stopped the glycolytic pathway, disabling PCa cells of using any of these metabolic pathways. CRPC cells treated with BPTES presented lower levels of acidification attributed to other sources than glycolysis or mitochondrial TCA activity (Fig. 6.7.C). Interestingly, LNCaP cells, the PCa cell line more resistant to BPTES treatment (Fig. 6.5.), presented increased levels of extracellular acidification after inhibition of glutaminolysis, mitochondrial activity and glycolysis (Fig. 6.7.C). This indicates that these cells are capable of using diverse and alternative energy sources. In this sense, we evaluated the activity of ALT in response to glutaminase inhibition, as this enzyme is placed in the interplay of glycolysis and glutaminolysis, by its capability of generating both pyruvate and glutamate. Interestingly, BPTES-treated LNCaP cells displayed increased ALT activity, whereas an enzyme diminished activity was observed in PC3 cells (Fig. 6.8.). Similar findings were described in HeLa cells, with ALT expression being induced by glutamine deprivation or glutaminase inhibition, which was shown to sustain the TCA cycle by the conversion of glutamate to α-ketoglutarate [43]. The compensatory response of cancer cells upon inhibition of glutaminolysis was also demonstrated in pancreatic cells, which displayed increased ALT and production of glutamate, as well as no decrease on α-ketoglutarate levels indicating the reactivation of alternative sources of carbon [92]. Therefore, it is possible that the source for the extracellular acidification in LNCaP cells after inhibition of glutaminolysis, mitochondrial activity and glycolysis, may come from the reactivation of alternative carbon sources likely by ALT activity (Fig. 6.6.C).

Cancer cell growth is intimately associated with lipid synthesis, which is continuously sustained by the supply of citrate. Several pieces of evidence have shown that cancer cells can generate citrate for fatty acid synthesis from the reductive metabolism of glutamine [44,45]. Glutaminase inhibition decreased FASN expression in LNCaP cells, whereas augmented in PC3 cells (Fig. 6.9.). Moreover, CPT1A expression was diminished in LNCaP cells and increased in CRPC cells (Fig. 6.9.). These results indicate that CRPC cells have the capability to augment fatty acid synthesis and lipid oxidation in response to glutaminase inhibition. Accelerated lipid catabolism under inhibited glutaminolysis was also described in breast cancer cells and related with activation of survival mechanisms [93]. Moreover, a previous study reported that the inhibition of ASCT2 decreased fatty acid synthesis in LNCaP cells, but not in PC3 cells [94], which suggests that LNCaP cells, contrarily to PC3, are directly using glutamine for fatty acid synthesis.

Altogether, the results obtained herein indicated a pivotal role for glutamine in shaping PCa cells metabolism, namely glycolysis and lipid handling. Despite this adaptation, inhibition of glutaminolysis with BPTES significantly altered PCa cells fate. Nevertheless,

these findings show that it is crucial deeply understanding the metabolic response of cancer cells when specific metabolic pathways are targeted for therapy.

In conclusion, androgens potentiated glutamine metabolism in PCa cells, whereas the inhibition of glutaminase activity diminished cell viability and migration, and increased apoptosis, particularly in the CRPC (Fig. 6.10.). These outcomes sound quite exciting considering targeting glutaminase CRPC therapy. Using molecular and metabolic extracellular flux analysis, this study also provided evidence that the suppression of glutaminolysis in PCa cells has impact on glycolysis and lipid metabolism (Fig. 6.10.). Moreover, it was first shown that the inhibition of glutamine metabolism in combination with androgen blockade had a synergic effect suppressing the viability of LNCaP cells. The obtained results indicate that co-targeting of the AR axis and glutamine metabolism may provide additional benefit for treatment of androgen-sensitive PCa. However, the present findings also highlight the importance of fully understand cancer metabolic remodelling whenever specific metabolic routes are inhibited with therapeutic purposes.

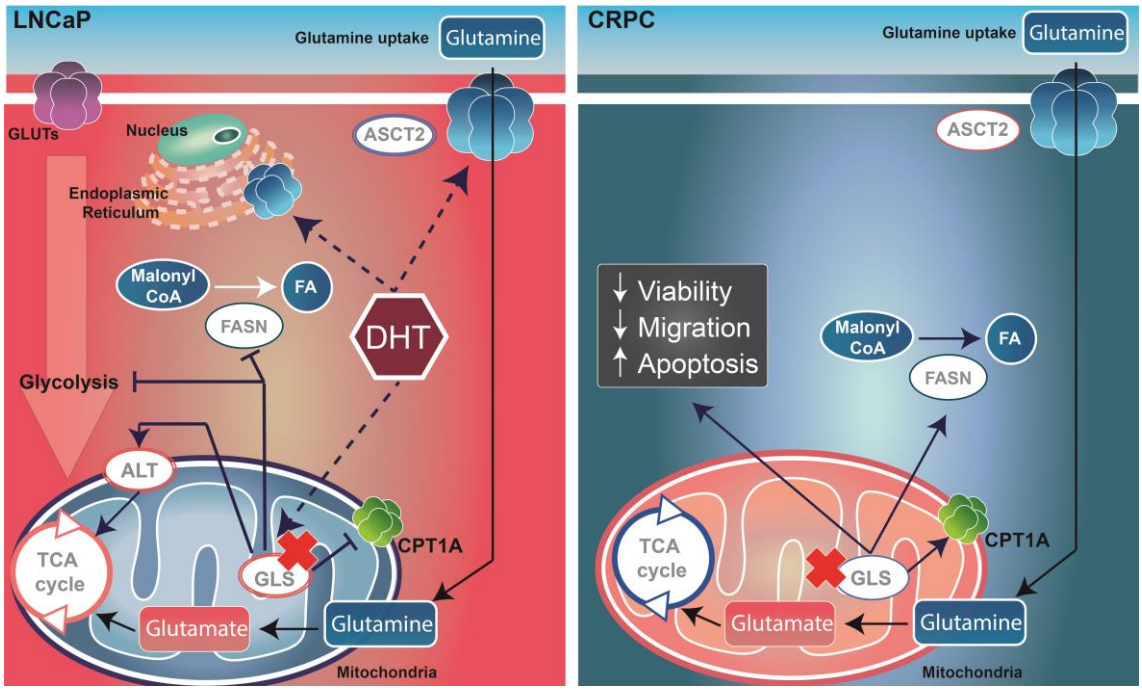

Figure 6.10. Glutaminolysis is an essential metabolic route in androgen-sensitive (LNCaP) and CRPC cells. DHT stimulates glutamine uptake, and the expression of ASCT2 and glutaminase (GLS) in LNCaP cells. DHT also triggered ASCT2 localization from the endoplasmic reticulum to the cell membrane. GLS inhibition suppressed glycolysis, and the expression of FASN (catalyses the conversion of malonyl CoA to fatty acids (FA)) and CPT1A in LNCaP cells. In contrast, FASN and CPT1A expression levels increased in CRPC cells. Augmented activity of ALT was found in LNCaP cells upon blockage of glutaminolysis. GLS inhibition decreased the viability and migration of CRPC, whereas augmenting apoptosis.

6.5. Acknowledgments

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalization in Axis I -Strengthening research, technological development and innovation (Projects No. 007491 and 029114) and National Funds by FCT-Foundation for Science and Technology (Project UID/Multi/00709/2013).

Cardoso H. J. and Figueira M.I. were recipient of FCT fellowships SFRH/BD/111351/2015 and SFRH/BD/104671/2014, respectively. P.A.M. is funded by FCT Investigator contract from the Foundation for Science and Technology (FCT), Portugal (Ref: IF/00614/2014) and FCT exploratory grant, Ref: IF/00614/2014/CP12340006. CBMR is financed by FCT Research Center Grant

6.6. Conflict of interest

The author declares that there is no competing interest.

6.7. References

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Chapter 7

The interplay between androgens and LDL-cholesterol modulating prostate cancer cells fate: enhanced proliferation and migration, and altered lipid metabolism

This Chapter was submitted for publication:

Cardoso HJ, Figueira MI, Carvalho TM, Vaz CV, Madureira PA, Socorro S. The interplay between androgens and LDL-cholesterol modulating prostate cancer cells fate: enhanced proliferation and migration, and altered lipid metabolism. Submitted to Andrology. 2019

Abstract

Androgens are the central stimulators of prostate cancer (PCa) cells growth and important metabolic regulators, stimulating lipid uptake, and *de novo* fatty acid synthesis. However, if exogenous lipid availability can influence the androgens actions in regulating lipid metabolism in PCa is unknown. On the other hand, the direct effect of LDL-supplementation modifying PCa cell fate remains underexplored. Also, it is unknown if the hormonal environment and the presence of androgens may alter the response of PCa cells to LDL-cholesterol. The present study investigated the effect of 5α-dihydrotestosterone (DHT) in regulating lipid metabolism, and its influence and that of LDL-cholesterol in modulating PCa cells viability, proliferation and migration. DHT upregulated the expression of fatty acid synthase (FASN) and carnitine palmitoyltransferase 1A (CPT1A) in androgen-sensitive PCa cells. The DHT effect stimulating FASN expression was maintained in lipid-depleted conditions and inhibited by fatostatin, which indicates the involvement of the transcription factor SREBP-1. LDL supplementation suppressed FASN expression regardless of the presence of DHT, whereas augmenting CPT1A levels. LDL-cholesterol enrichment increased PCa cells viability, proliferation, and migration dependently on DHT. Moreover, LDL and DHT co-administration increased the lipid droplets contents in PCa cells. Inhibition of LDL receptor or CPT1A abrogated the LDL effects promoting the viability of PCa cells. Overall, these findings showed that androgens deregulate lipid metabolism and enhance the effects of high-LDL availability, increasing PCa cells viability, proliferation and migration. This *in vitro* approach supports clinical and epidemiological data linking obesity with PCa, and first implicated androgens in this relationship.

7.1. Introduction

Androgens are widely recognized as the central stimulators of prostate cancer (PCa) cells survival and growth [1-3], exerting their actions through the androgen receptor (AR), a member of the steroid hormone receptor subfamily of ligand-activated nuclear transcription factors [4-6]. Therefore, androgen deprivation therapy (ADT), suppressing androgens levels and the AR-mediated effects, remains a standard for treatment of men with local high-risk and metastatic PCa [7, 8]. However, the disease progresses to a stage where tumor growth occurs despite the maintenance of reduced serum androgen levels; it is the so-called castration-resistant prostate cancer (CRPC). Advances in the understanding of mechanisms that contribute to the establishment of CRPC and the identification of androgens/AR actions and their molecular connections are of paramount relevance for the development of new therapeutic strategies for advanced PCa.

In the last years, we and others have described the role of androgens as metabolic regulators in PCa, modulating glycolysis, nucleotide and amino acid metabolism and cell lipid handling [5, 9-11]. Indeed, lipid metabolism, which is crucial for energy production and

membrane synthesis in PCa cells [12-14], is one of the main targets of the metabolic actions of androgens [15]. Androgens have been shown to stimulate lipid uptake, synthesis, storage and lipolysis from lipid droplets [16-18]. These outcomes are achieved by the regulation of expression and activity of several molecular targets. However, how androgens control lipid metabolism in prostate cells is not entirely understood, and there are several molecular partners involved in lipid handling that remain to be identified as androgens targets. Also, it is unknown if exogenous lipid availability would influence the effect of androgens regulating lipid metabolism.

On the other hand, clinical and epidemiological studies have been establishing a relationship between obesity and PCa, mainly by the fact that obese PCa patients develop aggressive forms of disease with accelerated progression to CRPC, poor prognosis and reduced survival rates [19-23]. Also, experimental *in vitro* and *in vivo* studies showed that diet-induced hypercholesterolemia induces PCa metastasis [24, 25]. However, the direct influence of exogenous LDL supplementation modifying PCa cell fate remains unknown, though this has been demonstrated in other types of cancer, namely, breast cancer and oesophageal squamous cell carcinoma [26-28]. Moreover, it is entirely unknown if the hormonal milieu driving cancer and the presence of androgens as lipid metabolism modulators may have a role altering PCa cells response to LDL-cholesterol.

The present study aimed to elucidate further the effect of androgens (5α-dihydrotestosterone, DHT) in regulating lipid metabolism, and the influence of these hormones and LDL-cholesterol modulating PCa cells viability, proliferation and migration. The expression of target regulators of lipid metabolism and lipid content in PCa cells under different LDL-cholesterol availability with or without DHT were also assessed.

7.2. Materials and methods

7.2.1. Chemicals

All chemicals, culture media, and antibodies unless otherwise stated were purchased from Sigma–Aldrich (St Louis, MO, USA).

7.2.2. Cell lines and treatments

Human prostate cell lines (PNT1A, LNCaP, DU145, and PC3) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). PNT1A are non-tumorigenic prostate epithelial cells. LNCaP cells, originated from a PCa lymph node metastasis, express the AR and are an androgen-sensitive model. DU145 and PC3 cell lines have origin in brain and bone metastasis of an undifferentiated grade IV prostate adenocarcinomas, and are considered to be non-sensitive to androgens [29, 30].

PNT1A, LNCaP, DU145, and PC3 cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/ streptomycin at 37 ºC in

an atmosphere equilibrated with 5 % CO_2. At 60% confluence, culture medium was replaced by phenol red-free RPMI1640 medium (R8755) containing 1% lipid-depleted FBS (LD-FBS, Biowest, Riverside, MO, USA) or 5% charcoal-stripped FBS depending on cells will be treated with lipids or androgens. Cells were maintained for additional 24 h and, then, exposed to 10 nM DHT or vehicle for 12, 24, 48 h. Alternatively, cells were exposed to Low density Lipoproteins (LDL) (100 µg/ml, Merck, Darmstadt, Germany) in the presence or absence of DHT. LDL-exposure experiments were repeated in LNCaP cells in the presence of 40 µM Etomoxir or human anti-LDLr antibody (5 µg/ml, AF2148, R&D Systems, Minneapolis, MN, USA) for 24 h and 48 h. Antibody was added to culture medium 1 h before LDL-stimulation. DHT-treated LNCaP cells in LD-FBS conditions were simultaneously cultured with 20 µM fatostatin. At different experimental conditions, cells were used for cell viability and migration assays, Ki-67 immunocytochemistry, and determination of lipid content, or, alternatively, harvested for protein extraction.

7.2.3. Cell Viability Assay

LNCaP (20.000 cells/well) and PC3 (5000 cells/well) cells were grown in 96-well plates, and cell viability was determined by the colorimetric MTT assay. After LDL, DHT and/or inhibitors stimulation, culture medium was removed and incubated with MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) at a final concentration of 0,5 mg/ml, in the dark for 4 h at 37 ºC. After incubation, MTT solution was carefully removed, and the formed formazan crystals were solubilized with 100 µL DMSO. The absorbance of the resultant purple coloured solution was measured at 570 nm using the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA). The value of absorbance is directly proportional to the number of viable cells in each experimental group.

7.2.4. Ki-67 immunocytochemistry

LNCaP cells (2.5 x10^5/well) were fixed with 4% PFA and permeabilized with 1 % Triton X-100 for 5 min at room temperature. Nonspecific staining was blocked by incubation with PBS containing 0.1% (w/v) Tween-20 (PBS-T) and 20% FBS for 1 h. After blocking, cells were incubated with the rabbit monoclonal anti-Ki-67 antibody (1:50, ab16667, Abcam, Cambridge, United Kingdom) for 1 h at room temperature. Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, Darmstadt, Germany) was used as secondary antibody. Specificity of staining was assessed by omission of the primary antibody. Cell nuclei were stained with Hoechst 33342 (5 µg/mL, Invitrogen) for 10 min. Lamellae were washed with PBS-T and mounted with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Images were acquired using the Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany). Proliferation was determined by the percentage of Ki-67-positive cells out of the total

number of Hoechst stained nuclei in 10 randomly selected ×40 magnification fields in each lamellae.

7.2.5. Migration Assay

Cell migration assay was performed using 8 μM pore size inserts (35224, SPL, Life Sciences, Naechon-Myeon Pocheon, South Korea). Briefly, LNCaP cells (3.0 $x10^5$ cells/well), in the presence or absence of LDL and DHT, were placed into the upper chambers in serum-free media. The lower chambers contained 20% LD-FBS. After 24 h, cells on the lower surface of membrane were fixed and stained with haematoxylin. Then, cells were counted in 10, randomly selected, ×40 magnification fields in each membrane.

7.2.6. Oil Red O Assay

LNCaP cells (5.0 $x10^5$ cells/well) seeded in 12-well plates and exposed to LDL (100 μg/ml) and DHT (10 nM) for 24 h, were then fixed with 4% PFA for 30 min. Cells were washed twice with distilled waster and rinsed with 60% isopropanol for 5 min. After washing, cells were stained with Oil Red O for 20 min. Representative microscope images were acquired. Oil Red O (lipid content) quantification was carried out by diluting dye with 100 % isopropanol for 5 min with gentle agitation. Absorbance was measured using xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad) at 492 nm.

7.2.7. Western Blot (WB)

Human prostate cells were homogenized in the appropriate volume of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail and 10 % PMSF , kept on ice for 20 min with occasional mixing, and then centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Total proteins were resolved by SDS-PAGE on 7.5 or 10% gels and electrotransferred to a PVDF membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with rabbit anti-CD36 (1:400, ab64014; Abcam), rabbit anti-fatty acid synthase (FASN, 1:1000, no.3180; Cell Signaling Technology, Danvers, MA, USA) , mouse anti-Carnitine palmitoyltransferase 1A (CPT1A, 1:1000, ab128568; Abcam), rabbit anti-AKT (1:1000, no.9272; Cell Signaling Technology), rabbit anti-phospho-AKT (1:1000, no.9271; Cell Signaling Technology), rabbit anti-p44/42 MAPK (Erk1/2) (1:1000, no.9102; Cell Signaling Technology), rabbit anti-phospho-p44/42 MAPK (Erk1/2) (1:1000, no.9101; Cell Signaling Technology), rabbit anti-phospho-c-Myc (1:1000, no.13748; Cell Signaling Technology), or mouse anti-E-cadherin (1:1000, sc-8426, Santa Cruz Biotecnhnology, Heidelberg, Germany) primary antibodies. Then, membranes were washed and incubated 1 h at room temperature

with the anti-rabbit IgG, HRP-linked (1:20000, no.7074; Cell Signaling Technology) or anti-mouse-IgGκ HRP-linked (1:20000, sc-516102, Santa Cruz Biotecnhnology) secondary antibodies. Protein expression was normalized using a mouse anti-ß-actin (1:1000, A5441) antibody. Membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and immunoreactive proteins were visualized with the ChemiDoc™ MP System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab software (Bio-Rad) and normalized by division with the respective ß-actin band density.

7.2.8. Statistical Analysis

The statistical significance of differences between experimental groups was evaluated by unpaired t -test with Welch's correction, or one-way ANOVA followed by Tukey post-hoc test, using GraphPad Prism v6.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean (S.E.M).

7.3. Results

7.3.1. Fatty acid metabolism regulators are differential expressed in PCa cells and regulated by androgens

The protein expression of fatty acid metabolism regulators (Fig. 7.1.A) in neoplastic and non-neoplastic prostate cell lines was evaluated by WB analysis (Fig. 7.1.B). The expression of the fatty acid transporter, CD36, was significantly lower in CRPC cell lines (DU145, PC3) relatively to the non-neoplastic PNT1A cells (0.66 ± 0.04 and 0.53 ± 0.07 fold variation, respectively, Fig. 7.1B), as well as in PC3 cells relatively to LNCaP cells (Fig. 7.1B). On the other hand, the expression of FASN (Fig. 7.1.A), an enzyme crucial to fatty acid synthesis, was significantly higher in all PCa cells relatively to PNT1A cells (2.76 ± 0.10, 1.43 ± 0.13 and 2.15 ± 0.11 fold variation for LNCaP, DU145 and PC3, respectively, Fig. 7.1.B). Among PCa cell lines, DU145 cells displayed significantly lower expression of FASN when compared to LNCaP or PC3 (Fig. 7.1.B); and LNCaP cells showed the highest FASN expression levels (Fig. 7.1.B).

CPT1A is a rate-limiting component in the carnitine-dependent transport of fatty acids across the inner mitochondrial membrane (Fig. 7.1.A), catalysing an important step in β-oxidation. CPT1 expression was significantly higher in LNCaP and PC3 cells (respectively, 2.39 ± 0.17 and 3.22 ± 0.31 fold variation relatively to PNT1A, Fig. 7.1.B), whereas DU145 cells displayed the lowest CPT1A expression among all PCa cell lines (0.69 ± 0.07 fold variation relatively to PNT1A, Fig. 7.1.B).

Androgens have been shown to play a role as metabolic regulators fuelling PCa cells growth and proliferation [5, 9, 11]. In this work, we evaluated the effect of DHT (10 nM) regulating the expression of CD36 (Fig. 7.2.A), FASN (Fig. 7.2.B) and CPT1A (Fig 7.2.C). DHT-treatment significantly decreased the protein expression of CD36 in PTN1A cells for 24 h of

treatment (0.2955 ± 0.05 fold variation to control, Fig. 7.2.A) and in LNCaP cells for 12 h, 24 h and 48 h (respectively, 0.72 ± 0.09, 0.63 ± 0.04 and 0.86 ± 0.02 fold variation to control, Fig. 7.2.A).

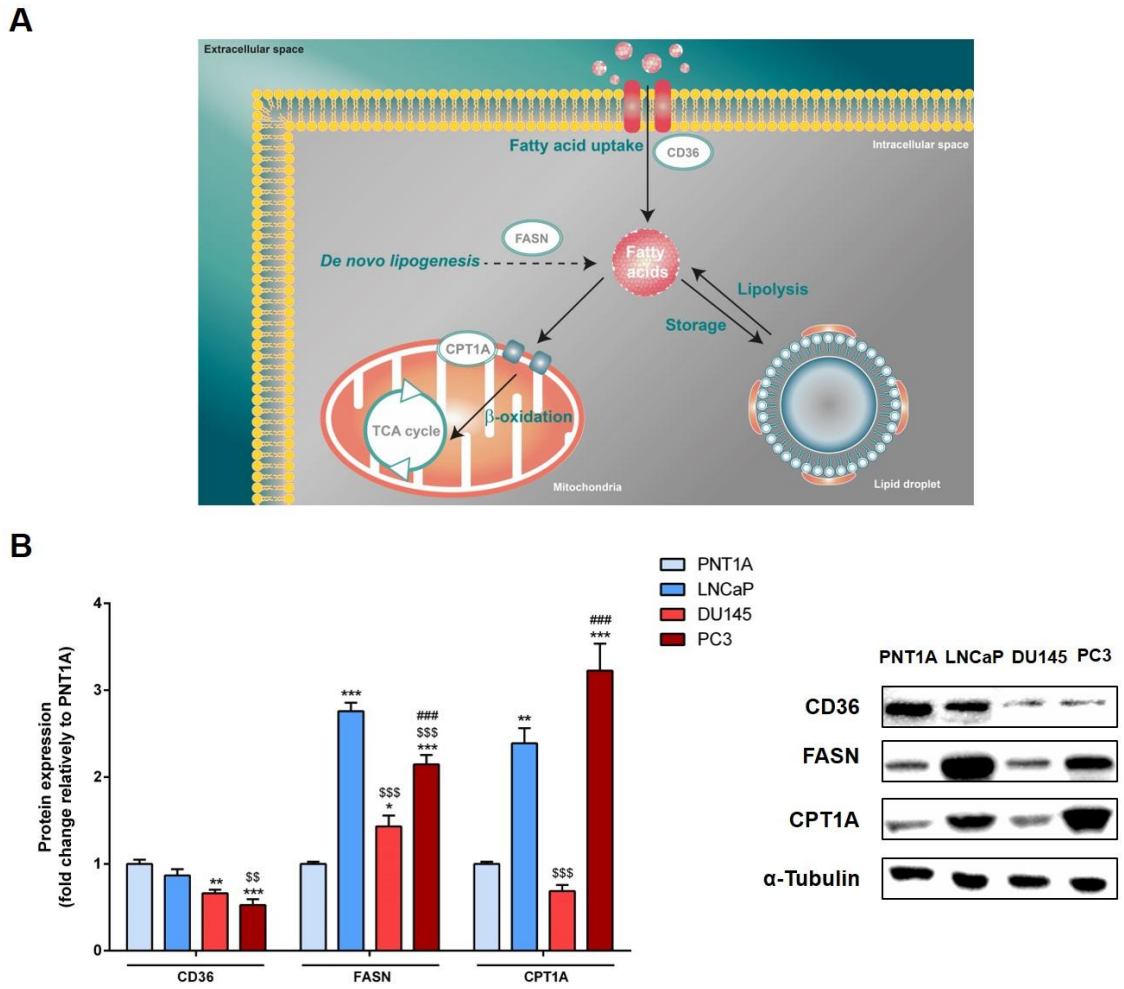

Figure 7.1. Lipid metabolism regulators, CD36, FASN and CPT1A, in PCa cell line models, LNCaP, DU145 and PC3 cells (A) Fatty acid uptake occurs through the membrane transporter CD36. In the intracellular space, fatty acids can be guided to ß-oxidation with the participation of CPT1A, an enzyme at outer mitochondrial membrane that allows the transport of fatty acid to mitochondrial matrix, which is a rate-limiting step in ß-oxidation. The products of lipid oxidation enter the tricarboxylic acid (TCA) cycle generating ATP. Alternatively, incorporated fatty acids can be stored in lipid droplets, being recruited by lipolysis when more energy substrates are needed. Cancer cells also have been shown to have the ability of de novo synthesise fatty acids, which depends on the activity of FASN. **(B)** Protein expression determined by WB analysis after normalization with α-tubulin. All results are expressed as fold-variation relative to the non-neoplastic PNT1A prostate cells. Error bars indicate mean ± S.E.M (n= 5) * p<0.05, ** p<0.01, *** p<0.001 when compared to PNT1A cells; $$ p<0.01; $$$ p<0.001 when compared to LNCaP; ### p<0.001 when compared to DU145 cells. Representative immunoblots are shown in the right panel.

On the other hand, DHT induced a significant increase on the expression of FASN in LNCaP cells treated for 24 h and 48 h (respectively, 1.37 ± 0.09 and 1.27 ± 0.09 fold variation to control, Fig. 7.2.B). CPT1A expression was significantly increased in PNT1A cells treated with DHT for 24 h and 48 h (respectively, 2.44 ± 0.55 and 1.59 ± 0.20 fold variation to control, Fig. 7.2.C), as well as, in LNCaP-treated cells for 24 h (1.63 ± 0.12 fold variation to control, Fig. 7.2.C).

Overall, no effect was seen on the expression of fatty acid metabolism regulators, CD36 (Fig. 2A), FASN (Fig. 7.2.B), and CPT1A (Fig. 7.2.C) in CRPC cells, DU145 and PC3, in response to DHT.

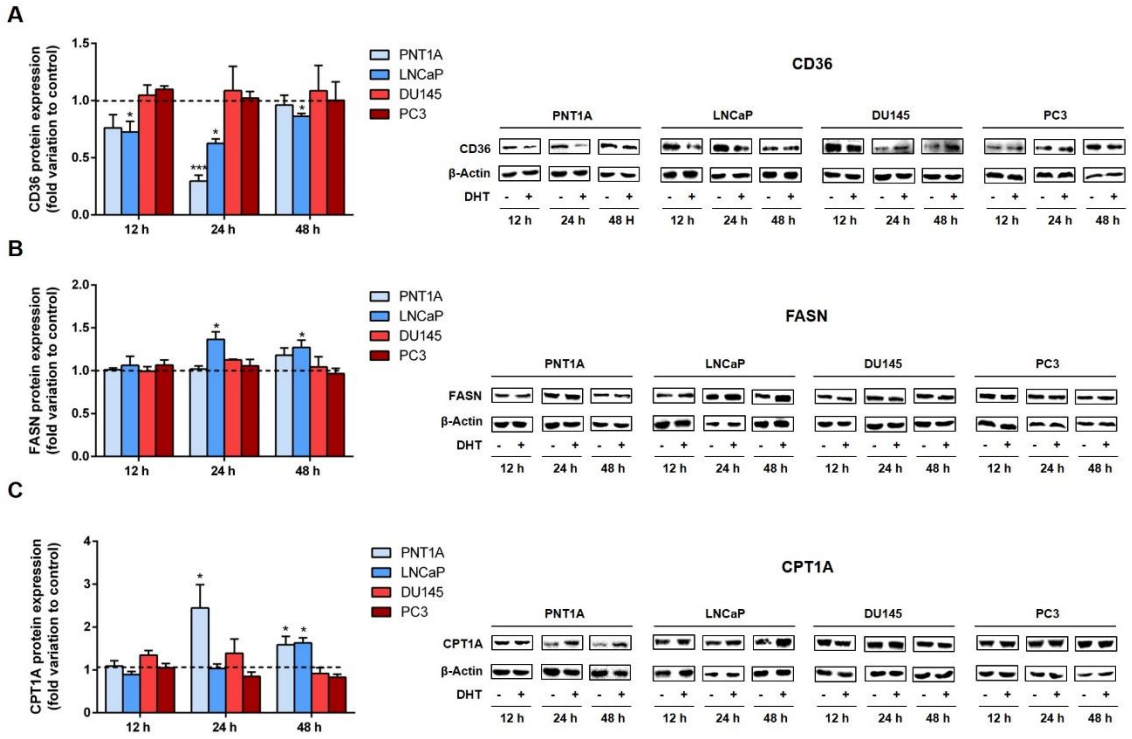

Figure 7.2. Effect of DHT (10 nM) on the expression of CD36 (A), FASN (B) and CPT1A (C) in neoplastic (LNCaP, DU145 and PC3) and non-neoplastic (PNT1A) prostate cells treated for 12, 24, or 48 h. Protein expression was analysed by WB after normalization with ß-actin. All results are expressed as fold-variation relative to the control untreated group (0 nM DHT, dashed line). Error bars indicate mean ± S.E.M (n= 5) * $p<0.05$, *** $p<0.001$ when compared to control. Representative immunoblots are shown in the right panels.

7.3.2. LDL-cholesterol increased PCa cells viability, proliferation and migration dependently on DHT

The effect of LDL-supplementation, alone or in combination with DHT, on PCa cells viability, proliferation and migration was investigated. LDL-stimulation significantly increased the viability of LNCaP and PC3 cells both for 24 h and 48 h of treatment (respectively 140 ± 1%, 167 ± 5% and 149 ± 7%, 186 ± 4% fold variation to LDL-untreated group, Fig. 7.3.A). Moreover, the viability of androgen-sensitive LNCaP cells was significantly increased in ~ 25 % when LDL was combined with DHT (175 ± 3 % fold variation to LDL-untreated group, for 24 h of treatment, Fig. 7.3.A).

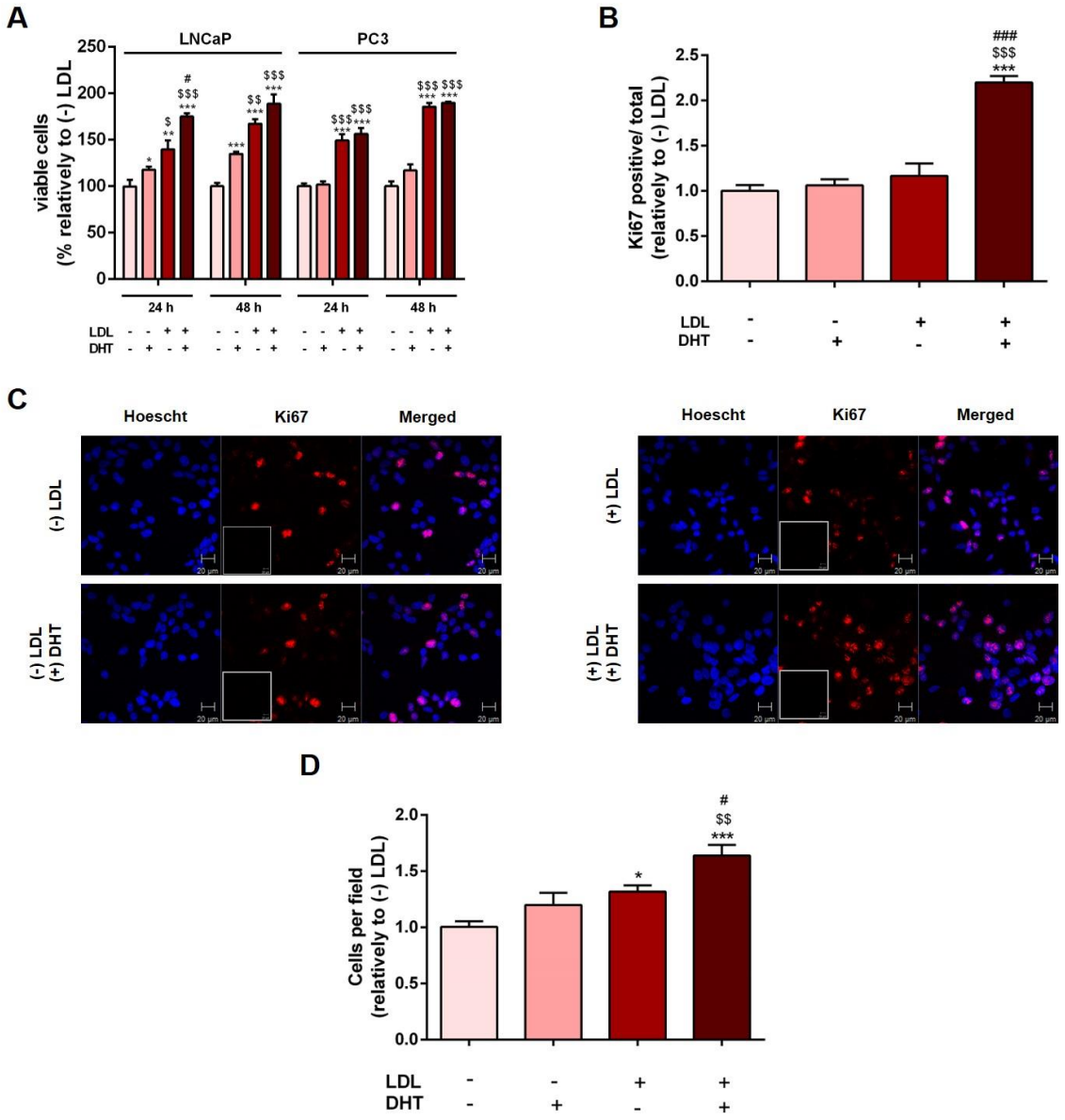

Figure 7.3. Effect of LDL (100 µg/ml) and DHT (10 nM) on PCa cells viability (A), proliferation (B, C) and migration (D). (A) Cell viability of LNCaP and PC3 cells was determined by the MTT assay after 24 and 48 h of treatment. **(B)** Proliferation of LNCaP cells was determined by the immunofluorescence analysis of Ki67 at 24 h. Data are expressed as the mean of Ki67-positive cells relatively to the total cell number (10 fields were assessed for each experimental condition). **(C)** Representative confocal microscopy images showing Ki67 labelling in the different groups. Images were obtained in the Zeiss LSM 710 laser scanning confocal microscope under 630 x magnification. Nuclei are stained with Hoechst 33342 (blue) and Ki67 positive staining is red. Negative controls for Ki67 obtained by omission of the primary antibody are provided as insert panels (-). **(D)** Migration of LNCaP cells was determined by a trans-wells assay in uncoated chambers. The upper chamber contained serum free medium and LNCaP cells in the presence or absence of LDL and DHT. Lipid depleted medium in the lower chamber was used as chemoattractant. Data are expressed as the mean number of migrating cells per 20x magnification field (10 fields were assessed for each experimental condition). All results are expressed as fold-variation relatively to the LDL-untreated group (LDL (-)). Error bars indicate mean ± S.E.M (n= 5) * p<0.05, ** p<0.01, *** p<0.001 when compared to LDL (-) group; $ p<0.05, $$ p<0.01, $$$ p<0.001 when compared to LDL (-)/DHT (+) group; # p<0.05, ### p<0.001 when compared to LDL (+) group.

Immunofluorescent labelling of the nuclear proliferation marker Ki-67 was used to confirm whether treatment with LDL plus DHT also improves LNCaP cells proliferation. Indeed, cell proliferation was significantly augmented in the LDL (+)/DHT (+) group (2.20 ± 0.07 fold variation to LDL-untreated group, Fig. 7.3B, 7.3.C). The enhanced effects of LDl in the presence of DHT were also observed in a trans-wells assay for assessing cell migration. LDL-supplementation significantly increased LNCaP cells migration (1.31 ± 0.06 fold variation to LDL-untreated group, Fig. 7.3.D), an effect that was ~ 25 % amplified in the presence of DHT (1.64 ± 0.10 fold variation to LDL-untreated group, Fig. 7.3.D).

Both LDL and DHT are known to influence PCa cells fate by modulating several intracellular signalling pathways. With this rationale, we analysed the expression of total and phosphorylated AKT and ERK isoforms (Fig. 7.4.). WB analysis showed that total AKT, a key downstream mediator regulating cell survival and proliferation, was decreased in the LDL (+)/DHT (+) group relatively to the group non-supplemented with LDL (0.75 ± 0.04 fold variation, Fig. 7.4.). However, p-AKT levels were only significantly increased in response to DHT (2.26 ± 0.40 fold variation in LDL(-)/DHT(+) relatively to LDL-untreated group, Fig. 7.4.). The DHT effect on p-AKT levels was not perceived when LDL was present (Fig. 7.4.).

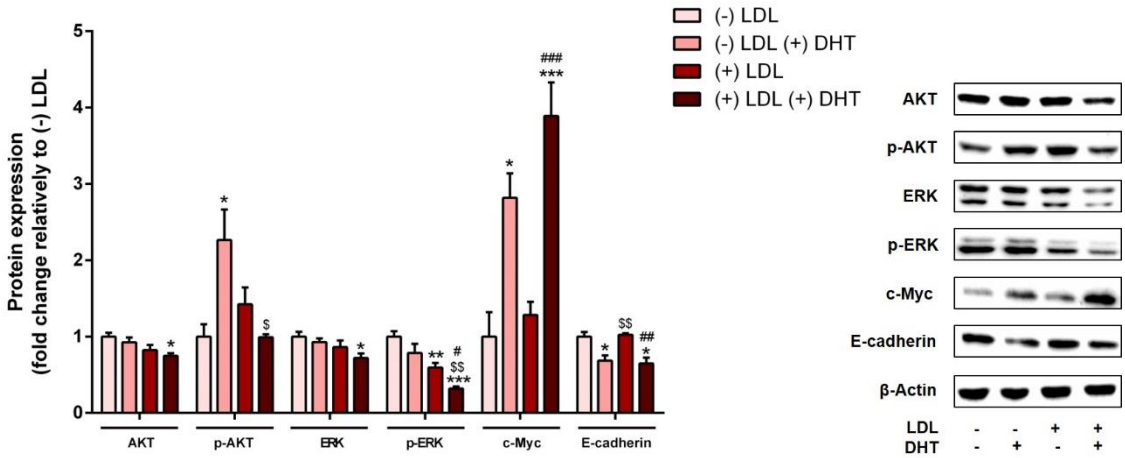

Figure 7.4. Effect of LDL (100 µg/ml) and DHT (10 nM) on the expression of AKT, p-AKT, ERK, p-ERK, c-Myc and E-cadherin in LNCaP cells treated for 24 h. Protein expression was analysed by WB after normalization with ß-actin. All results are expressed as fold variation relative to the LDL-untreated group (LDL (-)). Error bars indicate mean ± S.E.M (n= 5) * p<0.05, ** p<0.01, *** p<0.001 when compared to LDL (-) group; $ p<0.05, $$ p<0.01 when compared to LDL (-)/DHT (+) group; # p<0.05, ## p<0.01, ### p<0.001 when compared to LDL (+) group. Representative immunoblots are shown in the right panel.

Concerning ERK the presence of LDL significantly decreased p-ERK levels (0.59 ± 0.06 fold variation to LDL-untreated group, Fig. 7.4.). Both p-ERK and ERK expression decreased in the LDL(+)/DHT(+) treated group, an effect that was more pronounced for p-ERK (respectively, 0.32 ± 0.03 and 0.72 ± 0.06 fold variation to LDL-untreated group, Fig. 7.4.).

The oncogene c-Myc that is over-expressed in PCa is a potent driver of cell proliferative activity and has been indicated as a central player in the regulation of cell metabolism. The presence of LDL did not affect c-Myc expression, whereas DHT augmented its levels (2.82 ± 0.32 fold variation to LDL-untreated group, Fig. 7.4.). Moreover, upon

combining LDL with DHT, a further increase in c-Myc expression was observed (3.89 ± 0.44 fold variation to LDL-untreated group, Fig. 7.4.).

We also analysed if the enhanced migration of LNCaP cells in the LDL (+) and LDL (+)/DHT (+) experimental groups (Fig. 7.3.D) would be mediated by the loss of E-cadherin, a protein known to be supressed in the epithelial mesenchymal transition underliyng the emergence of metastatic carcinomas. E-cadherin expression was decreased in DHT-treated groups independently of the presence of LDL (0.68 ± 0.07 and 0.65 ± 0.08 fold variation to LDL-untreated group, Fig. 7.4.).

7.3.3. LDL/DHT effects are accompanied by altered fatty acid metabolism with accumulation of lipid droplets

Considering the effect of DHT modulating the expression of lipid metabolism regulators and the LDL actions affecting LNCaP cells fate dependently on DHT, we decided to investigate whether LDL availability wouldZ affect the response to DHT and lipid handling in prostate cells.

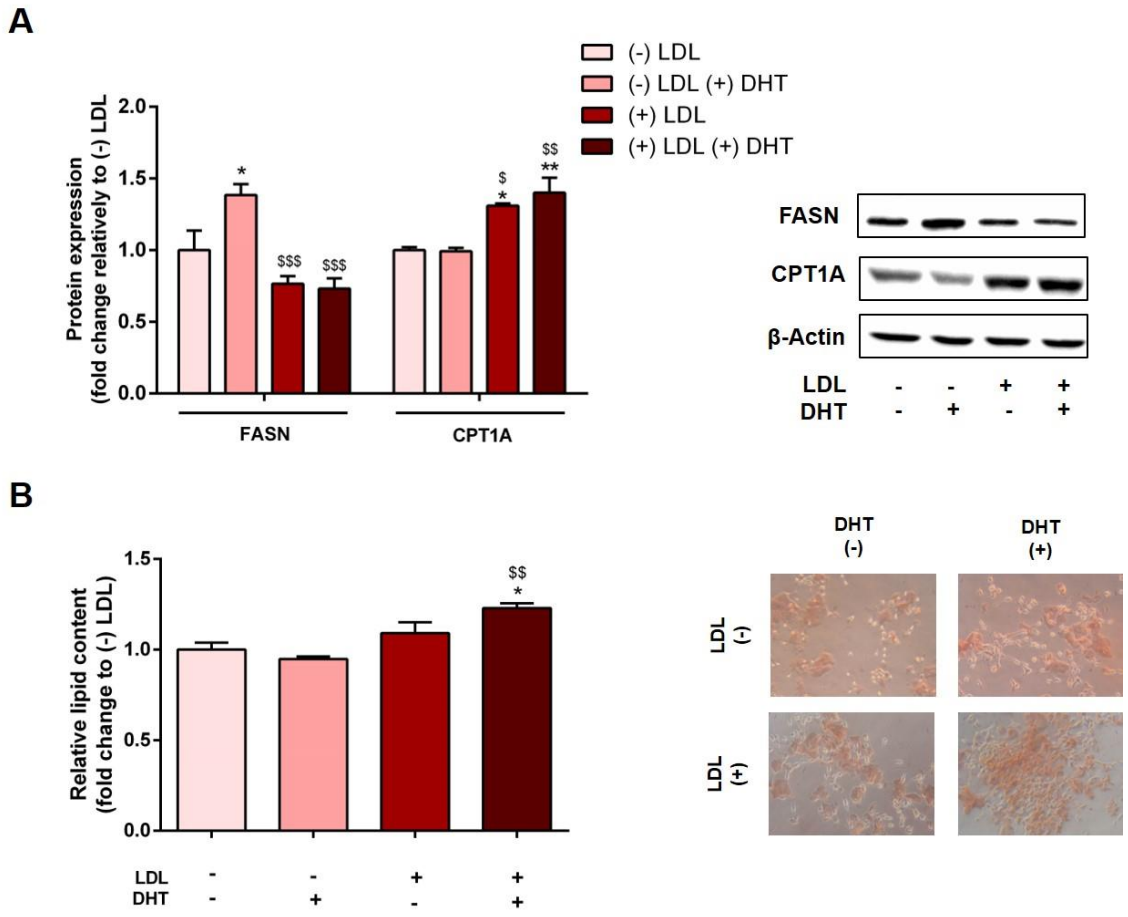

Figure 7.5. Effect of LDL (100 µg/ml) and DHT (10 nM) on the expression of FASN (A) and CPT1A (A), and accumulation of lipid droplets (B) in LNCaP cells treated for 24 h. (A) Protein expression was analysed by WB after normalization with ß-actin. Representative immunoblots are shown in the right panel. **(B)** Lipid droplet staining and quantification were made using the Oil Red-O assay. Representative images are shown in the right panel. All results are expressed as fold variation relative to the LDL-

untreated group ((-) LDL). Error bars indicate mean ± S.E.M (n= 5) * p<0.05, *** p<0.001 when compared to (-) LDL group; $$ p<0.01, $$$ p<0.001 when compared to (-) LDL (+) DHT group.

First, we analysed FASN and CPT1A expression, targeting fatty acid synthesis and oxidation, respectively. As expected, DHT-treatment for 24 h augmented FASN expression in the LDL (-) group (1.38 ± 0.08 fold variation, Fig. 7.5.A). LDL itself did not affect FASN expression levels, but its presence suppressed the DHT-stimulatory effects over FASN (Fig. 5A).

DHT did not affect CPT1A expression (Fig. 7.5.A). However, LDL-treated groups displayed increased expression of CPT1A (LDL (+), 1.31 ± 0.02 and LDL (+)/DHT (+), 1.40 ± 0.10 fold variation to LDL-untreated group, Fig. 7.5.A).

The accumulation of triglycerides and cholesterol esters in cytosolic lipid droplets protects lipids from harmful peroxidation and represent a lipid reservoir for maintenance and formation of cell membranes. We evaluated if LDL availability and DHT-treatment altered LNCaP cells capability for storing neutral lipids (Fig. 5B). The combination of LDL(+)/DHT(+) enhanced the relative number of lipid droplets relatively to the LDL-untreated groups (1.23 ± 0.03 fold variation to LDL-untreated group, Fig. 7.5.B).

7.3.4. The transcription factor SREBP mediates the DHT actions regulating fatty acid metabolism and p-AKT levels

As shown, DHT increased LNCaP cells viability (Fig. 7.3.A), and p-AKT and FASN expression (Fig. 7.4.) in lipid-depleted conditions (LDL(-)).The sterol regulatory element-binding protein-1 (SREBP-1) has been shown to be involved in the transcriptional activity of AR regulating FASN [31-33]. Next, we determined if the chemical inhibition of SREBP through a recognized inhibitor, Fatostatin, inhibited the effects of DHT in lipid depleted conditions. Exposure to fatostatin strongly diminished FASN expression in LNCaP DHT-treated cells (0.20 ± 0.05 fold variation to control, Fig. 7.6.). Moreover, fatostatin strongly blocked AKT and p-AKT expression (respectively, 0.07 ± 0.02 and 0.08 ± 0.02 fold variation to control, Fig. 7.6.).

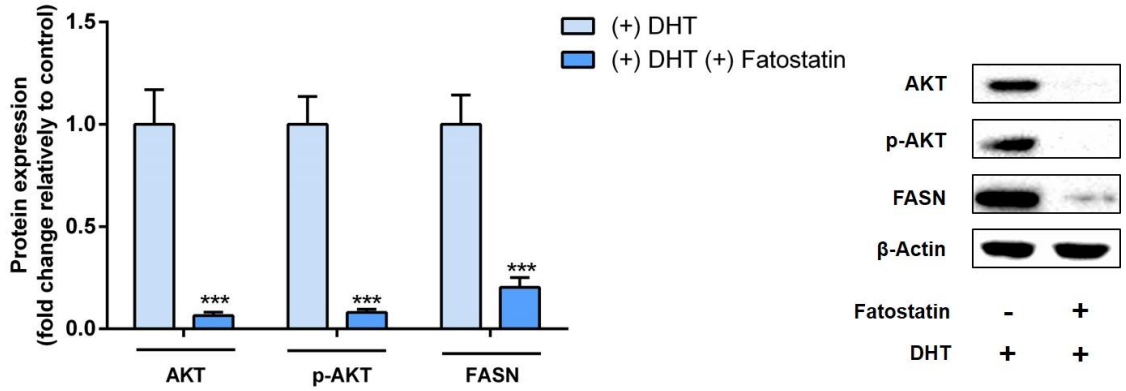

Figure 7.6. Effect of fatostatin (20 μM) suppressing DHT (10 nM) actions regulating the expression of AKT, p-AKT, FASN in LNCaP cells treated for 24 h in lipid-depleted conditions. Protein expression was analysed by WB after normalization with ß-actin. All results are expressed as fold variation relative to DHT-treated group. Error bars indicate mean ± S.E.M (n= 5) *** p<0.001. Representative immunoblots are shown in the right panel.

7.3.5. Anti-LDLR antibody and etomoxir blocked the LDL-effects increasing viability of PCa cells

Finally, we tested if blocking LDL entry into the cell or fatty acid oxidation would reverse LDL effects enhancing PCa cells viability (Fig. 7.7.). Cells were stimulated with LDL in the presence of anti-LDLR antibody, which resulted in decreased viability of both LNCaP and PC3 cells (respectively, 59 ± 18 and 79 ± 2% fold variation to control group, Fig. 7.7.A).

After 48 h of treatment, etomoxir, a well-known inhibitor of CPT1A, completely abolished the LDL effect increasing viability of LNCaP cells (Fig. 7.7.B). A ~ 78% reduction was observed when etomoxir was present, restoring LNCaP cells viability to that of the control.

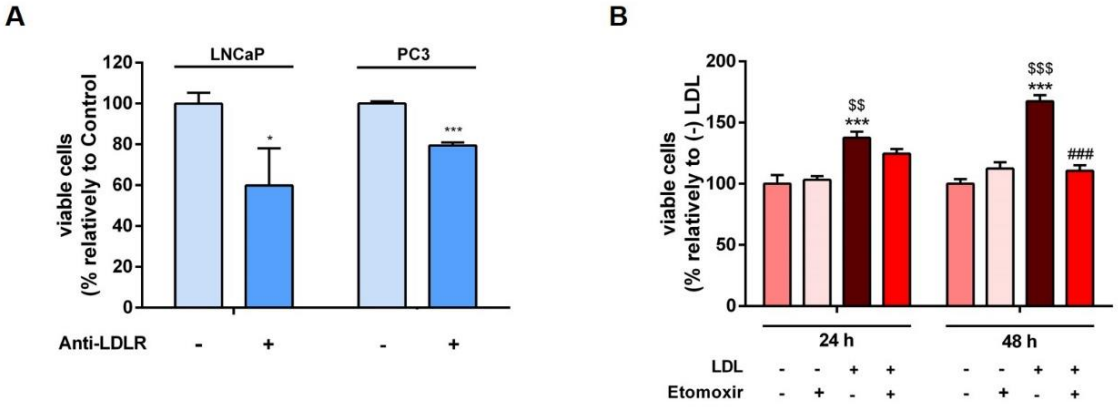

Figure 7.7. Effect of anti-LDLR antibody (5 µg/mL) (A) and etomoxir (40 µM) (B) over LDL actions sustaining LNCaP cells viability. (A) Cells were pre-treated with the anti-LDLR antibody for 2 h before exposure to LDL (100 µg/mL). **(B)** Cells were treated with LDL (100 µM/ml) in the presence or absence of etomoxir for 24 and 48 h. Cell viability was determined by the MTT assay. Error bars indicate mean ± S.E.M (n= 5) * $p<0.05$, *** $p<0.001$ when compared to the respective control group; $$ $p<0.01$, $$$ $p<0.001$ when compared to (-) LDL (+) etomoxir group; ### $p<0.001$ when compared to (+) LDL (-) etomoxir group.

7.4. Discussion

In this study, we investigated the effect of androgens in regulating lipid metabolism, and the influence of these hormones and LDL-cholesterol modulating PCa cells fate.

First, we characterized the basal expression of target regulators of lipid metabolism in our prostate cell line models; non-neoplastic (PNT1A) prostate cells, androgen-sensitive (LNCaP) PCa cells, and CRPC (DU145 and PC3) cells. Expression analysis of CD36, FASN and CPT1A targeted fatty acid transport, synthesis and oxidation (Fig. 7.1.A), respectively. CRPC cells displayed a decreased expression of the fatty acid transporter CD36 compared to non-neoplastic PNT1A and LNCaP cells (Fig. 7.1.B). These results first described the expression of CD36 in prostate cell line models dependently on their androgen-responsiveness. Interestingly, a differential increased expression of CD36 was found in estrogen receptor-positive breast cancer cells compared with estrogen receptor-negative [34], which indicates this transporter as a target of hormone regulation. Moreover, CD36 has been shown to be

overexpressed in pancreatic, gastric, and ovarian human cancer cases, and also in PCa [35-38].

FASN and CPT1A expression was significantly increased in PCa cell line models (Fig. 7.1.B), which follows other previous reports. FASN has little importance in non-neoplastic cells, but its overexpression has been identified as an early event in the development of PCa [39]. Moreover, increased expression and activity of FASN have been associated with PCa progression, more aggressive phenotypes of disease, and CRPC bone metastasis [39-42]. Concerning CPT1A, the studies evaluating its expression in human PCa cases or cell lines are almost non-existent, but a report demonstrated that CPT1A is highly abundant in PCa compared with benign tissues, especially if considering high-grade tumours [43]. This is in accordance with our results showing the highest expression of CPT1A in PC3 cells (Fig. 7.1.B), the most aggressive PCa cell line model under study.

Androgens are widely recognized as the main stimulators of prostate cell proliferation [44, 45], and also have been indicated as important regulators of cell metabolism, influencing several metabolic pathways, including glycolysis and lipid metabolism [5, 9, 11]. Indeed, androgen exposure has been shown to modulate lipid handling in PCa cells, with effects on lipogenesis, fatty acid uptake and lipid storage [15]. However, it is unknown whether conditions of different lipid availability would shape the effect of androgens regulating lipid metabolism in prostate cells. Also, there are several molecular partners involved in lipid handling that remain to be identified as androgens targets. Herein, we analyzed the effect of DHT in regulating the expression of FASN, CPT1A and CD36 (Fig. 7.2.).

Our results confirmed the effect of DHT-treatment enhancing the expression of FASN in the androgen-sensitive LNCaP cells (Fig. 7.2.B). FASN has been shown as a classical target of the androgens actions regulating lipid *de novo* synthesis in PCa cells [5, 46, 47]. Accordingly, androgen deprivation was shown to cause a marked reduction in FASN expression that was reverted by the administration of testosterone [48]. The results obtained in this study also showed that the DHT effects increasing FASN expression in LNCaP cells were maintained in lipid-depleted conditions, but abrogated in the presence of LDL (Fig. 7.5.A). These findings indicate that high LDL availability turn-off the production of fatty acids by PCa cells. On the other hand, results showed that LNCaP cells could maintain the capability of synthesizing *de novo* fatty acids in lipid-depleted conditions if stimulated with DHT. Moreover, the effect of DHT in regulating FASN expression in LNCaP cells was linked to the transcription factor SREBP-1, a central player in the control of lipid metabolism and a mediator of androgens actions regulating lipid handling [49-51]. Fatostatin, a well-known inhibitor of SREBP, suppressed the DHT effects up-regulating FASN protein levels in lipid-depleted conditions (Fig. 7.6.). Similar results were found in breast cancer cells with fatostatin decreasing FASN expression only in the absence of lipids [52].

CPT1A mediates a rate-limiting step in ß-oxidation by converting fatty acids to acylcarnitines, which enables the translocation to the intermembrane space of mitochondria and energy production. DHT exposure upregulated CPT1A expression levels in PNT1A and

LNCaP cells (Fig. 7.2.C), which indicates that androgens can stimulate lipid oxidation both in non-neoplastic and neoplastic prostate cells. However, the DHT effects upregulating CPT1A in LNCaP cells occurred only in conditions of LDL availability, being absent in lipid depleted conditions (Fig. 7.2.C, 7.5.A). Previous reports indicated that lipid mitochondrial metabolization through ß-oxidation is regulated by the AR [53, 54], but our findings show that androgens can stimulate lipid oxidation at the early step of their translocation to mitochondria. This is quite relevant since several studies have described that lipid utilization by the mitochondria is crucial for cell survival, activation of proliferation pathways and resistance to treatment [25, 55-57].

CD36, also known as fatty acid translocase, is a major transporter for fatty acids uptake. Moreover, CD36 was shown to play a relevant role in oncogenic signalling and, consequently, in cancer progression in several types of cancer, including prostate [34, 38, 58]. DHT-treatment diminished the expression levels of CD36 in PNT1A and LNCaP cells (Fig. 7.2.A). At least for our knowledge, this is the first report identifying the cell membrane fatty acid transporter CD36 as an androgen target gene. Other authors also showed that androgen exposure increased medium and long-chain fatty acids, cholesterol, and low-density lipoproteins uptake [59, 60]. However, several transporters can mediate lipid uptake in PCa cells [59]. A study indicated that the augmented uptake of lipids in response to androgens was associated with the increased expression of membrane fatty acid-binding protein (FABPm) [60]. In this way, androgens actions stimulating lipid uptake would result from the balance between CD36 and FABPm expression pattern [60]. Overall, the obtained findings broadened the role of androgens as central regulators of lipid metabolism.

Next, we analysed the effect of LDL altering PCa cells fate on the dependency of DHT. LDL exposure augmented LNCaP and PC3 cells viability, an effect that was amplified in LNCaP cells in the presence of DHT (Fig. 7.3.A). As a proof of concept, we evaluated LDL actions in the presence of anti-LDLr antibody. Inhibition of LDLr decreased LNCaP cells viability to the control levels (Fig. 7.7.A), demonstrating the importance of LDL uptake in promoting PCa cell viability. The information on LDLr actions in PCa is scarce, but it was shown to accelerate breast cancer cell growth in a mouse model of hyperlipidaemia [61]. LDLr inhibition also was shown to sensitize pancreatic cancer cells to chemotherapeutic drugs [62], which is demonstrative of the relevance of lipids viability for cancer cells growth.

Moreover, LNCaP cells proliferation was highly promoted by LDL whenever DHT was present (Fig. 7.3.B, 7.3.C). The information on LDL effects over PCa cell fate is limited, though a study showed that LDL exposure was related to an increased number of cancer cells [63]. However, high LDL availability has been shown to promote cell proliferation in several cancer types, namely, breast cancer and oesophageal squamous cell carcinoma [26-28]. The presence of androgens potentiating the effects of LDL in AR-positive LNCaP cells is a new finding that is supported by the existent reports demonstrating the role of androgens increasing LDL uptake [59].

LDL/DHT effects modulating LNCaP cells behaviour were underpinned by alterations on the protein fingerprint of key targets of survival and oncogenic pathways (Fig. 7.4.), namely, the AKT pathway that plays an important role in the survival of PCa cells, and has been associated with a poor clinical outcome [64-66]. The results obtained herein showed a significant increase of p-AKT in response to DHT in lipid-depleted conditions (Fig. 7.4.), which was linked with increased cell viability (Fig. 7.3.A). Increased p-AKT in DHT-treated LNCaP cells was also concomitant with the increased expression of FASN (Fig. 5A) and completely blocked by SREBP-1 inhibitor fatostatin (Fig. 7.6.). These findings are in line with the report of AKT activation, and the involvement of the PI3K/AKT→SREBP-1→ FASN pathway, and fatty acid synthesis, to satisfy the energy demands of cancer cells, their growth and invasion [67].

The ERK pathway is another important driver of cell proliferation and survival. The expression of the active p-ERK decreased with LDL supplementation and dependently on co-administration of DHT (Fig. 7.4.), whereas LDL or DHT alone had no effect. In breast cancer cells, LDL-cholesterol enhanced p-ERK expression levels [26]. Another study also showed a decreased expression of p-ERK with DHT treatment in LNCaP cells, which we verified only in the LDL(+)/DHT(+) group, probably because a completely lipid-free medium was used [68]. Nevertheless, the relationship between ERK activity in PCa is somehow controversial. Some authors have demonstrated a decline in ERK activity in advanced tumours, whereas others showed activation of this pathway in malignancy [69-72].

c-Myc is one of the oncogenes overexpressed in PCa being extremely important for cancer progression and establishment of the CRPC phenotype [73-75]. Also, coordinated action of c-Myc and AR has been proposed in PCa development [73, 74]. Furthermore, c-Myc has been indicated as a central regulator of metabolic alterations in cancer cells, namely in glucose and glutamine metabolism [76, 77]. This study showed an increased expression of c-Myc with LDL exposure in the presence of DHT (Fig. 7.4.). LDL alone had no effect, but DHT also increased c-Myc expression (Fig. 7.4.). It was demonstrated that c-Myc is associated with the deregulation of lipid metabolism in PCa cells and triple-negative breast cancer [68, 78]. Interestingly, the increased expression of c-Myc in the LDL(+)/DHT(+) group occurred concomitantly with the accumulation of intracellular lipids (discussion below). Further studies are needed to confirm the relationship of c-Myc with LDL/DHT actions in lipid metabolism.

Our results also showed that LDL had a stimulatory effect over LNCaP cells migration (Fig. 7.3.D), which was potentiated by DHT. This was accompanied by the altered expression of EMT markers, namely, E-cadherin, a key cell-to-cell adhesion molecule associated with cancer cell migration and invasion. Loss of E-cadherin has been shown facilitating PCa metastasis and enhancing PCa chemoresistance [79-81]. LDL exposure in the presence of DHT decreased expression of E-cadherin (Fig. 7.4.). Moreover, DHT alone also decreased E-cadherin expression, which is in line with the concept of androgens as enhancers of PCa cell migration and metastasis [82-84]. Our findings are also supported by a study describing the decreased expression of E-cadherin using the same DHT concentration [85]. Concerning other

EMT markers, such as vimentin and N-cadherin, their protein expression was undetectable (data not shown). Indeed, LNCaP cells have been shown to present low levels of vimentin [86, 87], being negative for N-cadherin [88].

Overall, this study first showed the interplay of LDL and androgens enhancing viability, and the proliferative and migration abilities of androgen-sensitive PCa cells (Fig. 7.8.).

It has been shown that cancer cells have the capability of metabolic adaptation in response to lipids availability [89]. When cell lipids are in excess, cholesterol is converted to cholesteryl esters that can be stored as lipid droplets [90, 91]. High LDL availability in the presence of DHT increased storage of lipids in LNCaP cells (Fig. 5B). The LDL stored in lipid droplets would be a reservoir to satisfy the cell growth needs, and maybe is supporting the enhanced proliferative activity and migration of LNCaP cells (Fig. 7.3.A, 7.3.C). Moreover, LDL supplementation, besides suppressing FASN expression and the lipid *de novo* synthesis, increased CPT1A levels (Fig. 5A) driving fatty acids to β-oxidation. The rate of mitochondrial fatty acid oxidation is regulated by CPT1A as it mediates the transport of fatty acids across the inner mitochondrial membrane. Etomoxir is a small-molecule widely used as an inhibitor of fatty acid oxidation by its irreversible inhibition of CPT1A [92]. CPT1A inhibition with etomoxir reverted the LDL effects promoting LNCaP cells viability (Fig. 7B), which indicates that the LDL incorporated in lipid droplets is being routed for lipolysis with fatty acids production and β-oxidation, to sustain cell viability (Fig. 7.8.).

Obesity and hypercholesterolemia have been associated with more aggressive stages of PCa [19-23, 93], with periprostatic adipocytes having a significant impact on cancer cells invasiveness [94]. Also, it has been indicated that cholesterol, as a steroidogenesis precursor, can fuel intratumoral androgen synthesis accelerating prostate tumors growth [95]. On the other hand, treatment with inhibitors of cholesterol synthesis, as simvastatin, was shown to reduce LNCaP tumors xenografts growth and androgen steroidogenesis [96]. These data, together with the cooperative relationship found here between LDL and androgens promoting PCa cell growth, open new doors to target cholesterol availability together with ADT for treatment of PCa.

In conclusion, this study showed that androgens regulate a wide range of lipid metabolism targets, and promote the LDL effects increasing cell viability, proliferation and migration of PCa cells (Fig. 7.8.). These findings support clinical data linking obesity and PCa, and first implicated androgens in this relationship. Furthermore, they sustain the development of future strategies for PCa treatment targeting obesity and the AR simultaneously.

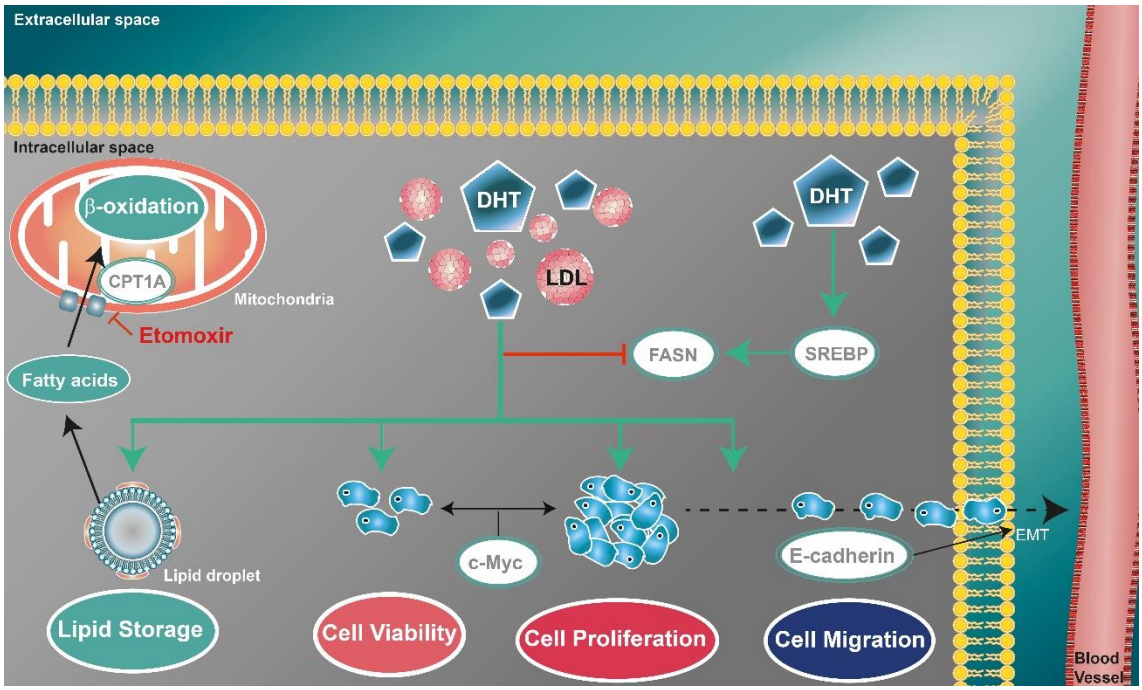

Figure 7.8. DHT and LDL-cholesterol actions in modulating PCa cells fate and lipid metabolism. LDL promoted viability, proliferation and migration of PCa cells dependently on the presence of DHT. These effects were underpinned by the altered expression of the oncogene c-Myc, and decreased expression of E-cadherin, which is a protein downregulated in the epithelial mesenchymal transition (EMT). The combination of LDL and DHT augmented the lipid droplets content. Blocking fatty acid ß-oxidation with etomoxir, an inhibitor of CPT1A, reverted the effect of LDL promoting cell viability, which indicates that the LDL incorporated in lipid droplets is being routed for fatty acids production and oxidation. DHT increased the expression of FASN, an effect also observed in lipid-depleted conditions and mediated by the transcriptional factor SREBP. The presence of exogenous LDL, regardless of the presence of DHT, turned-off FASN expression.

7.5. Acknowledgments

None.

7.6. Funding

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project No. 007491) and National Funds by FCT-Foundation for Science and Technology (Project UID/Multi/00709/2013). Cardoso HJ and Figueira MI were recipient of BID/ICI-FCS/CICS/Santander and FCT fellowships, respectively, SFRH/BD/111351/2015 and SFRH/BD/104671/2014.

7.7. Conflict of Interest

The authors declare that they have no conflicts of interest.

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Chapter 8

Tyrosine Kinase Inhibitor Imatinib Modulates the Viability and Apoptosis of Castrate-Resistant Prostate Cancer Cells Dependently on the Glycolytic Environment

This Chapter was submitted published as:

Cardoso HJ[#], Vaz CV[#], Carvalho TM, Figueira MI, Socorro S. Tyrosine Kinase Inhibitor Imatinib Modulates the Viability and Apoptosis of Castrate-Resistant Prostate Cancer Cells Dependently on the Glycolytic Environment. Life Sci. 1;218:274-283. doi: 10.1016/j.lfs.2018.12.055.

[#] Contributed equally

Abstract

Aims: The tyrosine kinase inhibitor imatinib has been used in prostate cancer treatment with outcomes that did not follow the in vitro findings. The glycolytic environment has been shown to influence the efficacy of anti-cancer drugs. This study aimed to evaluate the effect of imatinib on cell viability, apoptosis, and metabolism in cell line models of castrate-resistant prostate cancer (CRPC) under hyperglycemic and hypoglycemic conditions.

Main Methods: DU145 and PC3 CRPC cell lines were exposed to 20 μM imatinib under 5 mM (hypoglycemia) or 30 mM glucose (hyperglycemia) for 48-72h. Cell viability was assessed by the MTS assay. The expression of apoptosis regulators and glycolytic metabolism-related proteins was analysed by Western blot, and the activity of caspase-3 and lactate dehydrogenase (LDH) was determined spectrophotometrically. Glucose consumption and lactate production were determined using biochemical assays.

Key findings: Imatinib decreased CRPC cells viability, whereas increasing apoptosis; effects only observed in hyperglycemic conditions. Glucose consumption and lactate production were significantly increased in imatinib-treated DU145 and PC3 cells, and independently of glucose availability. Accordingly, LDH expression and activity were significantly increased in response to imatinib.

Significance: Higher glucose availability improved the effectiveness of imatinib suppressing survival and growth of CRPC cells. It was also shown that imatinib treatment stimulated the glycolytic metabolism of CRPC cells. This study first demonstrated that a glucose-enriched environment intensifies the effect of imatinib, which stimulates the interest for testing this compound into the clinical setting, namely in hyperglycemia conditions (diabetic patients) or in co-administration with inhibitors of glycolytic metabolism.

Keywords: apoptosis, imatinib, glycaemia, glycolytic metabolism, prostate cancer

8.1. Introduction

The advanced metastatic prostate cancer (PCa) is characterized by the loss of androgen responsiveness, reaching the stage of disease so-called castration-resistant prostate cancer (CRPC) (1). Clinically this means the failure of classical androgen ablation therapies and corresponds to a usually lethal form of PCa (2, 3).

Imatinib mesylate is a potent and selective inhibitor of receptors tyrosine kinase that has been used to treat gastrointestinal stromal tumors and leukemias (4, 5). Imatinib also has been tested in PCa treatment, but the results obtained were modest with this compound seeming to be ineffective controlling prostate-specific antigen levels and reducing tumor size (6, 7). Contrastingly, *in vitro* and animal experimentation studies showed that imatinib, alone or in combination with other drugs, has cytotoxic effects and sensitizes PCa cells to chemo- or radiotherapy (8-10). Our research group investigated the cytotoxic effects of imatinib in cell line models of CRPC, DU145 and PC3 cells (11). Imatinib was effective decreasing viability

and increasing apoptosis of DU145 cells, whereas displaying opposite effects in PC3 cells (11). These contradictory results were related to the distinct expression of c-KIT receptor isoforms (11), but also indicated that imatinib responses might depend on cell-specific or environmental conditions.

The glycolytic environment has been shown to influence the efficacy of various anti-cancer drugs in different types of cancer (12, 13). Although imatinib actions have been linked with alterations in glucose metabolism (14, 15), the effect of this tyrosine kinase inhibitor dependently on the glycolytic environment has not been examined.

On the other hand, the dependence on glycolysis is a recognized feature of cancer cells. This behavior, known as the Warburg's effect, is characterized by the use of glucose for lactate production instead of driven it to oxidative phosphorylation, what happens even in the presence of oxygen (16). The lactate produced is exported into the extracellular space, acidifying the tumor microenvironment and promoting cancer cells growth and dissemination (17). CRPC cells have been shown to display an increased glycolytic metabolism relatively to androgen-sensitive cells, with the glycolytic pattern being associated with progression and aggressiveness of disease (18-20).

Overall, the gathered information lead us to hypothesize that the imatinib effects controlling of PCa cells growth may depend on the glycolytic environment. The present study investigated the effect of imatinib in two cell line models of CRPC under conditions of different glucose availability (hyperglycemic *vs.* hypoglycemic). Alterations in cell viability, apoptosis and glycolytic metabolism were assessed. This included the expression analysis and activity measurement of several apoptosis regulators (Bcl-2, Bax, caspase-8, caspase-9 and caspase-3 proteins) and glycolytic metabolism-related proteins, namely glucose transporters (GLUTs), phosphofructokinase-1 (PFK1), lactate dehydrogenase (LDH) and monocarboxylate transporter 4 (MCT4).

8.2. Materials and methods

8.2.1. Cell lines and treatments

The human CRPC cell lines, DU145 and PC3, were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and penicillin (100 U /ml)/streptomycin (125 µg/ml)/amphotericin B (0,25 µg/ml) (SC-3690, Santa Cruz Biotechnology, Heidelberg, Germany), at 37 ºC in an atmosphere equilibrated with 5 % CO_2. Cells were maintained up to 60% confluence in all experiments and then the culture medium was replaced by glucose-free RPMI (R1383, Sigma-Aldrich) supplemented with 5 mM (hypoglycemia) or 30 mM of glucose (hyperglycemia) (21, 22). After additional 24 h, cells were exposed to a cytotoxic concentration (20 µM) of imatinib mesylate (CAS 220127–57-1, Santa Cruz Biotechnology) for 48 to 72 h, as recently described (10, 11). The experimental groups were as follow: i) 5 mM glucose without imatinib (imatinib (-)

hypoglycemic group); ii) 5 mM glucose with imatinib (imatinib (+) hypoglycemic group); iii) 30 mM glucose without imatinib (imatinib (-) hyperglycemic group); and iv) 30 mM glucose with imatinib (imatinib (+) hyperglycemic group). For 72 h of treatment, control and treated-cells were harvested for protein extraction, and cell culture medium was collected for measurement of glucose and lactate concentration.

8.2.2. Cell viability assay

DU145 and PC3 cells were grown in 96-well plates, and cell viability was determined by the colorimetric CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) at 48 and 72 h after treatment with imatinib in the presence of 5 mM or 30 mM glucose. The conversion of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2M) tetrazolium compound to the colored formazan product was detected at 490 nm in the xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA, USA). The relative number of viable cells in each experimental condition was calculated by normalizing the absorbance to that of the experimental group without imatinib (-) in hypoglycemic condition (5 mM glucose).

8.2.3. Quantification of glucose and lactate

The concentration of glucose and lactate in the cell culture medium was determined by spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain) as described previously (18). The glucose consumption and lactate production were calculated relatively to initial glucose and lactate concentrations at 0 h of treatment. All measurements were normalized to the total number of cells in each experimental condition.

8.2.4. Protein extraction

DU145 and PC3 cells were homogenized by pipetting in 60 µl of radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris, 1 mM EDTA) supplemented with 1 % protease inhibitors cocktail (Sigma-Aldrich) and 10 % PMSF (Sigma- Aldrich), kept on ice for 20 min with occasional mixing, and then centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration was determined by the Bradford assay (Bio-Rad).

8.2.5. Western blot (WB)

Total proteins were resolved by SDS-PAGE on 12.5 % gels and electrotransferred to a PVDF membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with rabbit anti-Bcl-2 (1:1000, no. 2876; Cell Signaling Technology), rabbit anti-Bax (1:500, no. 2772, Cell Signaling Technology, Danvers, MA), rabbit anti-caspase-9 (1:500, H-170: sc-8355; Santa Cruz Biotechnology), mouse anti-caspase-8 (1:500, D-8: sc-5263; Santa Cruz Biotechnology), rabbit anti-GLUT1 (1:1000, CBL242, Millipore), rabbit anti-GLUT3 (1:2500, sc-30107, Santa Cruz Biotechnology), rabbit anti-PFK1 (1:1000, sc-67028, Santa Cruz Biotechnology), rabbit anti-

LDH (1:10,000, Ab52488, Abcam, Cambridge, United Kingdom) or rabbit anti-MCT4 (1:1000, sc-50329, Santa Cruz Biotechnology) primary antibodies. Protein expression was normalized using a mouse anti-ß-actin (1:1000, A5441, Sigma-Aldrich) antibody. Membranes were incubated with ECL substrate (Bio-Rad) for 5 min, and immunoreactive proteins were visualized with the ChemiDoc™ MP System (Bio-Rad). Band densities were obtained according to standard methods using the Image Lab software (Bio-Rad) and normalized by division with the respective ß-actin band density.

8.2.6. Caspase-3 activity assay

The enzymatic activity of caspase-3 was determined spectrophotometrically at 405 nm by detecting the presence of the yellow product p-nitro-aniline (pNA), upon cleavage of caspase-3 substrate (Ac-DEVD-pNA). In brief, 50 µg of total protein extract was incubated overnight at 37°C with reaction buffer (25mM HEPES, 0.1% 3CHAPS, 10% sucrose, and 10mM DTT, pH 7.5) and 200 µM of Ac-DEVD-pNA. The amount of generated pNA was calculated by extrapolation with a standard curve.

8.2.7. LDH activity

The enzymatic activity of LDH was determined using a commercial assay kit (Spinreact) following the manufacturers' instructions. LDH catalyses the reduction of pyruvate by NADH and the rate of decrease in concentration of NADH is proportional to the catalytic concentration of LDH present in the cells. The NADH concentration is measured spectrophotometrically at 340 nm using a xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad).

8.2.8. Statistical analysis

The statistical significance of differences between experimental groups was evaluated by one-way ANOVA followed by *Bonferroni post-hoc test*, using GraphPad Prism v6.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered statistically significant. All experimental data are shown as mean ± standard error of the mean (S.E.M).

8.3. Results

8.3.1. Imatinib decreased DU145 and PC3 cells viability under hyperglycemic conditions

The cytotoxic effects of imatinib in DU145 (Fig. 8.1.A) and PC3 (Fig. 8.1.B) cells under hypoglycemic (5 mM) and hyperglycemic (30 mM) conditions were evaluated by the MTS assay. Upon 48 h and 72 h of treatment with imatinib, the viability of DU145 cells was significantly decreased in the hyperglycemic group, 73,6 ± 9,8 and 77,4 ± 9,0 *vs.* 114,2 ± 8, 9 and 127,2 ± 10,7 % in the imatinib (-) hyperglycemic condition, respectively (Fig. 8.1.A). Moreover, DU145

viability in the imatinib (+) hyperglycemic group at 72 h was significantly decreased relatively to the imatinib (+) hypoglycemic group (~ 42% reduction). Also, the viability of PC3 cells was markedly reduced after 48 h and 72 h of treatment with imatinib in hyperglycemic conditions (73,9 ± 15,3 *vs.* 103,1 ± 4,7 (P<0,05) and 65,6 ± 11,0 *vs.* 102,7 ± 2,9 (P<0,01), respectively) (Fig. 8.1.B). All the subsequent analysis on gene expression and cell metabolism were performed for 72 h of treatment.

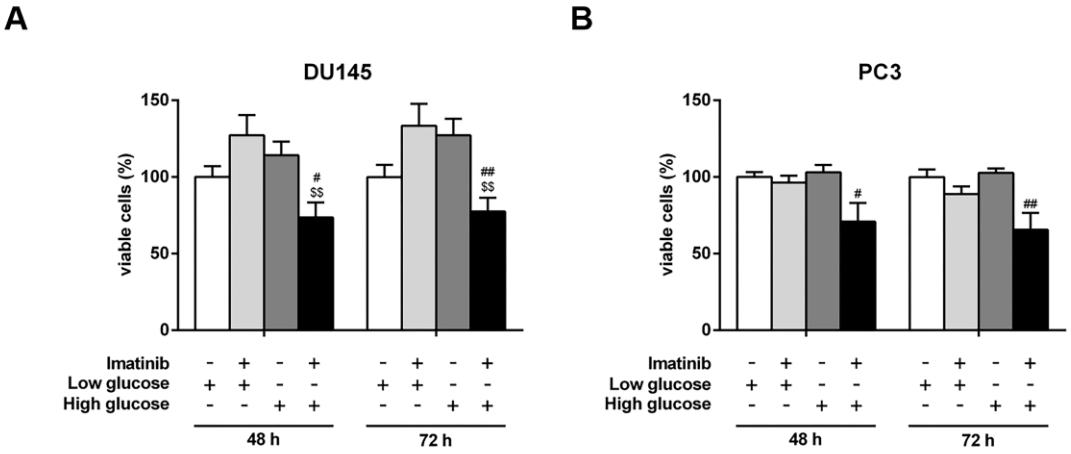

Figure 8.1. Viability of DU145 (A) and PC3 (B) cells after treatment with imatinib (20 μM) at different glucose concentrations (5 and 30 mM) for 48 and 72 h determined by the MTS assay. Results are expressed as fold-variation relatively to imatinib (-) with low glucose (5 mM glucose). Error bars indicate mean ± S.E.M (n = 5). $ p<0.05; $$ p<0.01; when compared with the imatinib (+) low glucose; # p<0.05; ## p<0.01; when compared with the imatinib (-) high glucose.

8.3.2. Hyperglycemic conditions improved the pro-apoptotic effect of imatinib

In order to determine whether the diminished viability/proliferation of DU145 and PC3 cells in response to imatinib treatment is a consequence of augmented apoptosis the expression levels and activity of several apoptosis regulators were evaluated.

Bax and Bcl-2 proteins are, respectively, pro- and anti-apoptotic members of the Bcl-2 family of apoptosis regulators involved in the regulation of the intrinsic pathway of apoptosis (23). Imatinib significantly decreased the expression of Bcl-2 in DU145 cells cultured in high glucose concentrations comparatively with the imatinib (-) 30 mM glucose (0,07 ± 0,02 *vs.* 0,17 ± 0,01, Fig. 8.2.A) and imatinib (+) 5 mM glucose groups (0,07 ± 0,02 *vs.* 0,16 ± 0,02, Fig 8.2A). In PC3 cells, Bcl-2 expression was also decreased in response to imatinib under hyperglycemic conditions (0,27 ± 0,04 *vs.* 0,45 ± 0,04 in the 30 mM glucose imatinib (-), P<0,05, Fig. 8.2.B). The Bax protein was only detected in PC3 cells and its expression was increased in hyperglycemic groups relatively to hypoglycemic conditions regardless of the presence of imatinib (P<0,05, Fig. 8.2.C).

Caspase-9 is the well-known activator of the intrinsic pathway of apoptosis, responsible for cleavage of the apoptosis effector caspase-3 (24). However, caspase-9 expression remained unchanged in DU145-treated cells (Fig. 8.2.D) whereas in PC3 it was

augmented in the imatinib (-) group under hyperglycaemia relatively to the imatinib (-) in hypoglycaemia (0,60 ± 0,04 *vs.* 0,43 ± 0,04 P<0,05 1, Fig. 8.2.E).

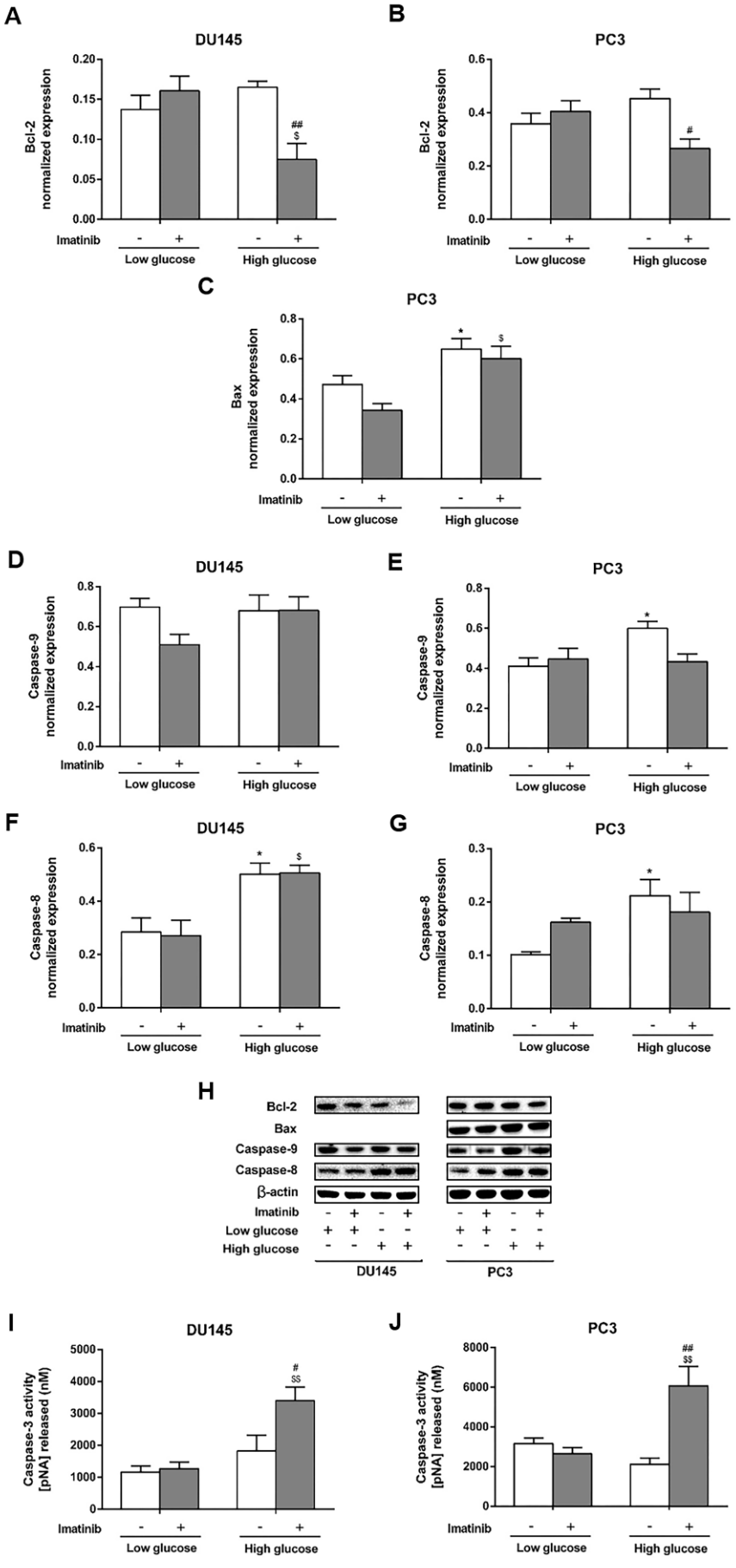

Figure 8.2. Effect of imatinib (20 μM) on the expression of apoptosis regulators and caspase-3 activity in DU145 and PC3 cells under hypoglycemic (5 mM) and hyperglycemic conditions (30 mM) for 72 h of treatment. Protein levels of Bcl-2 (A, B), Bax (C), caspase-9 (D, E) and caspase-8 (F, G) were determined by WB analysis after normalization with ß-actin. Representative immunoblots are shown in panel H. Caspase-3 activity (I, J) was measured spectrophotometrically by the release of the product pNA. Error bars indicate mean ± S.E.M (n= 5). * p<0.05; when compared with the imatinib (-) low glucose; $ p<0.05; $$ p<0.01; when compared with the imatinib (+) low glucose; # p<0.05; ## p<0.01; when compared with the imatinib (-) high glucose.

Caspase-3 can also be activated by caspase-8, the main mediator of the extrinsic pathway of apoptosis (25). Hyperglycemia increased the expression of caspase-8 in DU145 cells (P<0,05, Fig. 8.2.F) independently of the presence of imatinib; caspase-8 expression was also augmented in PC3 cells under 30 mM glucose (P<0,05, Fig. 8.2.G).

Concerning the activation of caspase-3, at the convergence of intrinsic and extrinsic pathways and a remarkable end-point of apoptosis (26), it was observed that imatinib treatment significantly increased the activity of caspase-3 in both DU145 and PC3 cells. However, this effect only occurred in the hyperglycaemic conditions; 3396 ± 435,7 *vs.* 1824 ± 491,2 in imatinib (-) hyperglycaemic or 1274 ± 209,2 in imatinib (+) hypoglycaemic groups in DU145 cells (Fig. 8.2.I); 6075 ± 990,2 *vs.* 2123 ± 311,7 in imatinib (-) hyperglycaemic or 2642 ± 321,3 in imatinib (+) hypoglycaemic groups in PC3 cells (Fig. 8.2.J).

8.3.3. The glycolytic metabolism of DU145 and PC3 cells is altered by glycaemia conditions and imatinib

The glycolytic environment has been shown to modulate the response to therapy in different cancer cell types (12, 13). With this rational we investigated the glycolytic metabolism of DU145 and PC3 cells under treatment with imatinib in hyperglycemic and hypoglycemic conditions. Glucose consumption and lactate production were determined spectrophotometrically and WB analysis was performed to evaluate the protein levels of key transporters and enzymes involved in glucose metabolism.

Imatinib treatment increased glucose consumption in DU145 cells under hypoglycaemic conditions (57,5 ± 1,3 *vs.* 36,3 ± 0,4 in imatininb, (-) P<0,001, Fig. 8.3.A) whereas no differences were observed in hyperglycaemia. In PC3 cells glucose consumption was significantly augmented in response to imatinib both in hypoglycaemia and hyperglycaemia conditions (24,8 ± 0,5 *vs.* 15,5 ± 0,7 in imatinib (-) and 11,5 ± 0,4 *vs.* 5,6 ± 1,1 imatinib (-), respectively), Fig 8.3.B). On the other hand, it is note of worth that glucose consumption in DU145 and PC3 cells was significantly decreased in hyperglycemia conditions comparatively with the same experimental groups in hypoglycemia (Fig. 8.3.A and 8.3.B), with the exception of DU145 imatinib (-).

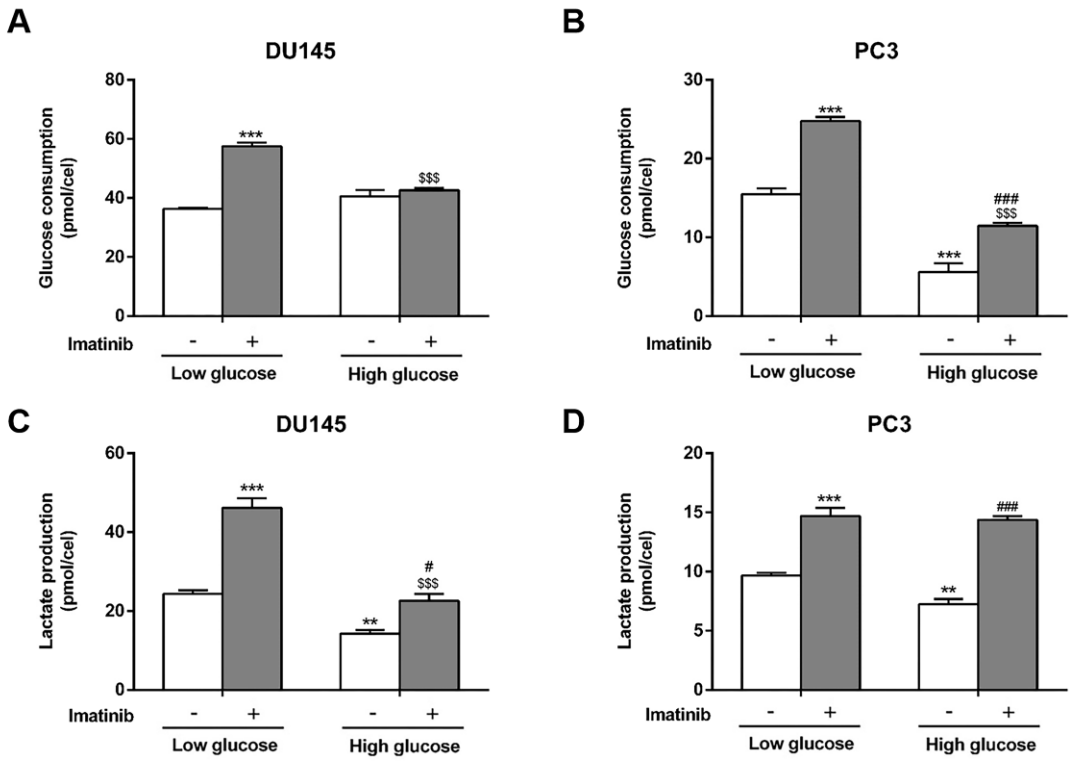

Figure 8.3. Glucose consumption (A, B) and lactate production (C, D) in DU145 and PC3 cells treated with imatinib (20 μM) under hypoglycemic (5 mM) or hyperglycemic conditions (30 mM) for 72 h. Error bars indicate mean ± S.E.M (n= 5).** p<0.01; *** p<0.001 when compared with the imatinib (-) low glucose; $$$ p<0.001 when compared with the imatinib (+) low glucose; # p<0.05; ### p<0.001 when compared with the imatinib (-) high glucose.

Regarding lactate production, it was increased in imatinib-treated cells in both glycemic conditions. Lactate production in DU145 cells in response to imatinib in hypoglycaemia varied from 24,4 ± 0,9 to 46,2 ± 2,4 (P<0,001) whereas in hyperglycaemia conditions the variation was from 14,3 ± 0,9 to 22,6 ± 1,7 (P<0,05) (Fig. 8.3.C). In PC3 cells the imatinib treatment also increased the lactate production regardless of glycemic conditions (14,7 ± 0,7 in imatinib (+) *vs.* 9,7 ± 0,2 in imatinib (-) in 30 mM glucose and 14,4 ± 0,3 in imatinib (+) *vs.* 7,2 ± 0,5 in imatinib (-) in 5 mM glucose, P<0,001 Fig. 8.3.D). Moreover, the lactate production was decreased in hyperglycemic conditions relatively to hypoglycemia; 14,3 ± 0,9 *vs.* 24,4 ± 0,9 in imatinib (-) and 22,64 ± 1,68 *vs.* 46,17 ± 2,40 in imatinib (+) in DU145 cells (P<0,01 and P<0,001, respectively, Fig. 8.3.C); 7,2 ± 0,5 *vs.* 9,7 ± 0,2 in imatinib (-) in PC3 cells (P<0,01, Fig. 8.3.D).

The transport of glucose across the plasma membrane, the first step of glycolytic process, is mediated by GLUTs, namely, the GLUT1 and GLUT3, which were previously identified in PCa cells (19, 27). The expression of GLUT1 was significantly decreased in hyperglycemic groups both in DU145 (0,09 ± 0,03 *vs.* 0,31 ± 0,05 in imatinib (-) and 0,08 ± 0,02 *vs.* 0,50 ± 0,09 in imatinib (+), Fig. 8.4.A) and PC3 (0,05 ± 0,01 *vs.* 0,31 ± 0,01 in imatinib (-) and 0,06 ± 0,02 *vs.* 0,17 ± 0,04 in imatinib (+), Fig. 8.4.B) cells. Also, a diminished expression of GLUT1 upon treatment with imatinib was observed in PC3 cells under hypoglycaemia (0,17 ± 0,04 *vs.* 0,31 ± 0,01, P<0,01, Fig. 8.4.B). Relatively to GLUT3 the results were the opposite, with the hyperglycemic conditions being associated to the

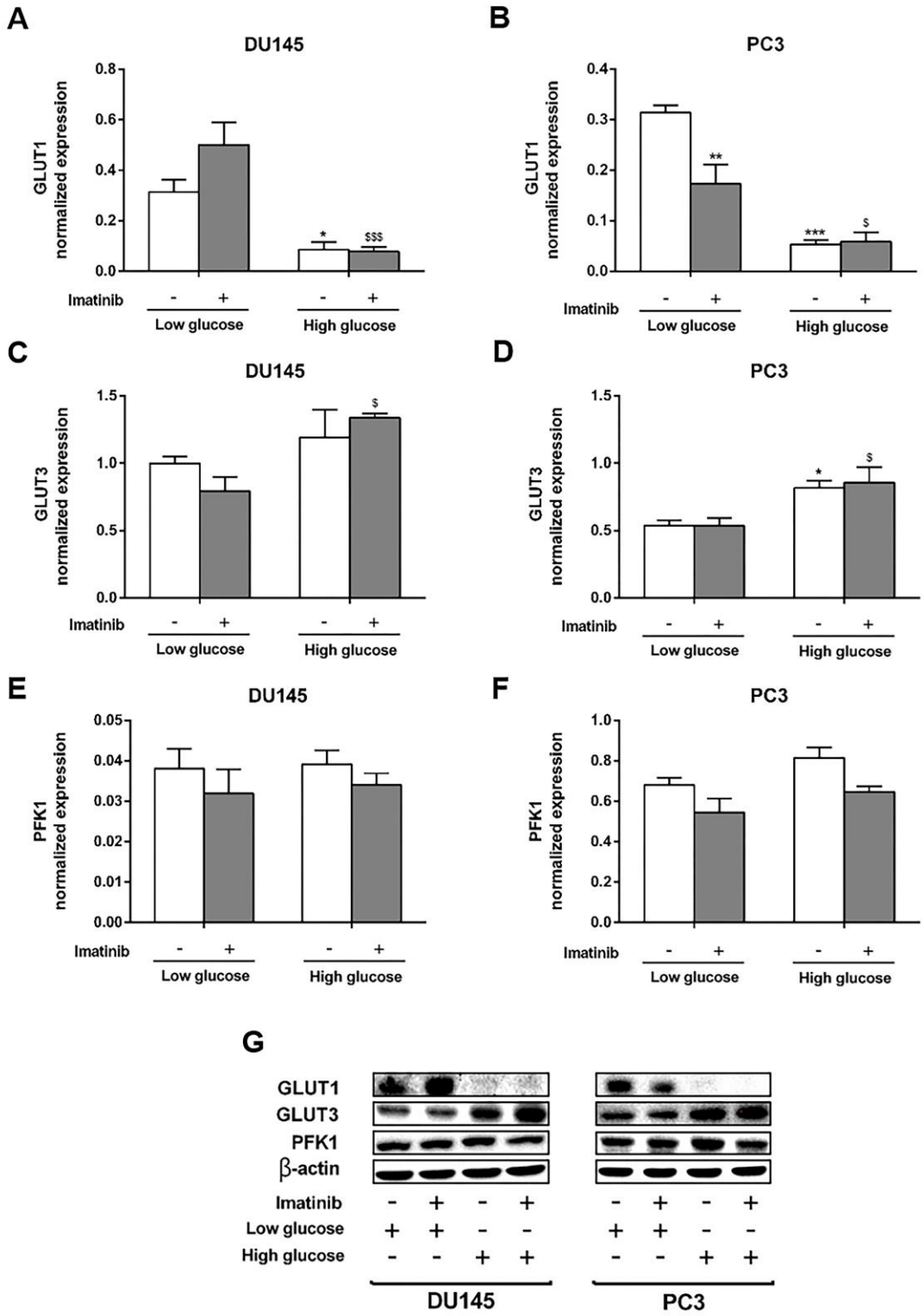

Figure 8.4. Protein levels of glycolytic metabolism-associated proteins, GLUT1 (A, B), GLUT3 (C, D) and PFK1 (E, F) in DU145 and PC3 cells treated with imatinib (20 μM) under hypoglycemic (5 mM) or hyperglycemic conditions (30 mM) for 72 h. Expression was determined by WB analysis after normalization with β-actin. Representative immunoblots are shown in panel G. Error bars indicate mean ± S.E.M (n= 5). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the imatinib (-) low glucose; $ $p<0.05$; $$$ $p<0.001$ when compared with the imatinib (+) low glucose.

increased expression of GLUT3. GLUT3 expression in imatinib-treated DU145 cells varied from

0,79 ± 0,10 in hypoglycaemia to 1,34 ±

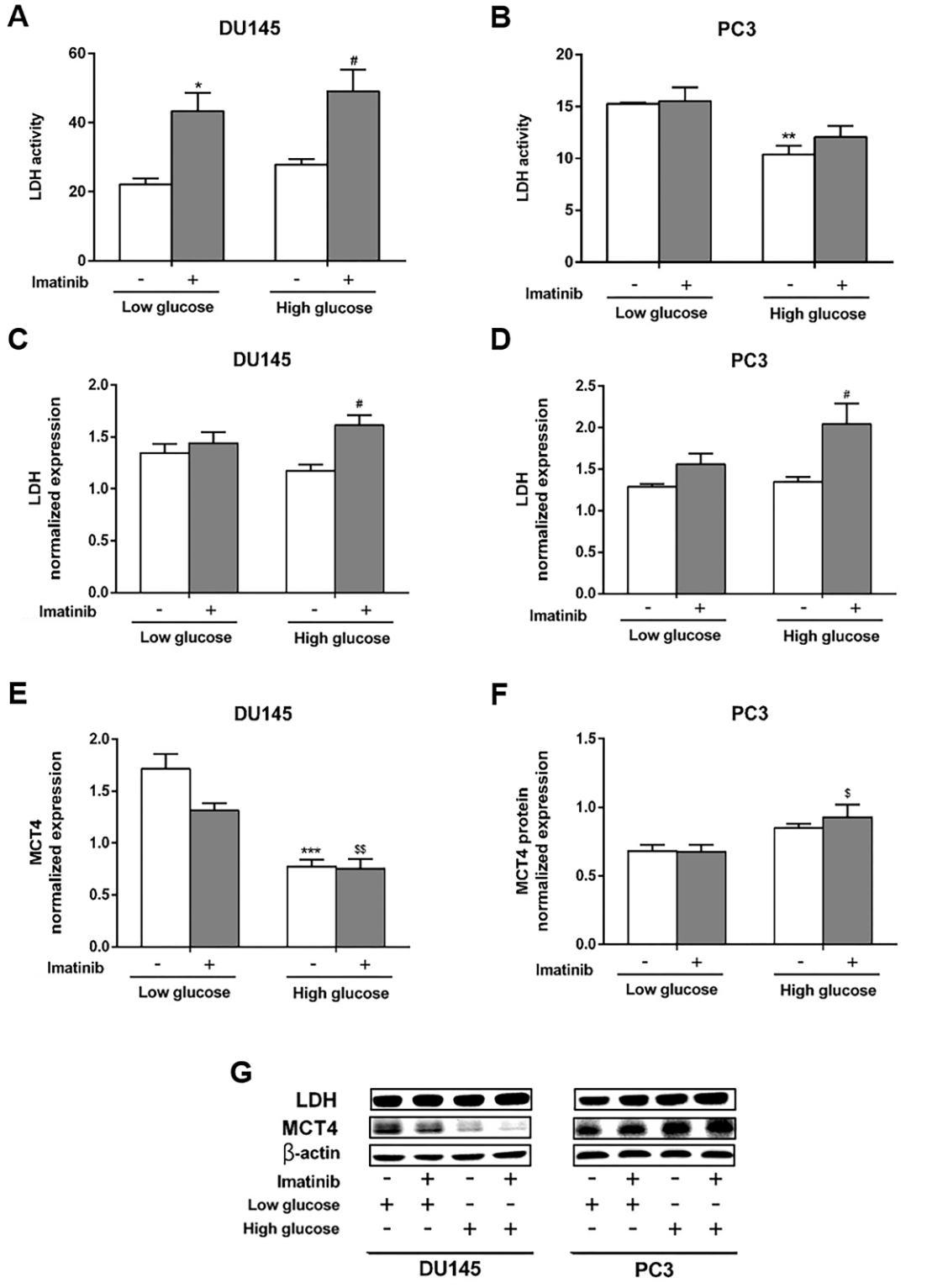

Figure 8.5. LDH activity (A, B) and protein levels of LDH (C, D) and MCT4 (E, F) in DU145 and PC3 cells treated with imatinib (20 μM) under hypoglycemic (5 mM) or hyperglycemic conditions (30 mM) for 72 h. Representative immunoblots are shown in panel G. Error bars indicate mean ± S.E.M (n= 5).* $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared with the imatinib (-) low glucose; $ $p<0.05$; $$ $p<0.01$; when compared with the imatinib (+) low glucose; # $p<0.05$ when compared with the imatinib (-) high glucose.

0,03 in hyperglycaemia (P<0,05, Fig. 8.4.C). A similar pattern was observed in PC3 cells (Fig. 8.4.D): imatinib (-), 0,54 ± 0,04 in hypoglycemia *vs.*0,82 ± 0,05 in hyperglycemia (P<0,05); imatinib (+), 0,53 ± 0,06 in hypoglycemia *vs.* 0,86 ± 0,11 in hyperglycemia (P<0,05).

An important step in the glycolytic flux is the conversion of fructose 6-phosphate into fructose 1,6-bisphosphate by PFK1, which is often considered a rate-limiting step in glycolysis (28). However, no differences were observed in the expression of PFK1 among experimental groups in DU145 (Fig. 8.4.E) and PC3 (Fig. 8.4.F) cells. Other crucial enzyme in the glycolytic process is LDH, which mediates the production of lactate from pyruvate upon glucose metabolization (17). LDH activity in DU145 cells, was increased in the imatinib (+) group in hyperglycaemia (49,1 ± 6,3 *vs.* 27,9 ± 1,6, P<0,05, Fig. 8.5.A). In the PC3 cell line, no effect of imatinib was observed but LDH activity diminished in the imatinib (-) group in hyperglycaemia (10,4 ± 0,8 vs. 15,3 ± 0,18, P<0,01, Fig. 8.5.B). We also identified an augmented expression of LDH in imatinib-treated DU145 and PC3 cells under hyperglycemia (respectively, 1,6 ± 0,1 *vs.* 1,2 ± 0,1, P<0,05 (Fig. 8.5.C) and 2,0 ± 0,3 *vs.* 1,4 ± 0,1, P<0,05 (Fig. 8.5.D)).

The lactate produced is exported to the extracellular space by a member of the MCTs family, the MCT4 that has been associated with PCa progression and poor prognosis (29, 30). In the DU145 cell line, MCT4 expression was significantly decreased in hyperglycemic groups (0,8 ± 0,1 *vs.* 1,7 ± 0,1 in imatinib (-) and 0,8 ± 0,1 *vs.* 1,3 ± 0,1 in imatinib (+), Fig. 8.5.E). However, in imatinib-treated PC3 cells the expression of MCT4 was significantly increased in the hyperglycaemia group (0,9 ± 0,1 *vs.* 0,7 ± 0,1 in hypoglycaemia, Fig. 8.5.F).

8.4. Discussion

The present study investigated the effect of imatinib on viability, apoptosis and glycolytic metabolism in two cell line models of CRPC under conditions of different glucose availability.
We observed that imatinib was effective in decreasing the viability of DU145 and PC3 cells, which was restricted to the 30 mM glucose groups (Fig. 8.1.). The diminished viability of DU145 and PC3 cells in response to imatinib in hyperglycemic conditions was accompanied by the altered expression and activity of apoptosis regulators of both the intrinsic and extrinsic pathways (Fig. 8.2.). This included the down-regulation of the anti-apoptotic Bcl-2 protein that prevents the release of cytochrome C into the cytosol and consequently inhibits apoptosis (31); and the up-regulation of the pro-apoptotic protein Bax, at least in PC3 cells. Hyperglycemic conditions, independently of imatinib treatment, were also characterized by the increased expression of caspase-8 and caspase-9, the initiator caspases of the intrinsic and extrinsic pathways of apoptosis (32), respectively. However, glycaemia itself had no effect on DU145 or PC3 cells viability (Fig. 8.1.).

Both caspase-8 and -9 can activate caspase-3, the known effector protein of the apoptotic process, which activity is considered a measure of the apoptosis rate (32) (33). The

results obtained demonstrated that imatinib treatment, though not affecting caspase-8 and caspase-9 expression, strongly increased caspase-3 activity in DU145 and PC3 cells (Fig. 8.2.I and 8.2.J). Therefore, it is expected that caspase-8 or caspase-9 activity would be increased in response to imatinib despite no changes on protein expression were detected. Moreover, the effect of imatinib enhancing caspase-3 activity was only perceived in conditions if high-glucose availability, which is in accordance with the observed down-regulation of Bcl-2 and up-regulation of Bax. These findings showed that apoptosis is augmented in response to imatinib (high glucose), and are in line with the diminished cell viability observed in the imatinib (+) hyperglycemic groups (Fig. 8.1.).

Altogether, our results revealed that imatinib is more effective inducing apoptosis and suppressing viability of CRPC lines in conditions of hyperglycemia. The exact mechanisms by which hyperglycemia potentiates imatinib-induced apoptosis of PCa cells are still unknown, but in breast cancer MCF-7 cells it was shown that this glycemic condition increased the cytotoxicity of carboplatin and 5-fluorouracil by reducing the expression of P-glycoprotein and increasing oxidative stress levels (13).

Glycaemia and the glycolytic status of the tumor microenvironment have been shown to influence the response of PCa cells to other therapeutic targets. For example, hyperglycaemia was shown to induce chemoresistance of PCa cells to the taxane docetaxel (21). Nevertheless, at least for our knowledge, this is the first study addressing the effect of the tyrosine kinase inhibitor imatinib under conditions of different glucose availability. The present findings highlight for the complex functional basis of imatinib therapy in PCa, which seems to depend on the environmental conditions and cell-specificities. Previous studies also have reported the distinct effects of imatinib controlling proliferation and apoptosis of DU145 and PC3 cells (11).

Despite not always in agreement with the *in vitro* findings, several epidemiological association studies have been indicating diabetes as a protective or a good prognostic factor in PCa (34-40). Our results showed that higher levels of glucose, the main serum alteration in diabetic patients, potentiated the effects of imatinib decreasing PCa cell viability and survival, which raises the curiosity about the efficacy of imatinib for treatment of castration-resistant diabetic patients.

Recent evidence has also highlighted for the fact that imatinib treatment provokes alterations in blood glucose levels of cancer patients with/without diabetes (41, 42). Imatinib, as well as Dasatinib, another tyrosine kinase inhibitor widely used for treatment of leukaemia, were related with scenarios of hypoglycaemia (14, 15, 41). Moreover, modifications of fasting glucose in chronic myeloid leukaemia patients have been considered as the first sign of resistance to imatinib (43). It was also shown that chronic myeloid leukaemia cells resistant to imatinib have increased glycolytic activity and lactate production relatively to sensitive-imatinib cells (44), which is typical of cancer cells with more aggressive phenotypes (19). Taking into account the influence of imatinib over glucose handling, we

evaluated its effect modulating the glycolytic metabolism of CRPC cells in conditions of hypo- and hyperglycemia.

Glucose consumption was augmented in DU145 and PC3 cells treated with imatinib (Fig. 8.3.A and 8.3.B), with results more pronounced in low glucose groups. This trend was somehow expected since high glucose availability has been shown to diminish glucose consumption in different cell types (45-47).

The uptake of glucose from the extracellular medium is carried out by members of GLUTs family, which in PCa cells has been indicated to be a task of GLUT1 and GLUT3 (19, 27). GLUT1 expression was diminished in DU145 cells in hyperglycemic conditions (Fig. 8.4.A) comparatively with the 5 mM glucose imatinib-treated groups, which is in accordance with the reduced consumption of glucose observed in these conditions. However, despite the increased glucose consumption observed in imatinb (+) groups, no significant alterations were found on GLUT1 or GLUT3 expression (Fig. 8.4.), which may suggest that other GLUTs can be involved. A likely candidate would be the GLUT12, as this transporter was recently identified as an androgen target gene and the GLUT responsible for the androgenic control of glucose uptake and PCa cell growth (48).

Also, it is curious the observed shift on GLUT1 and GLUT3 expression in response to glucose availability; GLUT1 expression was decreased in high glucose conditions whereas GLUT3 levels were increased. These results are supported by other studies showing the differential expression of GLUTs dependently on glucose concentrations (49, 50). At the moment, it is not clear how imatinib facilitates entry of glucose into cancer cells, and how imatinib treatment contributes to decreasing blood glucose levels in cancer patients (14, 15, 41). However, in light of the results obtained herein in PCa cells is liable to suggest that the increased glycolytic activity driven by imatinib may sustain the diminution of blood glucose concentrations.

Another crucial step in the glycolytic process is the irreversible conversion of fructose 6-phosphate to fructose 1,6-bisphosphate, a reaction catalysed by PFK1, but no differences could be observed on PFK1 expression levels, related with imatinib treatment or glycaemic conditions.

The glycolytic flux ends with the conversion of pyruvate to lactate through the activity of LDH, and the export of lactate to the extracellular space. Following the effects observed on glucose consumption, imatinib treatment increased lactate production in DU145 and PC3 cells (Fig. 8.3.) under different glycemic conditions. Moreover, the augmented lactate production was accompanied by the enhanced expression of LDH in both cell lines (hyperglycemia, Fig. 8.5.C and 8.5.D), and by the increased enzyme activity in DU145 (Fig. 8.5.A).

The MCTs are the molecular partners involved in the export of lactate to the extracellular space, which in the case of PCa cells has suggested to be a role of the MCT4. MCT4 has been shown to be expressed in DU145 and PC3 cells and in tumour samples of CRPC patients (19, 30, 51, 52). Despite the alterations in lactate production, imatinib treatment

did not show to alter MCT4 expression levels (Fig. 8.5.E and 8.5.F). Nevertheless, hyperglycemia conditions induced a down-regulation of MCT4 expression in DU145 cells, which was consistent with the lower production of lactate when cultured in 30 mM glucose comparatively with the culture medium containing 5 mM glucose.

Overall, imatinib stimulated glucose consumption and lactate production in CRPC, independently of glucose availability, due to the increased expression and activity of LDH. These findings are particularly relevant considering the previous studies showing that the increased expression of LDH and export of lactate are associated with the resistance of cancer cells to treatment (53). Indeed, increased levels of lactate in the tumor microenvironment have been shown to favor tumor growth, invasion, and aggressiveness, as well as, suppressing the immune system (53-55). Co-treatment of CRPC cells with imatinib and an LDH inhibitor might be the next step to investigate the effectiveness of this tyrosine kinase inhibitor for treatment of PCa in hyperglycemia conditions.

8.5. Conclusion

It was demonstrated that imatinib suppressed the viability of DU145 and PC3 cells whereas inducing apoptosis, which was restricted to a glucose enriched environment (Fig. 8.6.). This was the first study addressing the effect of this compound under conditions of different glucose availability and allowed to conclude that hyperglycaemia increases the effectiveness of imatinib suppressing growth and survival of CRPC cells. The obtained results also highlighted for the complexity of the functional basis underlying the response to imatinib therapy. In addition, imatinib treatment stimulated glucose consumption and lactate production, which can be linked with cancer cells aggressiveness and mechanisms of resistance. Altogether, the present work provided a set of pre-clinical findings that raise the curiosity about the use of imatinib for treatment of CRPC diabetic patients, as well as of its administration simultaneously with inhibitors of glycolytic metabolism.

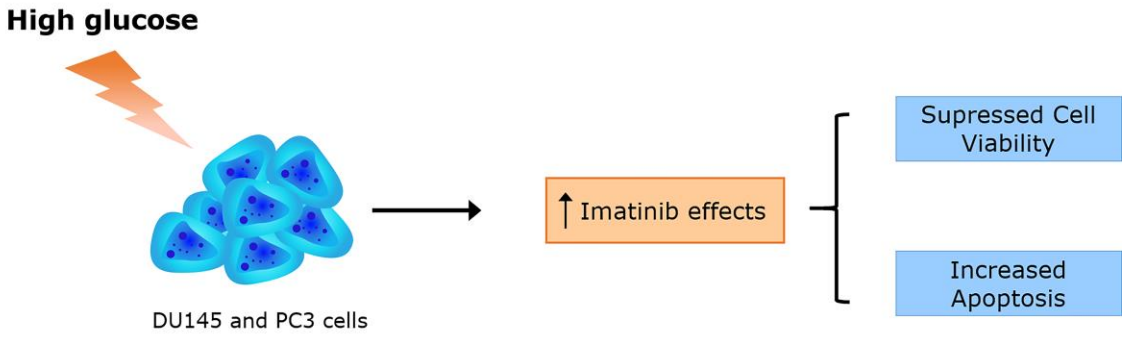

Figure 8.6. The glycolytic environment modulated the effect of the tyrosine kinase inhibitor imatinib in CRPC cells. High glucose availability potentiated imatinib actions suppressing viability and enhancing apoptosis of DU145 and PC3 cells.

8.6. Acknowledgments

None.

8.7. Funding

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project No. 007491) and National Funds by FCT-Foundation for Science and Technology (Project UID/Multi/00709/2013). Cardoso HJ and Figueira MI were recipient of BID/ICI-FCS/CICS/Santander and FCT fellowships, respectively, SFRH/BD/111351/2015 and SFRH/BD/104671/2014.

8.8. Conflict of Interest

The authors declare that they have no conflicts of interest.

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Chapter 9

Summarizing discussion and final remarks

Summarizing discussion

Despite the important advances in diagnosis and treatment, PCa remains a preoccupant disease, with high levels of prevalence and mortality worldwide. Basic and clinical research has contributed enormously in the understanding of the molecular basis of disease, which allowed important improvements in the diagnosis and treatment of PCa. However, the management of PCa continues to be a limitation for clinicians and a relevant scientific question. The identification of biomarkers for i) early detection of disease; ii) to distinguish indolent from aggressive disease, and iii) to predict treatment responses and progression of disease, still is need. Also, the understanding of the mechanistic driving the progression of PCa from the androgen-sensitive to the CRPC remains an unmet medical need. Currently, it is known that the initiation of PCa depends on genetic alterations, environmental pressures, lifestyle, and complex modifications in the expression or activity of several regulators of cell survival, proliferation and energy metabolism. Also, it is accepted that the establishment of CRPC is related with the reprogramming of PCa cells metabolism, as well as with resistance to ADT in consequence of disrupted AR signalling or augmented intra-prostatic secretion of androgens. Nevertheless, effective treatment approaches for PCa, slowing the progression to CRPC (usually occurring on average after 38 months of ADT) and better managing of metastatic stages of disease (lethal on average after 21 months) continue to be warranted.

Many years ago (1920s) Otto Warburg was pioneered in describing the tumour cells' capacity to increase glycolytic activity consuming high amounts of glucose and producing lactate as an energy source, the so-called Warburg effect. More recently, in 2011, the metabolic reprogramming in association with an over-activity of several oncogenes was considered a hallmark of cancer. The emergence of metabolic alterations as a cancer hallmark reawaken the research interest on cancer cell metabolism, which also has been explored in the context of therapy. The response of cancer cells altering energy metabolism pathways represents an adaptive advantage providing the resources needed to sustain survival and uncontrolled growth. Nowadays, the great discovery of Warburg that cancer cells have specificities in the metabolism of glucose was amplified, with the metabolic reprogramming involving a complex network of metabolites, growth factors, hormones, and oncogenic signalling pathways. Besides glucose, glutamine and fatty acids are major nutrients for cancer cells. Glutamine is used as an energy substrate, but also plays a role feeding glycolysis and the TCA cycle. Fatty acids, and lipids in general, are needed by cancer cells for membrane biogenesis, energy production and cell signalling. Moreover, the tumour microenvironment also has been shown to have a critical role in the metabolic reprograming, existing a cross-talk between cancer cells and other cells types in the tumour stroma.

In the case of prostate, it was demonstrated that the unique metabolism of non-neoplastic prostate cells, producing and secreting high amounts of citrate (in consequence of a truncated TCA cycle), is "corrupted" in cancer. In PCa cells, the TCA cycle and OXPHOS are reactivated, as well as other metabolic pathways such as glycolysis, glutaminolysis and fatty

acid β-oxidation. However, unlike other cancer types, PCa displays a tenue Warburg Effect that only is more evident in aggressive stages of disease. Also, for this reason, other metabolic pathways have emerged as critical routes in PCa development. The present thesis contributed to clarifying the metabolic peculiarities of PCa cells, and how these features can be influenced by the sex steroid hormones androgens, as well as by nutrient availability.

In Chapter 5 of this thesis, it was characterized the reliance of androgen-sensitive (LNCaP) and CRPC cells (DU145 and PC3) on specific metabolic pathways. We confirmed that CRPC cells presented enhanced activity of glycolytic pathway and higher capacity to use glycolysis when mitochondria was inhibited (Fig. 9.1.). Moreover, we observed the functionality of PCa cells mitochondria using glucose, glutamine and fatty acid metabolites. The CRPC PC3 cells were the cancer cell line with less dependency on all metabolites and displayed the highest capacity using glutamine and glucose when the other pathways were inhibited. On the other hand, LNCaP cells were the ones more dependent on glucose, glutamine and fatty acids, exhibiting greater difficulty to readapt to the inhibition of the respective metabolic pathway. These findings demonstrated that CRPC cells have a higher

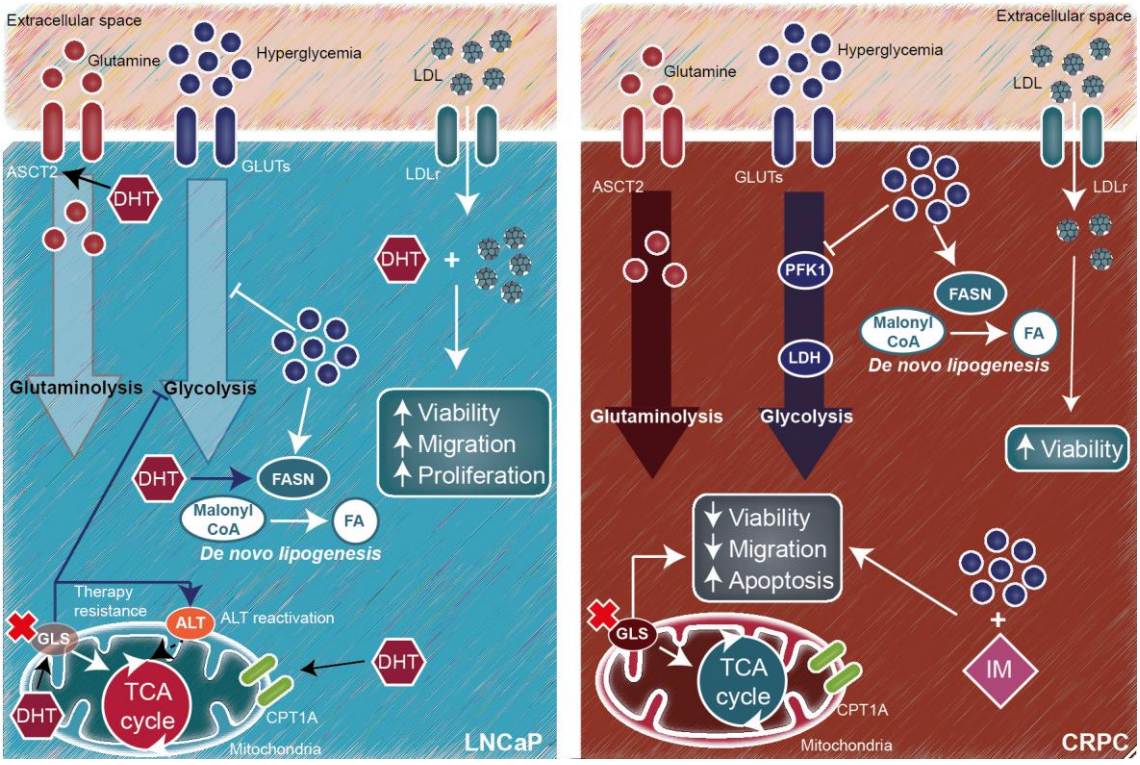

Figure 9.1. An integrative view of the metabolic specificities of androgen-sensitive LNCaP cells and castrate-resistant prostate cancer (CRPC) cells. Glutaminolysis and glycolysis are over-activated in CRPC cells relative to LNCaP (darker arrows). 5α-dihydrotestosterone (DHT) enhance the expression of different metabolic regulators, such as glutamin transporter (ASCT2), glutaminase (GLS), fatty acid (FA) synthase (FASN), and carnitine palmitoyltransferase 1A (CPT1A) in LNCaP cells. Androgens have been shown to be major stimulators of FA de novo synthesis from maloyl-CoA by increasing the expression and activity of FASN. Low-density lipoprotein (LDL)/LDLr increase the viability of PCa cells, and the combination with DHT has a synergic effect in promoting the viability, proliferation and migration of LNCaP cells. Inhibition of GLS was effective in decreasing the viability, growing and migration of CRPC cells, whereas increasing apoptosis. However, LNCaP cells were less sensitive to the GLS inhibition, likely by the reactivation ALT and decrease of glycolysis. Hyperglycaemia shifts the metabolism in the direction of FA increasing FASN expression, with a decrease in the utilization of glycolysis. Moreover, conditions of high glucose availability (hyperglycaemia) augment the effectiveness of tyrosine kinase inhibitor imatinib mesylate (IM) decreasing the viability, and increasing apoptosis, in CRPC cells.

ability to readapt to adverse circumstances of nutrients limitation, and to utilize alternative metabolic pathways to survive.

In Chapter 6, we investigated the relevance of glutaminolysis for PCa cells survival and growth and the effect of androgens in the regulation of glutamine metabolism. Differential expression of glutaminolysis regulators, glutamine transporter ASCT2 and glutaminase, was found in CRPC cells compared with LNCaP. CRPC cells displayed lower expression of ASCT2, whereas glutaminase expression was higher. These findings followed the expression pattern found in human PCa tissues and indicated the relevance of glutaminolysis in CRPC cells. Indeed, inhibition of glutaminase induced apoptosis, and decreased cell viability and migration of PCa cells, with effects more pronounced in CRPC cells, which raised the question considering targeting glutaminase for CRPC therapy. Androgen-sensitive LNCaP cells were resistant to glutaminase inhibition, at least for the lowest concentrations of inhibitor, which likely was related to the increased activity of ALT (Fig. 9.1.). Moreover, this study showed that blocking glutaminolysis had an impact on glycolysis and lipid metabolism in PCa cells. These results highlight the importance of fully understanding cancer metabolic remodelling whenever specific metabolic routes are inhibited with therapeutic purposes.

The development of resistance to ADT is a huge constraint in PCa treatment, which demands searching for new alternative and effective treatments. Some studies have tested the efficacy of ADT in combination with other anti-cancer drugs, namely docetaxel or cabazitaxel, though the benefits for patients were poor. Since previous studies have pointed out androgens as metabolic regulators in PCa we pursued to evaluated the androgenic regulation of glutaminolysis, and the effect of inhibiting this metabolic pathway simultaneously with the administration of an anti- androgen. In Chapter 6, it was also shown that androgens upregulated ASCT2 and glutaminase expression both *in vitro* and *in vivo*. In addition, DHT treatment induced the ASCT2 localization at LNCaP cells membrane, which was underpinned by enhanced glutamine consumption. Altogether, these findings broaden the role of androgens as central regulators of glutamine metabolism in PCa cells. On the other hand, co-targeting the AR axis and glutamine metabolism concomitantly showed a synergic effect suppressing the viability of PCa cells. The present findings opened interesting perspectives for the exploitation of targeting glutaminolysis and the AR in future preclinical and clinical studies.

In the context of metabolic regulation, androgens have been shown to stimulate lipid metabolism by modulating the expression and activity of several molecular targets, from lipid uptake, to synthesis and storage. However, there were knowledge gaps in these processes. Also, it was unknown if the androgenic regulation of lipid handling could be affected by increased lipid availability, as is the case in hypercholesterolemia and obesity. Chapter 7 showed that androgens upregulated FASN and CPT1A expression in prostate cells. In addition, the effect of DHT regulating FASN was dependent on SREBP activity (lipid-depleted conditions) and blocked by LDL availability. These findings supported the broad role of

androgens in the metabolic reprogramming of PCa cells creating a favourable environment for cell proliferation (Fig. 9.1.).

Obesity has been considered a risk factor for the development of aggressive PCa, with accelerated progression to CRPC, poor prognosis and reduced survival rates. Also, hypercholesteremia and abdominal obesity were related with PCa. However, the direct influence of LDL on PCa cell fate was unknown. The results presented in Chapter 7 demonstrated that LDL-cholesterol promoted PCa cells viability, proliferation, and migration dependently on the presence of DHT (Fig. 9.1.). LDL(+)/DHT(+) treatment also induced the accumulation of lipid droplets, and LDL itself, besides suppressing FASN expression, increased CPT1A levels indicating that fatty acids were being driven to β-oxidation. In turn, CPT1A inhibition with etomoxir reverted the LDL effects promoting LNCaP cells viability, which indicated that fatty acid β–oxidation was sustaining cell viability. This thesis results first showed the interplay of LDL and androgens enhancing viability, proliferation and migration of androgen-sensitive PCa cells. These findings are supported by the reports showing that high cholesterol levels fuel the intratumoral androgen synthesis. Furthermore, they sustain the development of future strategies for PCa treatment targeting obesity and the AR simultaneously.

Previously published studies demonstrated the influence of nutrient availability and tumour metabolic environment in the regulation of metabolic pathways, and conditioning PCa cells response. Some of these articles also showed that different glucose concentrations in the cell culture medium can modulate PCa cells sensitivity to anti-cancer drugs. Chapter 8 showed that the receptor tyrosine kinase inhibitor imatinib suppressed the viability of DU145 and PC3 CRPC cells whereas inducing apoptosis, restrictedly to a glucose-enriched environment. This allowed to conclude that hyperglycaemia increases the effectiveness of this drug in CRPC cells (Fig. 9.1.). Moreover, the glycolytic environment and lactate production could be related with imatinib resistance treatment. Overall, this chapter showed that hyperglycemia, the main serum alteration in diabetic patients, potentiated the effects of imatinib in CRPC cells, which raises the curiosity about the efficacy of this drug for treatment of castration-resistant diabetic patients. Gathered information strongly indicates that PCa treatment must be carefully managed concerning the metabolic environment and its interaction with drug efficiency.

Final Remarks

The work presented in this thesis confirmed the crucial actions of androgens in the regulation of metabolic pathways in PCa (glycolysis, glutaminolysis and lipid metabolism), identifying new molecular targets and highlighting the relevance of environmental influences (Fig. 9.1.). The androgenic effects were pivotal for PCa obtaining energy and, consequently, in the promotion of proliferation and metastasis.

On the relationship between PCa and obesity, the outcomes of this thesis demonstrated that cholesterol and androgens could be the "best friends" driving PCa carcinogenesis. This is of paramount importance in light of the increasing incidence of non-healthy eating habits and high cholesterol levels, and the epidemic obesity in Western societies, which in combination with the male sex hormones could be an "explosive" combination. The analysis of the epidemiological meaning of androgens and cholesterol circulating levels (high or low) and PCa risk is urgent. Furthermore, this work alerts for the particular attention clinicians should have when managing PCa disease in patients with high cholesterol levels.

Considering cancer cells metabolism, this dissertation was crucial in demonstrating the flexibility and capacity of PCa cells using different energy sources. Several metabolites are the food to feed the survival of cancer cells, which are capable of "eating" more than one aliment, adapting their habits considering specific nutrients scarcity or abundance. The findings obtained here also contributed to a better understanding of the role of lipids and glutamine in PCa cell viability, proliferation, highlighting for the molecular mechanisms underlying the metabolic support of cancer cell survival and growth. Moreover, the present thesis showed the differential utilization of metabolites in androgen-sensitive and CRPC PCa cells, a matter that deserves special attention in the future. Metabolism-based therapies for both androgen-sensitive and CRPC should be viewed with good expectations in light of these facts.

Another rationale of this work was the use of combined therapies as effective treatments for PCa. At this point, new windows were opened considering the inhibition of lipid and glutamine metabolism together with AR signalling or other metabolic active pathways. Future treatment approaches would encompass the utilization of cocktails of several metabolic inhibitors or/and indirect metabolism regulators (e.g. oncogenes, or hormones), to avoid the metabolic escape of PCa cells.

In conclusion, the metabolic environment and androgens are the perfect pair in the orchestration of PCa metabolic reprogramming and cancer development, and should continue being intensively studied with the aim of finding better PCa therapies.