UNIVERSIDADE DA BEIRA INTERIOR
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Mecanismos moleculares da progressão do cancro da bexiga e modulação metabólica induzida pelo extrato de chá branco

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O conteúdo do presente trabalho é da exclusiva responsabilidade da autora:

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(Vanessa Raquel Conde)

Dedicatória

Aos meu pais, por todas as oportunidades, esforço e apoio. “Obrigado”, nunca será suficiente.

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Resumo

O cancro da bexiga constitui uma das formas mais comuns de cancro. A maioria dos casos corresponde a tumores superficiais papilares, mas existe a possibilidade de estes evoluírem para um fenótipo muito mais agressivo e potencialmente fatal. É sabido que o metabolismo cancerígeno está intrinsecamente relacionado com elevado fluxo glicolítico, um fenómeno conhecido como efeito Warburg. Mesmo na presença de quantidades de oxigénio suficientes para realizar o processo de fosforilação oxidativa mitocondrial, estas células utilizam a glucose como principal fonte de energia, e exportam grandes quantidades de lactato. Deste modo, o estudo do metabolismo das células cancerígenas da bexiga e a forma como se associa com a progressão para estadios mais agressivos é fundamental para o desenvolvimento de novos métodos de diagnóstico e estratégias terapêuticas. Por outro lado, sabe-se que esta doença é influenciada por factores dietéticos, de entre quais o consumo de chá tem sido destacado em vários estudos. O chá, uma bebida obtida através da infusão de folhas de *Camellia sinensis*, é amplamente conhecido pelas suas propriedades anticancerígenas. De facto, vários estudos reportaram que o extrato de chá verde e alguns dos seus componentes podem causar apoptose, interrupções no ciclo celular e modular vias de sinalização específicas em células cancerígenas da bexiga. Foi também demonstrado que a ação destes componentes pode resultar na inibição da metastização e dos processos angiogénicos tumorais. Pensa-se que o chá branco, apesar de não estar tão estudado, possa possuir propriedades anticancerígenas mais intensas que os outros tipos de chá.

O primeiro objetivo deste trabalho foi analisar o metabolismo glicolítico de duas linhas celulares humanas de cancro da bexiga, representativas de diferentes estadios de progressão do cancro: RT4, representativas de um estadio primitivo, e TCCSUP, representativas de um estadio altamente invasivo. Com este propósito, foi estabelecido o perfil glicolítico das duas linhas celulares. Para tal, o meio extracelular foi analisado através de Ressonância Magnética Nuclear e os níveis de glucose, piruvato, alanina e lactato produzidos foram quantificados. Procedeu-se ainda à análise das expressões dos transportadores de glucose 1 e 3 (GLUT1 e GLUT3), do transportador de monocarboxilato 4 (MCT4) e das enzimas fosfofrutocinase 1 (PFK), glutamato-piruvato transaminase (GPT) e lactato desidrogenase (LDH) através da técnica de Western Blot. Com este estudo pretende-se contribuir para a identificação dos alvos moleculares terapêuticos para evitar ou contrariar a progressão do cancro da bexiga. Os nossos resultados demonstraram que, apesar de os níveis de consumo de glucose terem sido semelhantes em ambas as linhas celulares, os níveis de GLUT1, PFK e GPT estavam severamente reduzidos nas células TCCSUP, que representam um estadio altamente invasivo de cancro da bexiga. Além disso, estas células consumiam grandes quantidades de piruvato, levando à produção de grandes quantidades de lactato e alanina. O segundo objetivo deste trabalho consistiu no estudo preliminar dos efeitos de diferentes concentrações de extrato aquoso de chá branco na sobrevivência e no perfil glicolítico de ambas as linhas celulares, de

forma a obter novas perspetivas acerca dos mecanismos através dos quais o chá branco exibe os seus efeitos anticancerígenos. Pretende-se, por fim, possibilitar o desenvolvimento de novos suplementos alimentares ou farmacêuticos para combater o cancro da bexiga. Deste modo, as células foram tratadas com diferentes concentrações de extrato aquoso de chá branco durante 48 horas. Os efeitos citotóxicos do extrato de chá branco foram avaliados através de um ensaio com sulforrodamina B. Depois de identificadas as concentrações de mais apropriadas para o estudo, as células foram tratadas com essas concentrações durante 24 horas. As expressões de GLUT1, MCT4, PFK e LDH foram determinadas através de Western Blot. Os estudos acerca da citotoxicidade revelaram que as concentrações de 0.25 mg/ml e 1 mg/ml de extrato induziram significativa morte celular no estadio mais primitivo de cancro da bexiga, representado pelas células RT4, mas a indução de morte celular significativa nas células TCCSUP foi atingida apenas com a concentração de 1 mg/ml. De notar, os níveis de expressão do GLUT1, PFK, LDH e MCT4 não foram significativamente alterados com o tratamento em nenhuma das linhas celulares.

Os nossos resultados demonstram que a progressão do cancro da bexiga está associada a diversas alterações no metabolismo das células, particularmente no consumo de piruvato. Para além disso, verificámos que o consumo de glucose não é alterado na progressão de um estadio primitivo para um estadio altamente invasivo no cancro da bexiga, mas são produzidos níveis significativamente elevados de lactato e alanina, indicativos de um metabolismo mais acelerado. Estes factores podem indicar de que forma as células cancerígenas da bexiga respondem a ambientes agressivos, como estados de hipoxia. Além disso, os estudos de citotoxicidade revelaram que, apesar de o extrato de chá branco ser capaz de induzir morte celular em ambos os estadios de cancro da bexiga, é necessária uma concentração mais elevada para induzir a morte celular no estadio mais agressivo; isto sugere que as células de diferentes estadios de cancro da bexiga podem apresentar diferenças em termos de mecanismos de sobrevivência e/ou proliferação. Os nossos resultados preliminares indicam que esta indução de morte celular pelo extrato de chá branco não parece estar associada a alterações nos níveis de expressão de transportadores ou enzimas relacionadas com o processo glicolítico, mas são necessários mais estudos para comprovar estes resultados.

Este trabalho demonstra que existem alterações metabólicas significativas na via glicolítica das células cancerígenas da bexiga, à medida que o cancro progride, e que o metabolismo do piruvato tem um papel preponderante. Estes resultados fornecem evidências importantes de que o metabolismo, particularmente um *shift* do consumo de glucose para piruvato, está envolvido na progressão de um estadio primitivo para altamente invasivo no cancro de bexiga. Foi ainda demonstrado que o chá branco é capaz de induzir a morte celular tanto em estadios primitivos da doença como em estadios mais avançados, embora neste último as concentrações de chá branco necessárias sejam mais elevadas. Estes são factos importantes para o futuro desenvolvimento de novas estratégias terapêuticas para o cancro da bexiga.

Palavras-chave

Cancro da bexiga, TCCSUP, RT4, efeito Warburg, *Camellia sinensis*, chá branco.

Resumo Alargado

O cancro da bexiga constitui uma das formas mais comuns de cancro e pode surgir sob diversas formas, sendo o carcinoma de transição celular a mais comum. Cerca de 80% dos casos de cancro da bexiga são tumores superficiais papilares; os restantes 20% constituem tumores altamente invasivos e agressivos. Cerca de 10 a 15% dos tumores superficiais evoluem para um fenótipo muito mais agressivo e potencialmente fatal. As alterações sofridas pelas células saudáveis que originam células cancerígenas uroteliais são principalmente atribuídas a mutações genéticas e factores ambientais; no entanto, os mecanismos metabólicos responsáveis pela progressão do cancro da bexiga permanecem, na sua maioria, desconhecidos. É conhecido que o metabolismo cancerígeno está intrinsecamente relacionado com o elevado fluxo glicolítico, um fenómeno conhecido como o efeito Warburg. Mesmo na presença de quantidades de oxigénio suficientes para realizar o processo de fosforilação oxidativa mitocondrial, estas células preferem utilizar a glucose como fonte de energia e exportam quantidades excessivas de lactato. Este fenómeno já foi verificado em tumores urinários, refletido nos níveis elevados de intervenientes metabólicos da glicólise e que, nalguns casos, foram correlacionados com o aumento da malignidade dos tumores. O estudo do metabolismo das células cancerígenas da bexiga e a forma como se associa com a progressão para estados mais agressivos é fundamental para o desenvolvimento de novos métodos de diagnóstico, estratégias terapêuticas e até mesmo para o desenvolvimento de estratégias que possam ajudar a prever a sobrevivência dos doentes. Por outro lado, estudos demonstraram que o desenvolvimento desta doença é influenciado por factores dietéticos, dos quais o consumo de chá tem sido destacado. O chá, uma bebida obtida através da infusão de folhas de *Camellia sinensis*, é amplamente conhecido pelas suas propriedades promotoras da saúde. Dentre estas propriedades, os efeitos anticancerígenos do chá estão bem documentados. A planta *C. sinensis* pode originar quatro tipos diferentes de chá: chá branco, chá verde, chá *oolong* e chá preto. Todos os tipos de chá possuem atividade anticancerígena mas pensa-se que o chá branco, devido à sua composição fitoquímica rica em antioxidantes, possui propriedades anticancerígenas superiores. De facto, a atividade anticancerígena dos chás verde, oolong e preto está documentada; contudo, os estudos acerca dos efeitos anticancerígenos do chá branco, mesmo que previsíveis, são escassos. Particularmente na área do cancro da bexiga, vários estudos reportaram que o extrato de chá verde e alguns dos seus componentes podem causar apoptose, interrupções no ciclo celular e modular vias de sinalização específicas em células cancerígenas da bexiga. Além disso, a ação destes componentes também resultou na inibição da metastização e dos processos angiogénicos tumorais.

Deste modo, neste trabalho é feito o estudo do metabolismo glicolítico de duas linhas celulares humanas de cancro da bexiga, representativas de diferentes estadios de progressão do cancro: RT4, representativas de um estadio primitivo, e TCCSUP, de um estadio altamente

invasivo. Este estudo poderá contribuir para a identificação de alvos moleculares terapêuticos para evitar ou contrariar a progressão do cancro da bexiga. Também foram realizados estudos preliminares acerca dos efeitos de diferentes concentrações de extrato de chá branco na sobrevivência e no perfil glicolítico de ambas as linhas celulares, de forma a obter novas perspetivas acerca dos mecanismos através dos quais o chá branco exibe os seus efeitos anticancerígenos. Estes estudos poderão possibilitar o desenvolvimento de novos suplementos alimentares ou farmacêuticos para combater o cancro da bexiga.

Com estes propósitos, estabelecemos o perfil glicolítico das duas linhas celulares humanas de cancro da bexiga, RT4 e TCCSUP. Os níveis de glucose, piruvato, alanina e lactato produzidos foram analisados no meio extracelular através de Ressonância Magnética Nuclear. A expressão dos transportadores de glucose 1 e 3 (GLUT1 e GLUT3), do transportador de monocarboxilato 4 (MCT4) e das enzimas fosfofrutocinase 1 (PFK), glutamato-piruvato transaminase (GPT) e lactato desidrogenase (LDH) foi avaliada por Western Blot. Depois, as células foram tratadas com concentrações de extrato de chá branco de 0.025 mg/ml, 0.1 mg/ml, 0.25 mg/ml e 1 mg/ml durante 48 horas. A citotoxicidade foi avaliada através de um ensaio com sulforrodamina B. Depois de identificar as concentrações de chá branco responsáveis por uma morte celular significativa nas duas linhas celulares (1 mg/ml) e por não provocar morte significativa em nenhuma delas (0.1 mg/ml), as células foram tratadas com estas concentrações de extrato de chá branco durante 24 horas. A expressão do transportador GLUT1, do transportador MCT4 e das enzimas PFK e LDH foram também determinadas através de Western Blot. Os nossos resultados demonstraram que, apesar de os níveis de consumo de glucose terem sido semelhantes em ambas as linhas celulares, os níveis do GLUT1, da PFK e da GPT estavam severamente reduzidos na linha celular TCCSUP, representativa de um estadio altamente invasivo de cancro da bexiga. Além disso, estas células consumiam grandes quantidades de piruvato, levando à produção de grandes quantidades de lactato e alanina. O rácio lactato/alanina também foi significativamente superior nestas células, ilustrando um estado de maior *stress* oxidativo. Os estudos acerca da citotoxicidade induzida por extrato de chá branco revelaram que as concentrações de 0.25 mg/ml e 1 mg/ml de extrato induziram morte celular no estadio mais primitivo de cancro da bexiga, representado pelas células RT4, enquanto que nas células TCCSUP a morte celular induzida pelo extrato de chá branco apenas foi atingida na concentração de 1 mg/ml. Além disso, os níveis de expressão do GLUT1, da PFK, da LDH e do MCT4 não foram significativamente alterados com o tratamento com extrato de chá branco em nenhuma das linhas celulares.

Este trabalho demonstra portanto que a progressão do cancro da bexiga inclui muitas alterações no metabolismo das células, das quais o consumo de piruvato deve ser destacado. Também são apresentadas evidências de que o consumo de glucose não é alterado, mas são produzidos níveis significativamente elevados de lactato e alanina, indicativos de um metabolismo mais acelerado. Estes factores podem indicar de que forma as células

cancerígenas da bexiga respondem a ambientes agressivos, como estados de hipoxia. Além disso, os estudos de citotoxicidade revelaram que, apesar de o extrato de chá branco ser capaz de induzir morte celular em ambos os estadios de cancro da bexiga, é necessária uma concentração mais elevada para induzir a morte celular no estadio mais agressivo. Estes resultados sugerem que as células de diferentes estadios de cancro da bexiga podem apresentar diferenças em termos de mecanismos de sobrevivência e/ou proliferação, deste modo respondendo às ações do chá branco de formas diferentes. Os nossos resultados preliminares sugerem que esta indução de morte celular pelo extrato de chá branco não parece estar associada a alterações nos níveis de expressão de transportadores de glucose/lactato ou enzimas na glicólise e conversão de lactato, mas são necessários mais estudos para comprovar estes resultados.

Em conclusão, este trabalho vem demonstrar que existem alterações metabólicas significativas na via glicolítica das células cancerígenas da bexiga, à medida que o cancro progride, e que o metabolismo do piruvato tem um papel preponderante. Foi ainda demonstrado que o chá branco é capaz de induzir a morte celular, tanto em estadios primitivos da doença como em estadios mais avançados, embora neste último as concentrações de chá branco necessárias tenham sido mais elevadas. Estes resultados fornecem evidências importantes de que o metabolismo, particularmente um *shift* do consumo de glucose para piruvato, está envolvido na progressão de um estadio primitivo para altamente invasivo no cancro de bexiga. Por outro lado, fica claro que o chá branco possui propriedades anticancerígenas. Estas são descobertas relevantes para o futuro desenvolvimento de novas estratégias terapêuticas para o tratamento do cancro da bexiga.

Abstract

Bladder cancer is among the most common types of cancer and it can appear under different forms, being the transitional-cell carcinoma the most usual. The majority of the bladder cancer cases are superficial, low-grade tumors, but they may evolve to more aggressive and potentially fatal tumors. Cancer metabolism is intrinsically related to high glycolytic flux, a phenomenon known as the Warburg effect. Even in the presence of enough oxygen to sustain oxidative phosphorylation, these cells prefer to use glucose as main energy source, resulting in the export of very high levels of lactate. Knowledge of bladder cancer cells metabolism and how it is associated with progression to different and more aggressive states is still lacking and may help to develop new therapeutic approaches. Bladder cancer development is thought to be influenced by dietary factors, from which tea consumption has been highlighted. Tea is a beverage obtained from the infusion of the leaves or buds of the *Camellia sinensis* and is widely known for its anticancer properties. Particularly in bladder cancer, several studies reported that green tea extract and some of its components may cause cell apoptosis, cell cycle arrest, modulate cell specific pathways and inhibit metastization processes. White tea (WT), although not as well studied as the other types of tea, is thought to possess the highest anticancer properties among all types.

Herein we propose to study the glycolytic metabolism of two human urinary bladder cancer cell lines, representative of different cancer progression stages: RT4 (primitive stage) and TCCSUP (highly invasive stage). With these purpose, we established the glycolytic profile of the two human bladder cancer cell lines. Therefore, levels of glucose, pyruvate, alanine and lactate in extracellular media were measured by Proton Nuclear Magnetic Resonance. The expression of glucose transporters 1 and 3 (GLUT1 and GLUT3), monocarboxylate transporter 4 (MCT4), phosphofructokinase 1 (PFK), glutamic-pyruvate transaminase (GPT) and lactate dehydrogenase (LDH) was determined by Western blot. This may help to identify a molecular pharmacological/therapeutic target to counteract or avoid the progression of bladder cancer. Our results demonstrate that although glucose consumption levels were similar in both cell lines, the levels of GLUT1, PFK and GPT were severely reduced in the TCCSUP cell line, representative of the highly invasive cancer stage. Moreover, these cells consumed high quantities of pyruvate, yielding elevated amounts of lactate and alanine. We also propose to conduct preliminary studies on the effects of different WT extract concentrations on the survival and glycolytic profile of both cancer cell lines. This may yield new insights on the mechanisms through which WT exhibits its anticancer effects, raising hypothesis about its use, or of any of its components, in anticancer food supplements or drugs. For citotoxity studies, the cells were treated with different concentrations of WT extract during 48 hours. WT induced cytotoxicity was evaluated through a sulforhodamine B assay. After selecting two suitable WT extract concentrations, the cells were treated for 24 hours. The expression of GLUT1, MCT4, PFK and LDH was determined. Studies on WT cytotoxicity revealed that 0.25

mg/ml and 1 mg/ml of WT extract successfully induced cell death in the primitive cancer stage, represented by the RT4 cell line, but cell death induction on TCCSUP cell line was only achieved by 1 mg/ml WT extract. Moreover, expression levels of GLUT1, PFK, LDH and MCT4 were not significantly altered by the treatment with WT extract on RT4 or TCCSUP cell lines.

Our work demonstrates that bladder cancer progression includes several alterations in the cells' metabolism, from which pyruvate consumption seems to be a major factor. Also, compelling evidence is provided that glucose uptake is not altered and higher levels of alanine and lactate are produced, which are indicators of a more accelerated metabolism, and may indicate how bladder cancer cells respond to aggressive environments such as hypoxia. Moreover, our cytotoxicity studies revealed that, although WT extract is capable of inducing cell death in both stages of bladder cancer, a higher concentration of WT extract is necessary to achieve cell death in the highly invasive stage than in the primitive stage. These results illustrate that cells in different cancer stages may present differences in their survival and/or proliferative mechanisms. Our preliminary results suggest that this cell death induction by WT extract does not seem to be accompanied by alterations in the protein expression levels of glucose transporters or enzymes related to the glycolytic process, but more studies are needed to clarify these results. Nonetheless, our work clearly demonstrates the metabolic alterations that occur in the glycolytic machinery of bladder cancer cells, as the cancer progresses. Moreover, WT successfully induces cell death in primitive and more advanced bladder cancer stages. This provides important new insights on cancer metabolism and evidence regarding WT anticancer properties, both extremely important for the future development of new therapeutic strategies for bladder cancer.

Keywords

Bladder cancer, TCCSUP, RT4, Warburg effect, *Camellia sinensis*, white tea.

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Abbreviations

^{1}H NMR	Proton Nuclear Magnetic Resonance
Akt	Protein kinase B
ATP	Adenosine Triphosphate
Bad	Bcl-2-Associated Death promoter protein
Bax	Bcl-2-Associated X protein
Bcl-XL	B-cell lymphoma-extra large protein
BIM	Bcl-2–interacting mediator of cell death
BT	Black Tea
CIS	Carcinoma *in situ*
DMEM	Dulbecco's Modified Eagle Medium
EC	(-)-Epicatechin
ECG	(-)-Epicatechin-3-gallate
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin-3-gallate
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FBS	Fetal Bovine Serum
FKHR	Forkhead Transcription Factor
GLUT	Glucose Transporter
GPT	Glutamic-Pyruvate Transaminase
GT	Green Tea
H_2O_2	Hydrogen peroxide
HK	Hexokinase
LDH	Lactate Dehydrogenase
MCT4	Monocarboxylate Transporter 4
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
OT	Oolong Tea
PFK	Phosphofructokinase 1
PK	Pyruvate Kinase
PO	Polyphenol Oxidase
PP-60	Polyphenon-60
RIPA	Radio-Immunoprecipitation Assay buffer
ROS	Reactive Oxygen Species
SRB	Sulforhodamine B
TCC	Transitional Cell Carcinoma
WT	White Tea

I. Introduction

1. Bladder Cancer

1.1. General aspects

Bladder cancer is among the most common types of cancer and it can appear under different forms, being the transitional-cell carcinoma (TCC) the most usual (Pelucchi *et al.*, 2006; Crawford, 2008). The worldwide incidence and rates of urinary bladder cancer vary in the different world regions. In 2008, it was estimated 386,300 new cases and 150,200 deaths worldwide due to this type of cancer that mostly affects men (Jemal *et al.*, 2011).

Bladder tumors may be either superficial (classified as TIS, Ta or T1) or infiltrative (classified as T2, T3 or T4), according to histopathological characteristics (Oosterlinck *et al.*, 2002). More than 90% of all tumors in this site are TCCs that arise from epithelial bladder cells (Vaidya *et al.*, 2013; Shin *et al.*, 2014). In the context of TCC, there are two distinct pathways from which bladder carcinomas can arise. About 80% of the cases are originated by the papillary pathway, which originates superficial, low-grade papillary tumors; the other 20% are high-grade, invasive tumors formed by the non-papillary pathway. About 10 to 15% of the superficial tumors may evolve to a more aggressive and potentially fatal non-papillary phenotype, which invades the muscle wall of the bladder (figure 1) (Dinney *et al.*, 2004). In superficial, nonfatal tumors, the probability of recurrence is high, and in solid, high-grade tumors, death is a risk (Jung and Messing, 2000; McConkey *et al.*, 2010).

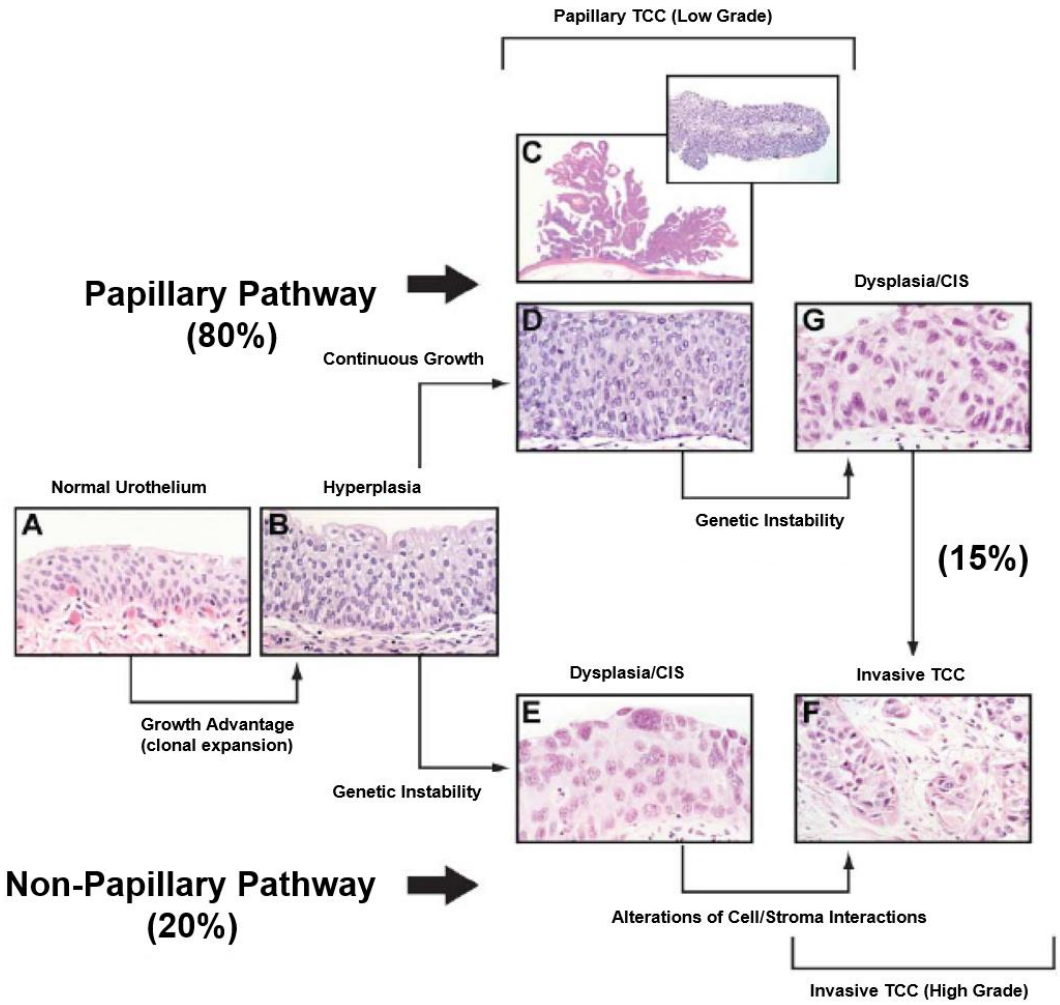


Figure 1. Main pathways of human bladder carcinogenesis. The bladder urothelium (A) begins to change when there is a clonal expansion of a preneoplasic cell (or more). The phenotype remains very similar to that of the normal tissue, only with an abnormally superior cell number (hyperplasia, B). Low grade superficial papillary tumors arise with the continuous growth of that clone (C and D). Genetic instability of the clones (with loss of tumor suppressor genes), whether in the papillary phase or soon in the hyperplasia state, leads to an intraurotherial dysplasia state/carcinoma *in situ* (CIS) in the tissue (E and G). At this point, the cells will ultimately begin to provoke alterations in their surrounding environment, leading to the development of invasive, high grade bladder carcinomas. Adapted from Dinney *et al.*, 2004.

The alterations suffered by normal, healthy urothelial cells that originate cancerous cells are mainly attributed to genetic mutations and environmental factors, such as exposure to cigarette smoke (Dinney*, et al.*, 2004; McConkey*, et al.*, 2010). However, the mechanisms responsible for bladder cancer progression remain largely unknown. Studying the specific molecular and metabolic pathways related to the initiation and progression of this disease is

crucial to develop new therapeutic strategies, as well as to identify possible biomarkers or triggers for tumor progression.

1.2. Bladder cancer and the Warburg effect

Carcinogenesis is the result of several genetic and metabolic alterations (Ramanathan *et al.*, 2005; Lopez-Lazaro, 2010), and it is widely known that the functioning of cancer cells is different from that of the normal ones. Cancer metabolism is intrinsically related to high glycolytic flux, a phenomenon known as the Warburg effect. After several years of studies, Warburg verified that cancer cells do not share the same metabolic preferences as normal cells (Warburg *et al.*, 1927; Warburg, 1956). In normal situations, cells obtain the majority of their energy requirements from oxidative phosphorylation, which occurs in the mitochondria; only a small part of the energy is obtained from the glycolytic pathway. Glycolysis is energetically less efficient than oxidative phosphorylation, only yielding two adenosine triphosphate (ATP) molecules per molecule of glucose metabolized, while oxidative phosphorylation yields 36 ATP molecules. So, usually this pathway is mainly utilized to convert pyruvate into acetyl-CoA that enhances Krebs cycle. This cycle generates the reduced form of the intermediary nicotinamide adenine dinucleotide (NADH), which will in turn be used to fuel the mitochondrial oxidative phosphorylation, maximizing ATP production (Oliveira *et al.*, 2014).

Glucose enters the cells by the action of specific glucose transporters (GLUTs), from which the high-affinity GLUTs 1 and 3 may be highlighted (Macheda *et al.*, 2005). In the cytosol, glucose molecules suffer enzymatic conversion to pyruvate, through a series of ten chain reactions that constitute the glycolytic pathway. From these reactions, it is important to highlight that there are three main points for the regulation of the glycolytic process. These are the irreversible conversions of glucose to glucose 6-phopshate by hexokinase (HK), of fructose 6-phosphate to fructose 1,6-biphosphate by phosphofructokinase 1 (PFK) and the last step, in which pyruvate kinase (PK) catalyzes the conversion of phosphoenolpyruvate into pyruvate (Xiong *et al.*, 2011; Oliveira, *et al.*, 2014). This series of reactions includes the final liberation of two ATP molecules per glucose molecule, as well as the reduction of two NAD^+ molecules to two NADH molecules (figure 2). The pyruvate formed in this pathway, aside from being converted to acetyl-CoA, may also be enzymatically converted to alanine or lactate. These processes are severely important in cancer cells and will be discussed in detail below.

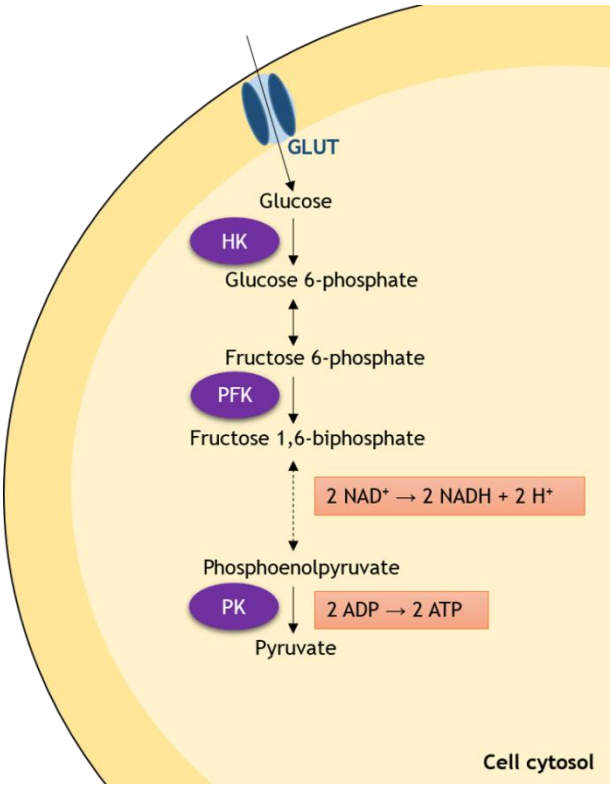


Figure 2. Schematic overview of the regulatory points and most important products formed in glycolysis. Glucose enters the cells by the action of membrane glucose transporters (GLUTs). In the cytosol, it is irreversibly converted to glucose 6-phosphate by hexokinase (HK). In the next step, conversion to fructose 6-phosphate occurs, which in turn will be irreversibly transformed in fructose 6-phosphate by phosphofructokinase 1 (PFK). Then, fructose 6-phosphate follows through a series of enzymatic reactions, which will result in the formation of two reduced nicotinamide adenine dinucleotide molecules (NADH) from two NAD^+ molecules. In the last step of this pathway, which is also the last regulatory point, phosphoenolpyruvate is irreversibly converted to pyruvate by pyruvate kinase (PK) and two adenosine triphosphate (ATP) molecules are formed.

However, cancer cells do not seem to share this usual and expected metabolic behavior. Instead, Warburg reported that, even in the presence of enough oxygen to sustain oxidative phosphorylation, these cells use glucose as main energy source and export high levels of lactate (Warburg, *et al.*, 1927; Warburg, 1956). Warburg and others also postulated that in cancer cells, mitochondrial respiration is either impaired, or less used. Additionally, some cancer cells present the ability to switch from glycolysis to oxidative phosphorylation, according to environmental factors (Rossignol *et al.*, 2004; Kaldma *et al.*, 2014; Oliveira, *et al.*, 2014). Several alterations in the intermediates of this pathway have been reported in cancer cells, such as deregulation of GLUTs and enzyme modulation, to sustain a high glycolytic flux (Osthus *et al.*, 2000; Atsumi *et al.*, 2002; Langbein *et al.*, 2006; Reis *et al.*, 2011; Ros and Schulze, 2013; Jin *et al.*, 2014). Of note, cancer cells are immortal, ever-

proliferating, highly replicable systems. In order to be able to duplicate their cellular contents, these cells have a high demand for biosynthetic intermediates. Glycolysis, besides converting glucose to pyruvate, is also a source of precursor biomolecules involved in several other metabolic pathways, which present very intrinsic relations with each other (figure 3) (Feron, 2009; Oliveira, *et al.*, 2014).

In the first step of the glycolytic pathway, glucose 6-phosphate may either follow the glycolytic way (see figure 1), or be converted to reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phosphate by the pentose phosphate pathway. Similarly, the yielded pyruvate may either be converted to lactate by lactate dehydrogenase (LDH); to acetyl-CoA by the pyruvate dehydrogenase complex; or to alanine by glutamic-pyruvate transaminase (GPT). Alanine can be used to incorporate proteins, or be exported (Feron, 2009). Lactate is exported from the cells, through the monocarboxylate transporters 4 (MCT4) (Feron, 2009).

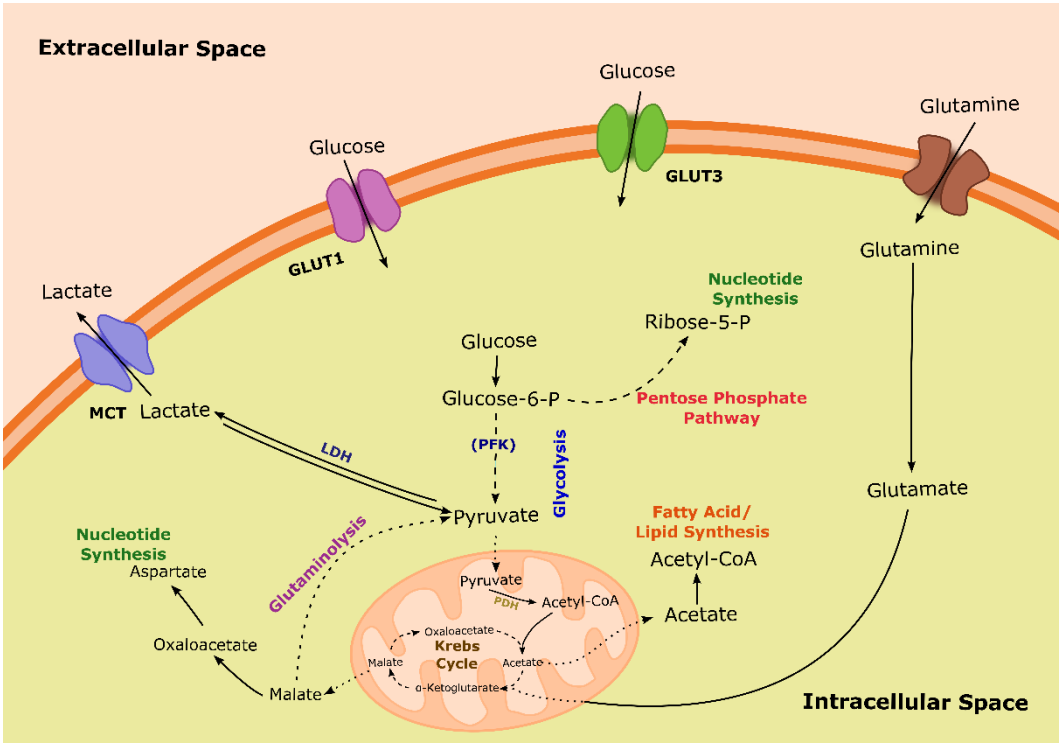


Figure 3. Schematic representation of the intrinsic relations between glycolysis and other metabolic pathways known to occur in cancer cells. Glucose, as well as glutamine, are the main substrates for these cells. The high-affinity glucose transporters 1 and 3 (GLUT1 and GLUT3) transport glucose to the cells, which is then converted to pyruvate. The first glycolysis step, catalyzed by the enzyme HK, converts glucose to glucose 6-phosphate, which can then enter the pentose phosphate pathway, where its carbons are transformed in ribose 5-phosphate and utilized as sources for nucleotide synthesis. The pyruvate derived from glycolysis may either be converted to lactate, which is exported from the cells by MCT4, to alanine or to acetyl-CoA, which participates in the Krebs cycle. Adapted from Oliveira *et al.* 2014.

As in many other cancer types, the Warburg Effect has also been verified in urinary bladder tumors, reflected in altered levels of glycolysis intervenients, such as pyruvate, enzymes and GLUTs (Langbein, *et al.*, 2006; Reis, *et al.*, 2011; Jin, *et al.*, 2014). In some cases, sub or overexpression of some of these intermediates of glycolysis has been correlated with an increase in tumor malignancy (Liao *et al.*, 2011; Reis, *et al.*, 2011; Jin, *et al.*, 2014). Moreover, it was reported that it is possible to distinguish between healthy subjects, patients with muscle-invasive bladder cancer and patients with non-muscle-invasive bladder cancer, based on their urines' metabolic profiles. These displayed several indicatives of excessive glycolytic and betaoxidative activities, such as high pyruvate and acetyl-CoA levels (Jin, *et al.*, 2014). However, there are not many studies available focused on the exact alterations in the metabolism of bladder cancer during cancer progression. The study of bladder cancer cells metabolism and how it is associated with progression to different and more aggressive states is essential to the development of new diagnosis methods, therapeutic strategies and even tools to help predicting the survival of the patients (Jin, *et al.*, 2014). Thus, more studies are needed to unveil the metabolic characteristics that ensure survival and proliferation of the bladder cancer cells, as well as their progression to more aggressive states. Herein we propose to study the glycolytic profile of two human urinary bladder cancer cells, representative of different cancer progression stages: RT4 (primitive stage) and TCCSUP (highly invasive stage). This may ultimately help to identify a molecular pharmacological/therapeutic target to counteract or avoid the progression of bladder cancer.

1.3. Risk and preventive factors

The study of possible therapeutic targets to bladder cancer is extremely important and may arise from unveiling the molecular mechanisms related or promoted by the progression of this disease. Many risk factors for the development of bladder cancer in humans have been identified (Pelucchi, *et al.*, 2006). These include smoking (Crawford, 2008; Kurahashi *et al.*, 2009; Kobeissi *et al.*, 2013), exposure to diesel and combustion fumes (Kobeissi, *et al.*, 2013), genetic components (Crawford, 2008; Kobeissi, *et al.*, 2013; Wang *et al.*, 2013) and liquid ingestion (Hemelt *et al.*, 2010; Wang, *et al.*, 2013). But not all factors are consensual. For instance, there are authors that discuss liquid ingestion as a risk factor while others who defend that it may be a preventive factor instead. This occurs because some authors consider that the ingestion of great volumes of liquids may increase the amount of carcinogenic components (present in those liquids) in contact with the bladder cells (Villanueva *et al.*, 2006) while others suggest that ingesting high quantities of liquids may help to dilute and expel potentially harmful metabolites present in the bladder (Michaud *et al.*, 1999). Noteworthy, the increased risk also depends on the ingested liquids, especially soft drinks (Wang, *et al.*, 2013). Similarly, some studies show that the regular intake of certain

beverages, such as milk (Hemelt, *et al.*, 2010) and tea (Wang, *et al.*, 2013), is responsible for the decrease of this risk.

There are several studies that establish a connection between tea consumption and decreased cancer risk (Kada *et al.*, 1985; Wang *et al.*, 1992; Suganuma *et al.*, 1999; Steele *et al.*, 2000; Cooper *et al.*, 2005; Pastore and Fratellone, 2006; Rieger-Christ *et al.*, 2007; Khan and Mukhtar, 2010; Mao *et al.*, 2010; Cross *et al.*, 2011; Dias *et al.*, 2013). Although some authors report beneficial effects of tea in cancer, studies on bladder tumors are very scarce and conclusions are still lacking. Therefore, preliminary works regarding the effects of treating bladder cancer cells with tea will also be presented in this work.

2. Tea: types, composition and health benefits

Camellia sinensis (L.), commonly known as the tea plant, has been used for many centuries in traditional medicine. The infusion prepared by using its leaves or buds is also known as tea. The main types of tea yielded from this plant are green tea (GT), white tea (WT), black tea (BT) and oolong tea (OT). This classification is based on differences in the manufacture and preparation processes, which result in distinctive chemical compositions. Tea's ability to stimulate the immune system and mitigate several diseases has raised great interest. Emphasis is given to cancer research area, where tea's beneficial properties have been verified, and several of its components have also been investigated separately (Taniguchi *et al.*, 1992; Wang, *et al.*, 1992; Huang *et al.*, 1997; Hong *et al.*, 2001; Higdon and Frei, 2003; Zaveri, 2006; Boehm *et al.*, 2009; Carvalho *et al.*, 2010; Mao, *et al.*, 2010; Darvesh and Bishayee, 2013).

2.1. Types of tea

C. sinensis can originate four types of teas, depending on the tea leaves' harvesting and processing (Pastore and Fratellone, 2006; Moderno *et al.*, 2009; Dias, *et al.*, 2013) (figure 4). Upon harvesting, the leaves suffer an enzymatic oxidation process, also called "fermentation" (Moderno, *et al.*, 2009; Dias, *et al.*, 2013). The enzyme involved in this process, polyphenol oxidase (PO), is the main responsible for the differences in the phenolic profiles of the several types of tea. Its action can be inactivated by quickly heating the leaves or buds, a post-harvesting technique commonly used in the production of GT and WT (Moderno, *et al.*, 2009; Dias, *et al.*, 2013).

GT, BT and OT are all obtained from *C. sinensis* mature dried leaves, but they possess different chemical compositions and consequently some very obvious organoleptic

differences, namely in taste, color and flavor. In the production of GT, the mature leaves are harvested and then steamed and rolled before drying, in order to inactivate PO and prevent oxidation. In this way, the chemical composition of GT remains similar to that of the *C. sinensis*' mature leaves. On the other hand, the production of BT (also known as "fermented" tea) includes rolling and crushing the leaves, which are then allowed to "ferment" for two hours, and heated afterwards. Production of OT (also known as "semi-fermented" tea) is similar to the latter; however, the leaves are only allowed to "ferment" for one hour before being heated (Moderno, *et al.*, 2009; Dias, *et al.*, 2013). Finally, there is the rarest and most expensive tea, the WT. This type of tea is produced from the tips or leaf buds not fully opened, which are quickly heated to prevent withering and oxidation (Moderno, *et al.*, 2009; Dias, *et al.*, 2013). Therefore, WT's chemical composition is similar to that of *C. sinensis*' buds and young leaves.

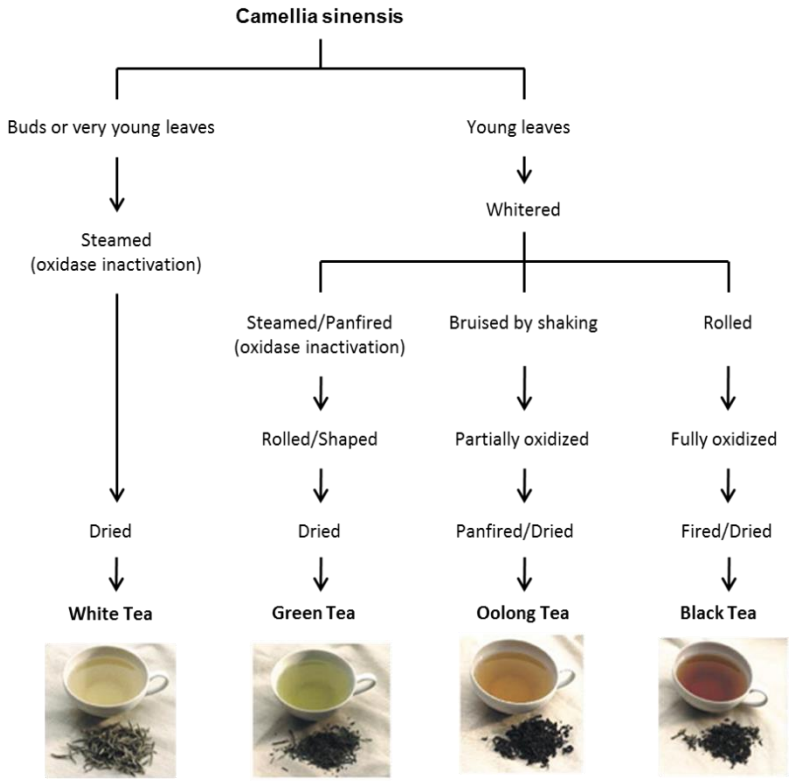


Figure 4. Processing methods that yield the different types of tea. White tea (WT) production requires steaming and drying immediately after harvesting, to prevent the action of polyphenol oxidase (PO). For the production of green tea (GT), the mature leaves are harvested and then quickly heated, to inactivate PO and prevent oxidation, after which they are rolled, dried and sorted. Production of black tea (BT) and oolong tea (OT) includes crushing and rolling the leaves after harvesting and withering, a process that disrupts cellular compartmentation and brings phenolic compounds into contact with PO. Then, the leaves are allowed to "ferment" for two hours, for BT, or one hour, for OT, before being heated. Adapted from Dias *et al.* (Dias, *et al.*, 2013).

2.2. Chemical composition

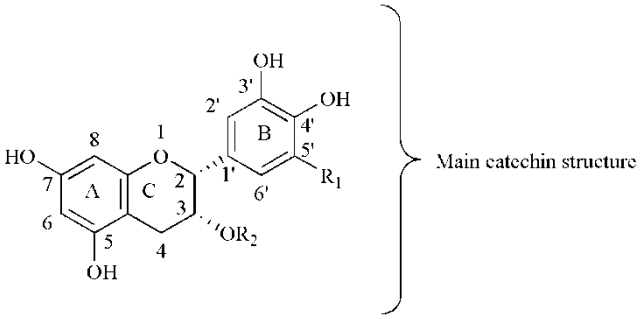
Tea's chemical composition is very complex, containing polyphenols, proteins, polysaccharides, free amino acids, minerals, trace elements, methylxanthines and organic acids, among many others (Cabrera *et al.*, 2006; Moderno, *et al.*, 2009; Dias, *et al.*, 2013). As referred above, the four types of tea present different chemical compositions, which are affected by several factors such as geographical origin, climate, growing conditions, harvesting practices, maturity stage of the plant and manufacturing processes (Lin *et al.*, 2003; Moderno, *et al.*, 2009; Dias, *et al.*, 2013).

Polyphenols

Polyphenols are the most abundant and active group of compounds present in tea, and are thought to be the most important source of the health benefits attributed to this beverage (Higdon and Frei, 2003). Flavonoids are amongst the major classes of phenolic compounds, from which is important to highlight the catechins, members of the flavan-3-ol family. Catechins present very high antioxidant capacity (Cooper, *et al.*, 2005; Pastore and Fratellone, 2006; Dias, *et al.*, 2013), as well as anti-inflammatory, antimicrobial, antimutagenic, antimetastatic and anticarcinogenic properties (Kada, *et al.*, 1985; Wang, *et al.*, 1992; Suganuma, *et al.*, 1999; Steele, *et al.*, 2000; Cooper, *et al.*, 2005; Pastore and Fratellone, 2006; Rieger-Christ, *et al.*, 2007; Khan and Mukhtar, 2010; Kumar *et al.*, 2010; Mao, *et al.*, 2010; Afaq and Katiyar, 2011; Cross, *et al.*, 2011; Hessien *et al.*, 2012; Li *et al.*, 2012; Dias, *et al.*, 2013). There are various catechins in tea, such as (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epigallocatechin-3-gallate (EGCG) (Pastore and Fratellone, 2006; Moderno, *et al.*, 2009), and their chemical structure is responsible for the health benefits attributed to them (especially the antioxidant power) (Yang *et al.*, 2007; Costa *et al.*, 2009; Aboul-Enein *et al.*, 2013; Bubols *et al.*, 2013). Moreover, EGCG is considered to be the most abundant and active catechin of tea, being also one of the most studied (Fernandez *et al.*, 2000; Pastore and Fratellone, 2006; Zaveri, 2006; Moderno, *et al.*, 2009; El-Shahawi *et al.*, 2012; Dias, *et al.*, 2013).

The main catechins are essentially comprised of three rings (the aromatic rings, A and B, linked to a dihydropyran heterocyclic ring, C) and are characterized by multiple hydroxyl groups on the A and B rings (Braicu *et al.*, 2013) (figure 5). Their chemical differences are due to the presence of different groups attached to those rings (Moderno, *et al.*, 2009; Braicu, *et al.*, 2013; Dias, *et al.*, 2013). In EC, we can find an ortho-di-hydroxyl group in the B ring and a hydroxyl group in the C ring; ECG contains a gallate moiety esterified in the C ring. EGC possesses a trihydroxyl group on the B ring, and EGCG possesses an esterified gallate on the C

ring (Braicu, *et al.*, 2013; Dias, *et al.*, 2013). GT and WT present higher catechin content, while OT and BT present in high quantities other phenolic compounds (Lin, *et al.*, 2003; Unachukwu *et al.*, 2010; Dias, *et al.*, 2013; Li *et al.*, 2013), which are formed by the action of PO. This enzyme is released during the crushing of the leaves for production of BT and OT and catalyzes the oxidation and polymerization of the catechins, producing theaflavins and thearubigins (Lin, *et al.*, 2003; Li, *et al.*, 2013).



	R1	R2
(-)-epicatechin	H	H
(-)-epigallocatechin	OH	H
(-)-epicatechin-3-gallate	H	Gallate group
(-)-epigallocatechin-3-gallate	OH	Gallate group

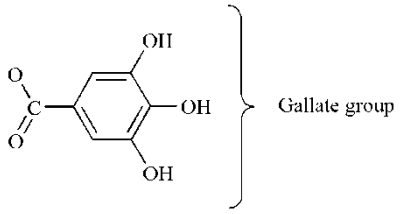
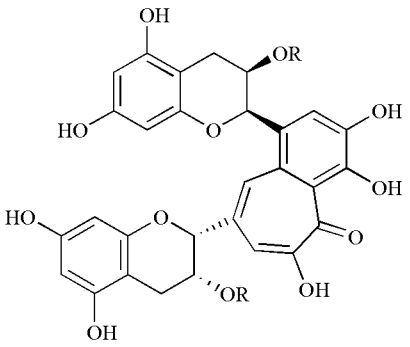


Figure 5. Chemical structures of the main tea catechins. These compounds are essentially constituted by two aromatic rings (A, B) and a dihydropyran heterocyclic ring (C). The flavan-3-ol epicatechin is constituted by an ortho-di-hydroxyl group in the B ring (at carbons 3' and 4') and a hydroxyl group in the C ring (at carbon 3), and its ester derivative epicatechin-3-gallate possesses an additional gallate moiety esterified in the C ring, at carbon 3. Epigallocatechin contains a trihydroxyl group on the B ring (at carbons 3', 4' and 5') and its ester derivative epigallocatechin-3-gallate additionally possesses an esterified gallate at the carbon 3 of the C ring.

Theaflavins (figure 6) possess a basic skeleton comprised of the bicyclic benzotropolone ring, and result from catechins' dimerization. In turn, thearubigins are still poorly chemically characterized. They are benzotropolone derivatives, and are thought to be the result of the

hydroxylation of theaflavins. Of note, the formation and characterization of their chemical structure needs further clarification (Li, *et al.*, 2013).



	R
Theaflavin	H
Theaflavin-3,3'-digallate	Gallate group

Figure 6. Chemical structures of the main theaflavins. Theaflavins result from the dimerization of the main catechins and are constituted by a skeleton comprised of the bicyclic benzotropolone ring. The majority of theaflavins are formed from an epicatechin and an epigallocatechin. Theaflavin-3,3'-digallate is produced by dimerization of epicatechin-3-gallate and epigallocatechin-3-gallate.

Catechins' chemical composition is very important, because it highly influences their beneficial properties. For example, their antioxidant properties, such as superoxide anion scavenging ability and quenching of hydroxyl radicals are highly correlated with the content of pyrogallol/hydroxyl groups and the presence of galloyl moieties, respectively (Nanjo *et al.*, 1999; Moderno, *et al.*, 2009). The number and position of the hydroxyl groups on the molecules are also thought to influence this antioxidant capacity (Braicu, *et al.*, 2013).

Methylxanthines

The methylxanthines contained in tea are caffeine, theophylline and theobromine. Caffeine (figure 7) is the main methylxanthine present in tea; its levels range between 1.0 and 3.5% in tea preparations (Fernandez, *et al.*, 2000; Lin, *et al.*, 2003).

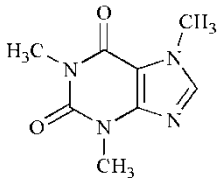


Figure 7. Chemical structure of caffeine. It is a naturally occurring tea purine derivative with three methyl groups at positions 1, 3 and 7.

Contrary to catechins and due to its chemical stability, caffeine levels are not affected by the "fermentation" process (Lin, *et al.*, 2003), although some studies indicate that different types of tea present different caffeine levels (Lin, *et al.*, 2003; Unachukwu, *et al.*, 2010; Dias, *et al.*, 2013). These discrepancies may be due to different extraction conditions, distinct analytical methods and the variability of the plants. Nonetheless, the anticarcinogenic properties of caffeine have been documented (Lu *et al.*, 2002; Hashimoto *et al.*, 2004). Particularly in studies of tea consumption by humans, the importance of caffeine in tea preparations was highlighted, since decaffeinated teas presented very low cancer inhibitory properties (Huang, *et al.*, 1997). However, data on the role of caffeine on tea-associated health benefits are scarce and much work needs to be done.

In the field of cancer research, the most studied types of tea are GT and BT. Nevertheless, since WT has the highest catechin concentration among all types, it is expected to possess great anticancer properties (Dias, *et al.*, 2013). However, to date, there are no studies focused on WT consumption and bladder cancer.

3. Tea and bladder cancer

3.1. Epidemiological studies

After ingestion by mice, EGCG widely distributes through the body, including the urinary bladder (Suganuma, *et al.*, 1999). Moreover, several studies reported the beneficial effects of GT or its components on bladder cancer cells. However, particularly in the human bladder cancer research area, the anticarcinogenic properties of tea, although predictable, still lack strong supporting evidence. There are several epidemiological studies performed in this area, based on the statistical analysis of questionnaires filled by patients or former patients, regarding their dietary and lifestyle habits in the years anteceding the cancer onset. In this context, some authors reported regular tea consumption to be either a risk factor for bladder cancer (Lu *et al.*, 1999) or a preventive factor (Zheng *et al.*, 1996; Bianchi *et al.*, 2000; Wang, *et al.*, 2013). However, the majority of these studies defend that tea consumption has no association with the disease triggering, development or outcome (Morgan and Jain, 1974; Claude *et al.*, 1986; Heilbrun *et al.*, 1986; Bruemmer *et al.*, 1997; Nagano *et al.*, 2000; Demirel *et al.*, 2008; Kurahashi, *et al.*, 2009; Hemelt, *et al.*, 2010; Kobeissi, *et al.*, 2013). Table 1 summarizes the main findings in some studies.

Table 1. Epidemiological studies regarding regular tea consumption and human bladder cancer. The types of tea, studies and results obtained are presented.

		Type of tea consumed	Risk factor	Protective factor	No association	Resume of the main findings
Epidemiological studies	Population-based cohort	**GT** (Kurahashi, *et al.*, 2009)			✖ (Kurahashi, *et al.*, 2009)	**na** (Kurahashi, *et al.*, 2009)
	Case-control	**GT** (Lu, *et al.*, 1999; Hemelt, *et al.*, 2010; Wang, *et al.*, 2013) **BT** (Claude, *et al.*, 1986; Lu, *et al.*, 1999; Demirel, *et al.*, 2008; Hemelt, *et al.*, 2010; Wang, *et al.*, 2013) **OT** (Lu, *et al.*, 1999) **Unspecified** (Morgan and Jain, 1974; Kobeissi, *et al.*, 2013)	✖ (Lu, *et al.*, 1999)	✖ (Wang, *et al.*, 2013)	✖ (Morgan and Jain, 1974; Claude, *et al.*, 1986; Demirel, *et al.*, 2008; Hemelt, *et al.*, 2010; Kobeissi, *et al.*, 2013)	**🡅 In patients that consumed GT or BT daily (1 cup or more), for a period over 30 years.** (Lu, et al., 1999) **🡇 In patients with daily consumption of GT and BT (1 cup or more).** (Wang, *et al.*, 2013) **na** (Morgan and Jain, 1974; Claude, *et al.*, 1986; Demirel, *et al.*, 2008; Hemelt, *et al.*, 2010; Kobeissi, *et al.*, 2013)
	Cohort	**GT** (Nagano, *et al.*, 2000) BT(Heilbrun, *et al.*, 1986; Nagano, *et al.*, 2000) **Unspecified** (Zheng, *et al.*, 1996)		✖ (Zheng, *et al.*, 1996)	✖ (Heilbrun, *et al.*, 1986; Nagano, *et al.*, 2000)	**🡇 In postmenopausal women who consumed more than 2 tea cups daily.** (Zheng, *et al.*, 1996) **na** (Heilbrun, *et al.*, 1986; Nagano, *et al.*, 2000)
	Population-based case-control	**Unspecified** (Bruemmer, *et al.*, 1997; Bianchi, *et al.*, 2000)		✖ (Bianchi, *et al.*, 2000)	✖ (Bruemmer, *et al.*, 1997)	**🡇 In subjects with low fluid intake who consumed more than 5 tea cups per day.** (Bianchi, *et al.*, 2000) **na** (Bruemmer, *et al.*, 1997)

Legend: GT – Green tea; BT – Black tea; OT – Oolong tea; 🡇 - Reduced number of cases of bladder cancer; 🡅 - Increased number of cases of bladder cancer; na – No statistically significant association.

However, and although there is relevant information provided by these studies, there are also some drawbacks to be considered. The use of questionnaires makes the studies highly dependent on the subjects' interpretation or past memory raising doubt about the veracity, due to the subjects' forgetting or deliberately tampering the facts. Besides, most of these studies also include a complicated analysis of data, ranging from type and duration of the beverages consumed, fruit and vegetable consumption, smoking status, among others. Moreover, some of the studies do not refer the type of tea investigated (Morgan and Jain, 1974; Zheng, *et al.*, 1996; Bruemmer, *et al.*, 1997; Bianchi, *et al.*, 2000; Kobeissi, *et al.*, 2013), which hinders any association between consumption of one tea type and bladder cancer development. Finally, most of the studies were performed in very different populations, which greatly vary in terms of age, countries and habits, making very difficult the extrapolation of results and conclusions.

All these drawbacks show how important it is to develop further studies regarding human bladder cancer. Since human studies are always very difficult to conduct, and epidemiological studies present the cons considered above, good strategies to study bladder cancer, as all well as many other diseases, lay in the use of animal models and *in vitro* approaches. Although there has to be some care in the extrapolation of the results obtained in these studies to humans (mainly due to different metabolization of tea's components), they may help unveil disease mechanistic and the exact effects of tea and its components in bladder cancer prevention and/or treatment.

3.2. *In vitro* and *in vivo* studies

The numerous and complex signaling pathways that exist in a cell are extremely important to maintain its homeodynamics and normal functioning. The disclosure of these pathways has become very important in the study of several diseases. Cancer cells normally display several differences in metabolism, gene expression and survival mechanisms, among others. Thus, as expected, tea and its components can inhibit carcinogenesis through a wide variety of mechanisms (Hou *et al.*, 2005; Yang and Wang, 2011; Yang *et al.*, 2011). Mainly acting through its polyphenol components, particularly catechins, it was demonstrated the GT's ability to prevent the initiation and growth of bladder tumors in rats (Sato, 1999; Sato and Matsushima, 2003; Chen *et al.*, 2011), inhibit bladder tumor development and invasion *in vitro* (in some cases showing positive synergistic effects with other substances) (Roomi *et al.*, 2006; Chen, *et al.*, 2011) and protect normal bladder cells, while killing the malignant ones (Coyle *et al.*, 2008; Chen, *et al.*, 2011). Although catechins alone are a powerful tool to oppose cancer development, GT extract and dried leaves can also be very helpful and a

practical way to treat cancer. They possess numerous phytocomponents with anticancer properties, being less expensive, widely available and safe (Sato, 1999).

Although many studies report the anticancer properties of tea or its components in several carcinogenic models, the exact mechanisms through which they exert these effects remain to be unveiled. This is due to the fact that, aside from the lack of many studies regarding this matter, the anticancer effects suggest interference in many different pathways and processes, ranging from apoptosis, metastization and cell cycle arrest, among many others. Table 2 presents a summary of studies in this field, highlighting the main findings.

Table 2. Summary of the main effects observed in several *in vivo* and *in vitro* studies focused on the effects of tea and its phytochemicals in bladder cancer.

	Tea/ compound tested	Effects observed							
		Tumor size	Metastization	Angiogenesis	Apoptosis	Cell cycle arrest	Morphological changes	Cell cytotoxicity	Chromosome damage
In vivo Studies	**EGCG** (Rieger-Christ, *et al.*, 2007; Chen, *et al.*, 2011; Hsieh *et al.*, 2011)	↓	↓	↓	↑	nd	nd	nd	nd
	GT polyphenols (Sagara *et al.*, 2010)	↓	↓	nd	nd	nd	nd	nd	nd
	EGCG (Kemberling *et al.*, 2003)	↓	nd	nd	nd	nd	nd	nd	nd
	Powdered dried GT leaves (Sato, 1999; Sato and Matsushima, 2003)	↓	↓	nd	nd	nd	nd	nd	nd
	GT (Sato, 1999)	↓	↓	nd	nd	nd	nd	nd	nd
	OT (Sato, 1999)	↓	↓	nd	nd	nd	nd	nd	nd
	BT (Sato, 1999)	↓	nd	nd	nd	nd	nd	nd	nd
In vitro Studies	**EGCG** (Kemberling, *et al.*, 2003; Chen *et al.*, 2004; Rieger-Christ, *et al.*, 2007; Chen, *et al.*, 2011)	↓	↓	nd	↑	↑	↑	nd	nd
	PP-60 + EGCG + ECG (after insult with H2O2) (Coyle, *et al.*, 2008)	nd	nd	nd	↓	nd	nd	nd	nd
	Lysine + proline + arginine + ascorbic acid + GT extract (Roomi, *et al.*, 2006)	nd	↓	nd	nd	nd	nd	nd	nd
	Methylxanthines (caffeine/pentoxifylline) + Thiotepa (Fingert *et al.*, 1986)	nd	nd	nd	nd	↑	nd	↑	↑
	GT extract (Lu *et al.*, 2005)	nd	↓	nd	nd	nd	nd	nd	nd

Legend: EGCG - epigallocatechin 3-gallate; GT - green tea; OT - oolong tea; BT - black tea; PP-60 - polyphenon-60; ↓ - Reduced/inhibited; ↑ - Increased; nd - not determined.

In vitro studies on bladder cancer cells have been performed using different cell lines. The main compound tested was EGCG and, as expected, its anticancer properties were reported. Treatment of bladder TCC cells with increasing concentrations of EGCG revealed time and dose-dependent decreases in cell survival, as well as several morphological changes, such as cellular shrinkage, pyknosis and cell surface blebbing (Kemberling, *et al.*, 2003; Chen, *et al.*, 2011). Moreover, studies on different bladder cancer cell lines treated with EGCG revealed growth inhibition and evidence of cell cycle arrest in the G0/G1 phase, most likely caused by interference in the cyclin D1-cdk4/6-Rb protein machinery (Chen, *et al.*, 2004). Significant reduction in the migration of cancer cells treated with EGCG was also reported. Treatment with this catechin most likely interfered in the p42/44 MAP kinase and protein kinase B (also known as Akt) pathways and reduced the expression of N-cadherin, ß-, and ɣ-catenin proteins (Rieger-Christ, *et al.*, 2007; Chen, *et al.*, 2011), all implicated in cellular migration. Induction of apoptosis in cancer cells treated with EGCG was also reported, most likely due to the decrease in heat-shock protein 27 and Bcl-2 protein levels (involved in the inhibition of cell apoptosis), increase in Bcl-2-associated death promoter (Bad) and Bcl-2-associated X (Bax) protein levels (which are known proapoptotic proteins) and increased activity of caspases 3 and 9, illustrating the potential activation of the apoptotic mitochondrial pathway by EGCG (Chen, *et al.*, 2011). The hypothesis that EGCG activates the apoptotic mitochondrial pathway in bladder cancer cells was also suggested by another study, in which the treatment of EGCG combined with gold nanoparticles resulted in reduced tumor cell viability, increased number of apoptotic bodies formed, decreased levels of antiapoptotic B-cell lymphoma-extra large (Bcl-XL) protein and increased levels of proapoptotic proteins such as Bad and Bax, as well as the expression levels of caspases 3 and 7 (Hsieh, *et al.*, 2011).

Besides EGCG, GT extract also showed positive effects *in vitro*. Treatment of bladder tumor cells with different concentrations of GT extract resulted in induction of cellular actin polymerization (a protein that forms the cells' microfilaments and is typically depolymerized in cancer cells), most likely through an increase in Rho activity, a regulator of actin stress fiber formation (Lu, *et al.*, 2005). Another study reported that treatment of bladder cancer cells with different concentrations of a mixture of GT extract, lysine, proline, arginine, L-ascorbic acid and N-acetyl cysteine resulted in significant inhibition of cell invasion and a dose-dependent decrease in secretion of metalloproteinases 2 and 9, which are enzymes typically secreted by highly metastatic cancer cells, that allow them to destroy components of the extracellular matrix and migrate to other locations in the tissues (Roomi, *et al.*, 2006). These results reinforce the hypothesis of cell motility interference by tea and its components.

Moreover, the antioxidant capacity of tea components is also thought to participate in its anticancer effects. For instance, treatment of normal/cancerous bladder cell lines exposed to hydrogen peroxide (H_2O_2, an oxidative agent) with GT extract (14% polyphenols), polyphenon-60 (PP-60, 60% pure catechins), ECG and EGCG revealed that the catechins and PP-60 were

able to improve cell survival, although the protection afforded against apoptosis induced by H_2O_2 was higher for normal bladder cells than in cancerous ones. Since H_2O_2 exerted its apoptotic effects mainly by inducing reactive oxygen species (ROS) generation, further studies hypothesized that these compounds may be able to modulate cellular gene expression (possibly by causing the induction of protein kinase C and downregulation of nuclear factor kappa beta). These alterations in cell signaling would suppress cell death mechanisms and counterbalance the production of ROS (Coyle, *et al.*, 2008).

Of note, tea catechins also possess the ability to generate ROS. The structure of these compounds is relatively unstable, and it is common for catechins to suffer oxidation processes. This oxidation can either be performed by catechins themselves (auto oxidation), or can be catalyzed by transition metals such as copper and iron (Lambert and Elias, 2010). A study has reported that the incubation of several carcinoma cell lines with EGCG resulted in the inhibition of cellular growth, caused by inhibition of phosphorylation and reduced protein levels of epidermal growth factor receptors; these effects were delayed with the addition of the enzyme superoxide dismutase, suggesting that they may be, at least partially, a result of the action of EGCG oxidation products (Hou, *et al.*, 2005). This fact is an important feature to consider not only when fighting a pathological state, but also due to toxicity that is observed when high doses of tea polyphenols are administered. For instance, moderate doses of polyphenols induce low ROS production and activate the nuclear factor Nrf2, which can then translocate to the cell nucleus and stimulate the expression of antioxidant enzymes (Na and Surh, 2008). On the other hand, excessive amounts of polyphenols will produce higher levels of ROS. Treatment of mice with a single oral dose of 1500 mg/kg EGCG reduced the animals' survival by 85% and the administration of daily doses of 500 and 750 mg/kg decreased survival by 20% and 75%, respectively. High doses of orally administered EGCG may induce hepatocyte toxicity and even mortality in mice, in a dose and time-dependent manner. These events were suggested to be caused by EGCG induction of oxidative stress (Lambert *et al.*, 2010). Therefore, these compounds can exhibit either antioxidant or pro-oxidant properties, which depend on their concentrations and are based on complex chemical interactions (Lambert and Elias, 2010; Yang, *et al.*, 2011; Braicu, *et al.*, 2013).

Importantly, although polyphenols are the major chemical components of tea, its beneficial effects may also be exerted by other constituents. Although only caffeine is present in tea, both caffeine and pentoxifylline showed positive synergistic effects on treatment of cancer cells with the alkylator drug Thiotepa, which is commonly used for treating bladder cancer. After treatment with these methylxanthines, survival of the cells previously treated with Thiotepa decreased significantly; further analysis revealed that these methylxanthines may prevent G2 cell cycle delay (a normal defense mechanism that allows the cells to repair their DNA after Thiotepa aggression), increasing lethal chromosomal aberrations and ultimately leading to cell death (Fingert, *et al.*, 1986).

These *in vitro* studies are extremely important, since they report the anticancer action of tea and its components on several cellular pathways (figure 8). As discussed above, the number of possible intracellular targets and mechanisms of action of tea and its components is very significant; therefore, more studies are needed, in order to unveil its exact effects on cancer cells. Of note, although these results are very promising, one must keep in mind that *in vitro* results are not always transposed *in vivo*; this is due to the fact that metabolism of these compounds on living systems may difficult their action (Lambert and Yang, 2003). Therefore, *in vivo* studies are absolutely necessary, in order to verify if tea and its components can indeed benefit bladder cancer therapeutics.

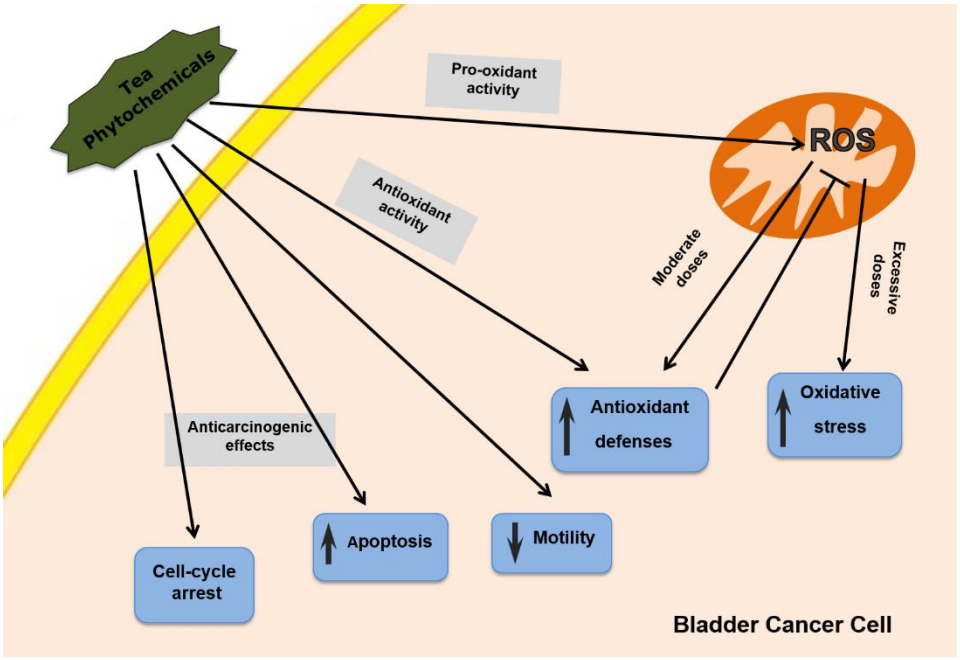


Figure 8. Schematic illustration of the main effects of tea components in a bladder cancer cell. Tea's phytocomponents can exert antioxidant or pro-oxidant activities, depending on its concentrations. When in high doses, they can induce excessive reactive oxygen species (ROS) production by mitochondria, contributing to oxidative stress increase. On the other hand, when in moderate doses, they contribute to low production of ROS, which will provoke a response in the cell, augmenting the endogenous antioxidant defenses. These compounds can also interfere in multiple cell signaling pathways, promoting cell cycle arrest, inducing cell apoptosis and decreasing cells ability to migrate.

In terms of animal carcinogenic models, most works studied GT consumption (mostly in substitution of drinking water) or EGCG administration effects (either mixed in drinking water or injected in the animals). *In vivo* studies of tea effects on bladder tumors were performed on mouse (Rieger-Christ, *et al.*, 2007; Sagara, *et al.*, 2010; Chen, *et al.*, 2011; Hsieh, *et al.*,

2011) and rat (Sato, 1999; Kemberling, *et al.*, 2003; Sato and Matsushima, 2003) models. Fortunately, some of the effects of tea components observed *in vitro* have been, to some extent, also reported *in vivo*. For example, EGCG was added to drinking water (0.05% w/v) and consumed by 6 weeks old mice, 7 days before subcutaneous injection of bladder cancer cells and 15 days after the referred injection. Results showed a significant decrease in tumor volume. Also, no side effects were observed, aside from a slight weight gain in the treated mice (Rieger-Christ, *et al.*, 2007). In another study using female mice with BBN-induced bladder cancer, which were fed with a solution of GT polyphenols (in a concentration of 0.5%), histological and immunohistochemical analysis of urinary bladder tissues revealed that a 24 weeks treatment with GT polyphenols reduced tumor growth and microvessel density. These results illustrate that these compounds also possess antiangiogenic effects, which may be responsible for the reduction in tumor growth, although no specific mechanistic studies were performed (Sagara, *et al.*, 2010).

A more recent study also demonstrated inhibition of tumor growth in mice fed with EGCG (in concentrations of 25 and 50 mg/kg per day) during a 42 days period after cancer induction. The mechanism of action proposed includes activation of intrinsic mitochondrial apoptotic pathway and is based on the *in vitro* studies discussed above (Chen, *et al.*, 2011). Significant reduction of tumor growth was also reported in bladder tumor-induced male mice treated with EGCG conjugated with gold nanoparticles. This result was accompanied by a decrease in cellular vascular endothelial growth factor expression, a protein known for stimulating vasculogenesis and angiogenesis. These findings suggest that, besides the apoptotic effects demonstrated by the conjugated treatment *in vitro*, the combination of EGCG plus nanoparticles may also be responsible for an angiogenesis inhibition *in vivo*, most likely through suppression of vascular endothelial growth factor. Nevertheless, the exact mechanisms are still unclear (Hsieh, *et al.*, 2011).

In a study using male rats with BBN-induced bladder cancer, animals treated with GT extract and powdered GT leaves displayed significant decrease in tumor volume. Moreover, histological observations revealed an improvement of histological grade of the induced bladder tumors and tendency to decreased depth of invasion (Sato, 1999). Ingestion of GT powdered leaves (2.5% mixed into a feeding pellet) before and after cancer induction resulted in a significant decrease in the number of tumors per rat and in mean volume per tumor, relatively to controls, as well as improvement of histological grade of the tumors (Sato and Matsushima, 2003). Treatment of rats with a solution of 200 μM EGCG also induced a significant decrease in tumor growth (Kemberling, *et al.*, 2003). The authors suggested that, while EGCG itself is effective as antitumor agent, its isolation and administration to patients would be difficult, mainly due to economic issues. Hence, the administration of GT leaves seems to be a better solution to counteract bladder cancer, providing an excellent quantity

of other beneficial compounds such as other catechins, caffeine, quercetin and vitamins (Sato, 1999; Sato and Matsushima, 2003).

Concerning *in vivo* and *in vitro* research studies, many promising results have been obtained (see Table 2). Indeed, tea extracts and components showed great anticancer activities. However, these studies present some cons that may hamper the extrapolation of the conclusions to human health applications, such as the fact that the concentrations of catechins (and other bioactive compounds) tested *in vitro* are normally much higher than those verified *in vivo*. Moreover, differences between animal species subjected to research and humans may also hamper the correct interpretation, extrapolation and practical application of the results and conclusions. Nevertheless, tea's health benefits seem undeniable, and its ability to inhibit tumor spreading and metastasis was verified in *in vitro* and *in vivo* carcinogenic models. The complex mechanisms by which tea and its components may exert their benefits are still poorly understood, and a general consensus has not been achieved yet. Further studies are needed, since studying tea's compounds and mechanisms of action may yield new insights in developing new therapeutic strategies for the treatment of bladder cancer.

II. Aims of the study

This work was focused in two main objectives.

The urinary bladder carcinoma is one the less studied types of cancer, in terms of metabolic profiles. Some studies have already identified that those cells also present the main metabolic feature of cancer cells, which is the high glycolytic flux, characteristic of the well-known Warburg effect. However, data on cancer progression and the associated alterations in the metabolism of cells from the different stages of the disease is still lacking. The study of bladder cancer cells metabolism and how it is associated with progression to different and more aggressive states is essential to the development of new diagnosis and therapeutic strategies. Therefore, the first objective of this work comprises the study of the glycolytic metabolism of two human urinary bladder cancer cells, representative of different cancer progression stages: RT4 (primitive stage) and TCCSUP (highly invasive stage). We hypothesize that both lines present distinct glycolytic profiles and that the detected differences may help to clarify the underlying mechanisms that ensure the progression of this type of cancer from one well-defined tumor to a more aggressive and invasive stage. Ultimately it may help to identify a molecular pharmacological/therapeutic target to counteract or avoid the progression of bladder cancer.

Additionally, this type of cancer is thought to be highly influenced by diet. It was reported that the regular consumption of tea may be a preventive factor against bladder cancer in humans. Moreover, several *in vivo* and *in vitro* studies identified the anticarcinogenic abilities of tea and its components. However, the molecular mechanisms by which tea and/or its phytochemicals exert the possible protective effects remain unclear. Although some studies showed the potential of tea to alter the metabolism of cancer cells, it remains to be tested in bladder cancer cells. Therefore, the second objective of this work was to develop preliminary studies to test the hypothesis that WT extract may exert its anticancer activities in these cells and in bladder cancer progression by modulating some metabolic intermediates of the glycolytic pathway.

III. Materials and Methods

1. Chemicals

The fetal bovine serum (FBS) was obtained from Biochrom (Berlin, Germany). ECF substrate was obtained from GE Healthcare (Weßling, Germany). All other chemicals were purchased from Sigma-Aldrich (St.Louis, MO, USA), unless specifically stated otherwise.

2. WT extract

WT was purchased on the Portuguese market (Diese, Bestlife - Comércio e Distribuição Lda., Portugal). Samples were subjected to infusion (1 g/100 mL distilled water; pH 5.5) at 100ºC during 5 min, according to the manufacturer's instructions. The resulting infusions were filtered with qualitative filter papers (VWR, catalog no. 516-0819, Leuven, France) in a vacuum system and freeze-dried overnight in a ScanVacCoolSafe Freeze-Dryer (Labogene, Lynge, Denmark), as previously described (Dias *et al.*, 2014). The mean extraction yields (g of lyophilized extract per 100 g of dried teas leaves) was 33.75%. The lyophilized extracts were kept in a desiccator, protected from light, until use.

3. Cell lines and experimental design

The two human urinary bladder TCC cell lines, TCCSUP and RT4, were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). RT4 cell line was established from a well-differentiated transitional papillary tumor, stage T2. These cells maintain transitional epithelial appearance, being large with defined margins (Rigby and Franks, 1970). TCSSUP cell line is derived from a highly invasive and anaplastic grade IV TCC of the bladder. These cells are small, with undefined boundaries and irregular growth pattern (Nayak *et al.*, 1977). Cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin solution, in an incubator with 5% CO_2 at 37ºC. The culture medium was substituted every 2 days, and cultures were split when a confluence of 80% or more was achieved. After 5 passages, the assay plates were left to achieve the desired confluence, after which they were treated with the respective mediums during the assay times for each particular study.

Glycolytic profile studies

After achieving the desired confluence of 75% or more, the cells were cultured during 48 hours in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution. At the end of this time, extracellular medium was collected for Proton Nuclear Magnetic Resonance (^{1}H

NMR) analysis and cells were trypsinized and frozen at -80ºC, for further protein extraction and Western Blot analysis.

Studies with cells exposed to WT

After achieving a confluence of 75% or more, the cells were cultured during 24 hours in one of the following mediums: DMEM supplement with 10% FBS and 1% penicillin-streptomycin solution, DMEM supplemented with 10% FBS, 1% penicillin-streptomycin solution and 0.1 mg/ml WT extract or DMEM supplemented with 10% FBS, 1% penicillin-streptomycin solution and 1 mg/ml WT extract. At the end of this time, extracellular medium was collected for ^{1}H NMR analysis and cells were trypsinized and frozen at -80ºC, for further protein extraction and Western Blot analysis.

4. ^{1}H NMR spectroscopy and spectral analysis

Samples of extracellular medium were collected from each culture flask and analyzed by ^{1}H NMR using our routine methods (Alves *et al.*, 2011; Rato *et al.*, 2012). In brief, spectra were acquired at 14.1 T, 25ºC, using a Bruker Avance 600 MHz spectrometer equipped with a 5-mm QXI probe and a z-gradient (Bruker Biospin, Karlsruhe, Germany), with solvent-suppression and at least 128 scans. The relative areas of 1H NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro NMR spectral analysis software (Acorn, NMR Inc., Fremont, CA, USA). ^{1}H NMR spectroscopy was performed in order to determine levels of production or consumption of glucose, lactate, alanine and pyruvate in both cell lines. The referred metabolites were identified in the spectra using the chemical shifts described in the literature: glucose, doublet located at 5.22ppm; lactate, doublet located at 1.33ppm; alanine, doublet located at 1.46ppm; pyruvate, singlet at 2.36ppm. A 10 mM sodium fumarate solution (singlet, 6.50 ppm) was used as internal reference.

5. Total protein extraction and quantification

Cells were removed from the assay plates and homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% protease inhibitor cocktail, 1% sodium orthovanadate 100 mM). Then, samples were allowed to stand on ice for 15 minutes, and later centrifuged at 14000.g for 20 minutes at 4ºC. The resulting pellet was discarded. Protein quantification was determined using the biuret protein assay commercial kit (Bibby Scientific UK), and following the manufacturer's instructions. In brief, protein quantification of the samples was calculated by measurement of absorbance at 540 nm. The shifts in absorbance values result from the formation of purple complexes by

samples containing more than two peptide bonds and the copper salts. Different bovine serum albumin concentrations were used as standard for calibration.

6. Western Blot

Western Blot was performed as previously described (Alves *et al.*, 2011) to quantify the expression of GLUT1, GLUT3, MCT4, LDH, PFK and GPT. In brief, samples containing 50ug protein were separated by electrophoresis in 12% polyacrylamide gels for 90 minutes, with 30mA per gel. Proteins were then electrotransferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hemel Hempstead, UK), and blocked in a 5% non-fat milk solution, for 90 minutes at room temperature. Membranes were incubated overnight with the primary antibodies rabbit anti-GLUT1 (1:500, CBL242, Merck Millipore), goat anti-GLUT3 (1:500, sc-31838, Santa Cruz Biotechnology), rabbit anti-MCT4 (1:1000, sc-50329, Santa Cruz Biotechnology), rabbit anti-LDH (1:5000, ab52488, Abcam), rabbit anti-PFK (1:500, sc-67028, Santa Cruz Biotechnology) or rabbit anti-GPT (1:200, sc-99088, Santa Cruz Biotechnology). A mouse anti-α-tubulin antibody (1:2500, T9026, Sigma Aldrich) was used as protein loading control. Immunodetection of the proteins was performed separately using the secondary antibodies goat anti-rabbit IgG-AP (1:5000, sc-2007, Santa Cruz Biotechnology) or goat anti-mouse IgG-AP (1:5000, sc-2008, Santa Cruz Biotechnology). Membranes were then incubated with the fluorescent substrate ECF for 5 minutes, and the reaction was detected with the Molecular Imager FX Pro plus Multimager system (Bio-Rad Laboratories). Band densities were measured using the Quantity One software (Bio-Rad Laboratories). The density values obtained were then normalized in relation to α-tubulin, by dividing the band volume by the respective α-tubulin band volume.

7. Sulforhodamine B (SRB) citotoxity assay

WT extracts' cytotoxicity effects were evaluated by the SRB assay. In brief, cells were previously treated with different WT extract concentrations mixed in the DMEM medium (0 mg/ml, 0.1 mg/ml or 1 mg/ml). After 48 hours, cells were fixated and incubated with SRB (Biotium, no. 80100, Hayward, USA) dye for 1h. Later, absorbance was measured at 492 nm. The shifts in absorbance values result from the binding of the dye to basic amino acids of the cellular proteins, providing an estimate of the total proteins, related to the cell number.

8. Statistical analysis

For the glycolic profile studies, statistical significance was assessed by unpaired t-test. For the WT SRB cytotoxicity assay, two-way ANOVA multiple comparisons were performed. For the WT metabolic studies, statistical significance was evaluated by one-way ANOVA and multiple t-tests. Statistical significance of all results was assessed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). All data are presented as mean±SEM. Differences with $P<0.05$ were considered statistically significant.

IV. Results

1. Bladder cancer progression from a primitive to a highly invasive stage is associated with decreased expression in GLUT1 and PFK

High glucose consumption is a common feature in cancer cells. Our results show that glucose consumption in both cell lines is very similar (figure 9A). Glucose enters the cells by the action of GLUTs, and it has been reported that their expression may be associated with increased malignancy (Reis, *et al.*, 2011). Although GLUT3 protein expression levels showed no significant differences between TCCSUP cell line (1.101±0.1128) and RT4 cell line (1.104±0.1460, figure 9C), GLUT1 expression was found to be significantly lower on cells from an invasive stage, TCCSUP cells (0.8280±0.2016), relatively to cells from a primitive stage, RT4 cells (1.639±0.3436, figure 9B). After glucose enters the cells it is metabolized and in that process, the irreversible conversion of fructose 6-phosphate to fructose 1,6-biphosphate by PFK is reported to be a rate-limiting control point (Underwood and Newsholme, 1965; Xiong, *et al.*, 2011; Ros and Schulze, 2013). Our results show that bladder cancer cells from a primitive stage, RT4, present a significant higher expression of PFK (2.432±0.7147) when comparing with bladder cancer cells from a highly invasive stage, TCCSUP, (1.008±0.4837, figure 9D).

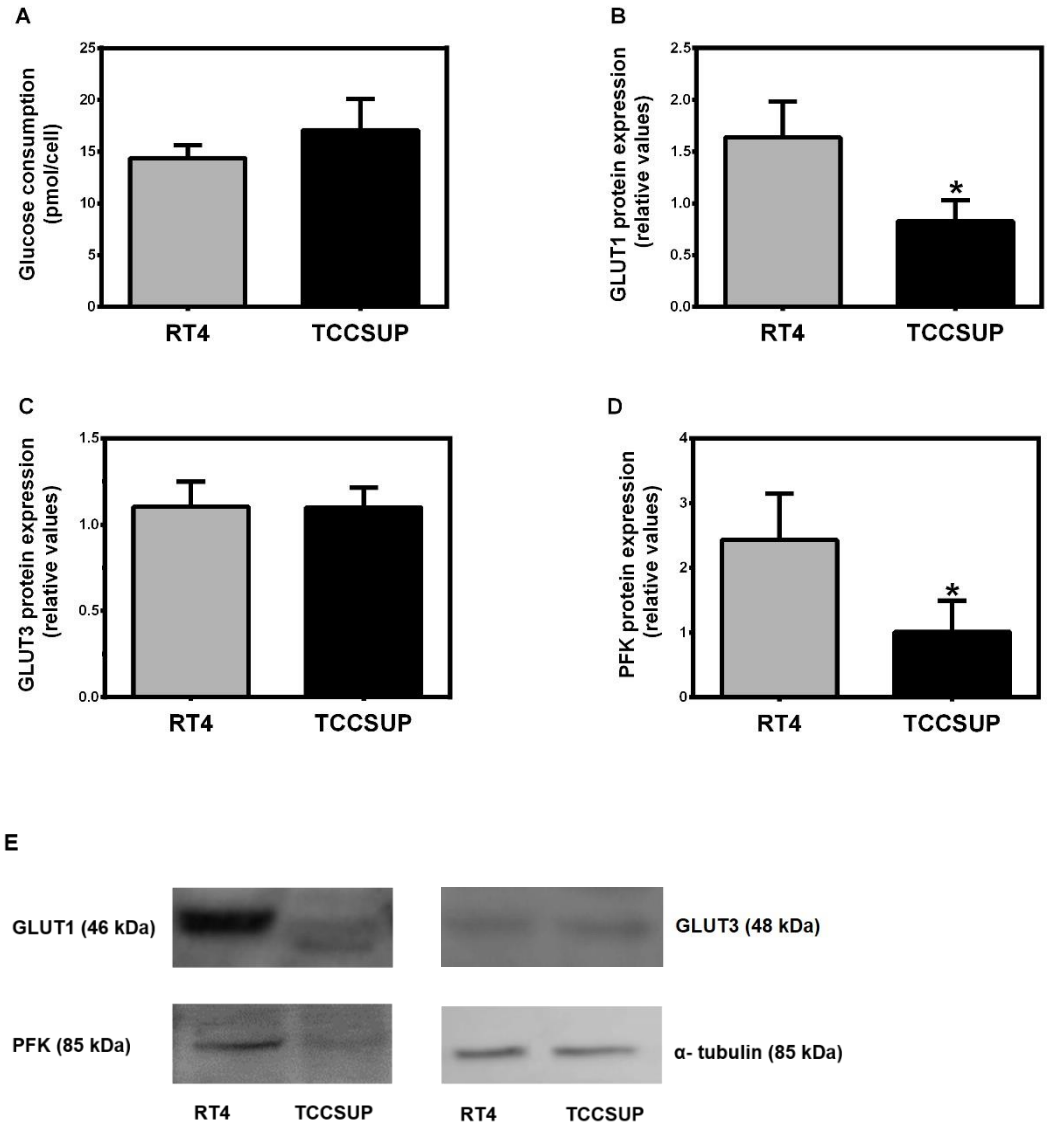


Figure 9. Glucose metabolism and uptake in bladder cancer cells representative of a primitive (RT4) and a highly invasive stage (TCCSUP). The consumption of glucose (Panel A) and protein expression levels of glucose transporter 1 (GLUT1, Panel B), glucose transporter 3 (GLUT3, Panel C) and phosphofructokinase (PFK, Panel D) are presented. Representative western blot bands are presented in panel E. Results are expressed as mean±SEM (n=6) and normalized in relation to α-tubulin. Significantly different results ($P<0.05$) are indicated as: * – relative to primitive bladder cancer cells (RT4).

2. Bladder cancer progression from a primitive to a highly invasive stage is associated with severe alterations in pyruvate metabolism

The glycolytic pathway culminates with the production of pyruvate from glucose taken from the extracellular medium. Pyruvate can be then be converted to lactate and/or alanine, through enzymatic conversion by LDH and GPT, respectively (Feron, 2009). Our results show that primitive stage bladder cancer cells, RT4, slightly produced pyruvate (0.0200±0.1319 pmol/cell) while highly invasive bladder cancer cells significantly consumed pyruvate (2.870±0.9404 pmol/cell, figure 10A). Pyruvate can be then converted to alanine or lactate. In fact, the production of alanine was found to be significantly higher in highly invasive bladder cancer cells (4.612±0.6463 pmol/cell) when compared with primitive stage bladder cancer cells (2.260±0.3203 pmol/cell, figure 10B).

Of note, the protein expression of GPT was found to be significantly decreased from 1.383±0.2026 in primitive bladder cancer cells to 0.7780±0.1263 in highly invasive bladder cancer cells (figure 10C). The pyruvate metabolism, as reflected in lactate/alanine ratio, is associated with NADH/NAD^+ equilibrium which in turn is indicative of the redox state of the cells (Williamson *et al.*, 1967; Vaz *et al.*, 2012). Our results show that this ratio is significantly higher in TCCSUP cells (27.03±1.115) when comparing with RT4 cells (20.23±2.106, figure 10D).

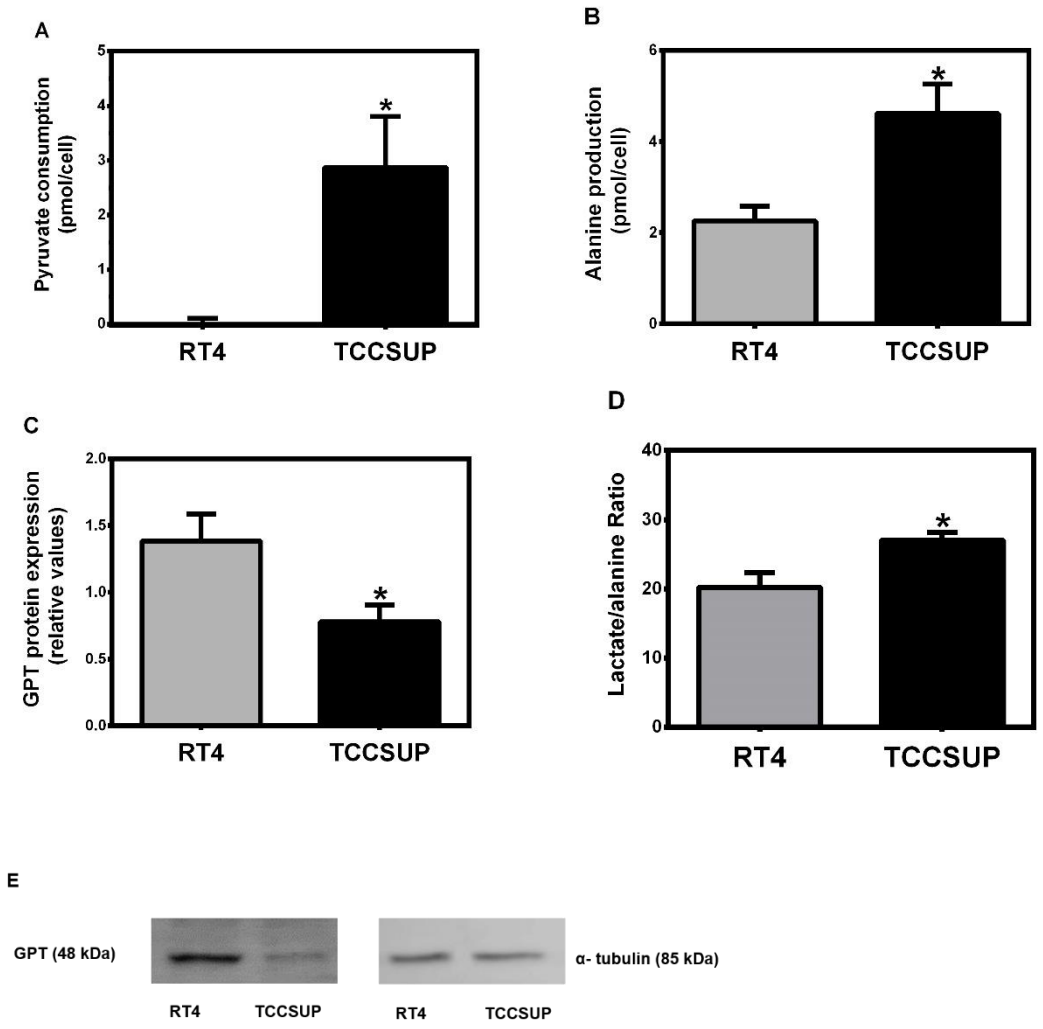


Figure 10. Pyruvate metabolism in bladder cancer cells representative of a primitive (RT4) and a highly invasive stage (TCCSUP). The consumption of pyruvate (Panel A), production of alanine (Panel B), the expression of glutamic-pyruvate transaminase (GPT, Panel C) and lactate/alanine ratio (Panel D) are presented. Representative western blot bands are presented in panel E. Results are expressed as mean±SEM (n=6) and normalized in relation to α-tubulin. Significantly different results ($P<0.05$) are indicated as: * – relative to primitive bladder cancer cells (RT4).

3. Production of lactate is stimulated in bladder cancer progression from a primitive to a highly invasive stage though LDH expression is decreased

The Warburg effect, which characterizes the metabolic behavior of cancer cells, is mainly based on the glucose uptake and consequent production of large amounts of lactate. Our results show that the progression of bladder cancer cells from a primitive to a highly proliferative stage is associated with stimulation of lactate production which increases from 45.69±3.755 pmol/cell in RT4 cells to 129.4±17.69 pmol/cell in TCCSUP cells (figure 11A). The lactate is produced by the action of LDH, which is responsible for the conversion of the pyruvate formed in glycolysis to lactate. The lactate is then exported from the cell by specific monocarboxylate transporters (Feron, 2009). Of note, our results show that bladder cancer cells from the highly proliferative stage, TCCSUP, presented a significantly lower levels of LDH expression (2.276±0.4898) than bladder cancer cells from the primitive stage, RT4, (4.947±0.2415, figure 11B). Our results also show that the expression levels of MCT4 are not altered in any of the cell lines representing the primitive and the highly proliferative stages (figure 11C).

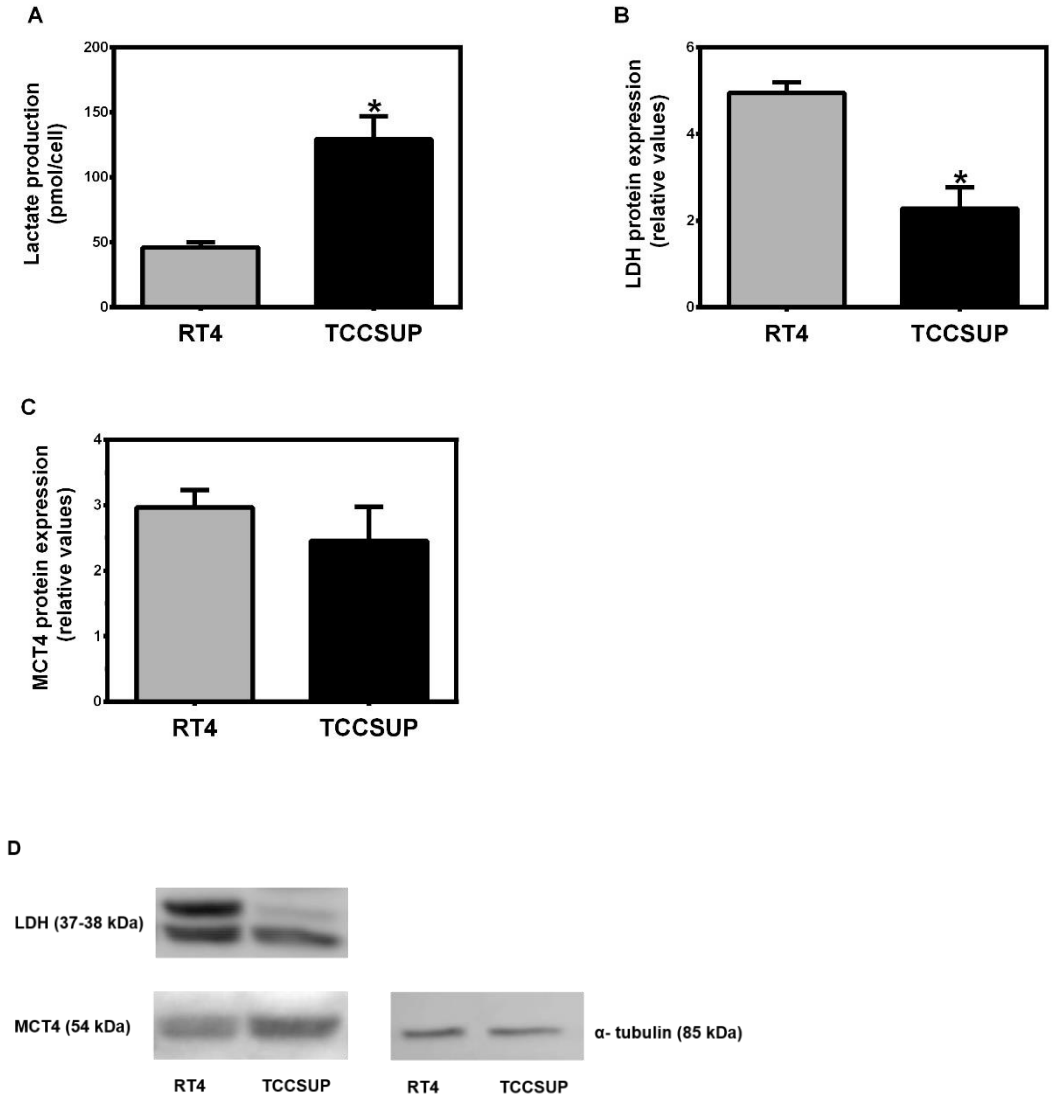


Figure 11. Lactate metabolism and transport in bladder cancer cells representative of a primitive (RT4) and a highly invasive stage (TCCSUP). The production of lactate (Panel A), the expression of lactate dehydrogenase (LDH, Panel B) and the expression of monocarboxylate transporter 4 (MCT4, Panel C) are presented. Representative western blot bands are presented in panel D. Results are expressed as mean±SEM (n=6) and normalized in relation to α-tubulin. Significantly different results ($P<0.05$) are indicated as: * – relative to primitive bladder cancer cells (RT4).

4. WT extract significantly reduces bladder cancer cell growth after 48 hours

Several studies reported tea's ability to induce apoptosis and prevent tumor growth in bladder cancer (Kemberling, *et al.*, 2003; Chen, *et al.*, 2004; Chen, *et al.*, 2011; Hsieh, *et al.*, 2011). However, WT's anticancer properties were never tested, to our knowledge, in bladder cancer cells. Therefore, to verify the effects of WT extract administration in both bladder cancer cell lines growth, SRB cytotoxicity assay was performed (figure 12). Our results demonstrate that in cells from a primitive stage of bladder cancer (RT4), significant cell growth decrease was verified when WT was added in the concentrations of 0.25 mg/ml (-67±45 % cell growth) and 1 mg/ml (-663±34 % cell growth), relatively to the other tested WT extract concentrations: 0.025 mg/ml (289±31 % cell growth) and 0.1 mg/ml (120±56 % cell growth). Regarding the TCCSUP cell line, which represents a highly proliferative stage of bladder cancer, 1 mg/ml of WT extract was able to significantly decrease cell growth (-434±28 % cell growth), relatively to the other tested WT extract concentrations: 0.025 mg/ml (193±94 % cell growth), 0.1 mg/ml (340±79 % cell growth) and 0.25 mg/ml (338±89 % cell growth).

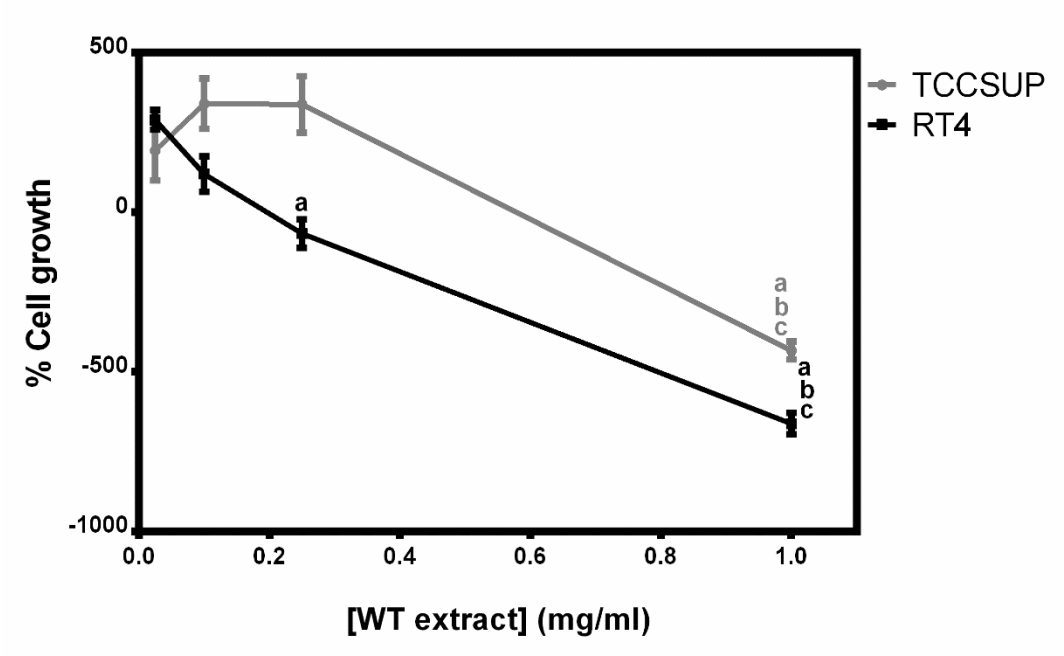


Figure 12. Effects of WT extract in the cell growth of bladder cancer cells from a primitive (RT4) and a highly proliferative (TCCSUP) stage, after 48h. Variation in cell growth (displayed as percentage) is plotted *vs* WT extract concentration (mg/ml). Results are expressed as mean±SEM (n=6 for each condition). Significant results ($P<0.05$) are indicated as: a - *vs* 0.025 mg/ml; b - *vs* 0.1 mg/ml; c - *vs* 0.25 mg/ml.

5. Exposure of cells from primitive and highly invasive stages of bladder cancer to WT extract does not influence GLUT1 and PFK levels

Our results showed that WT extract can affect cells growth, thus we aimed to identify some mechanisms or targets through which these effects are achieved. Therefore, since we have shown that bladder cancer cells from the primitive and the highly proliferative stage presented very distinct glycolytic profiles, we tested whether WT extract exerts its anticancer effects by modulating glucose metabolism in bladder cancer cells of different stages. The concentrations of the WT extract chosen were 0.1 mg/ml (one that, in the SRB assay, did not display evidence of significantly inhibiting cell growth in any of the cell lines) and 1 mg/ml (the one that displayed significant growth inhibition in both cell lines).

As previously discussed, excessive glucose consumption is an important feature in cancer cells. Moreover, the influence of WT on glucose metabolism is reported in several studies (Islam, 2011; Tenore *et al.*, 2013). Previous studies reported that WT can decrease glucose uptake in metabolic active cells with high glycolytic flux, altering the mRNA and protein expression levels of some glycolysis-related transporters (Martins *et al.*, 2014). In the bladder cancer cell line representative of a primitive stage (RT4), our results show that both concentrations of WT, 0.1 mg/ml (1.0058±0.18036-fold variation to control) and 1 mg/ml (1.1237±0.2502-fold variation to control), had no significant influence on GLUT1 expression, when compared to non-treated cells (1.000±0.1946, figure 13A). Similarly, in the cell line representative of a highly proliferative stage of bladder cancer (TCCSUP), treatment with 0.1 mg/ml (1.0698±0.2680-fold variation to control) and 1 mg/ml (0.8926±0.2412-fold variation to control) of the WT extract had no significant effects, in relation to non-treated cells (1.000±0.2918, figure 13A). Similar results were obtained when analyzing the expression levels of PFK. Treatment with 0.1 mg/ml (0.6560±0.1617-fold variation to control) and 1 mg/ml (0.8320±0.1891-fold variation to control) of WT extract showed no significant alterations in the PFK expression levels in bladder cancer cells representative of a primitive stage (RT4), when comparing to non-treated cells (1.000±0.2361, figure 13B). Also, in the TCCSUP cell line, representative of a highly proliferative stage, WT extract treatment did not influence PFK expression levels, neither in the concentration of 0.1 mg/ml (1.0123±0.1916-fold variation to control) nor at 1 mg/ml (1.3840±0.3314-fold variation to control), in relation to non-treated cells (0.9999±0.2858, figure 13B).

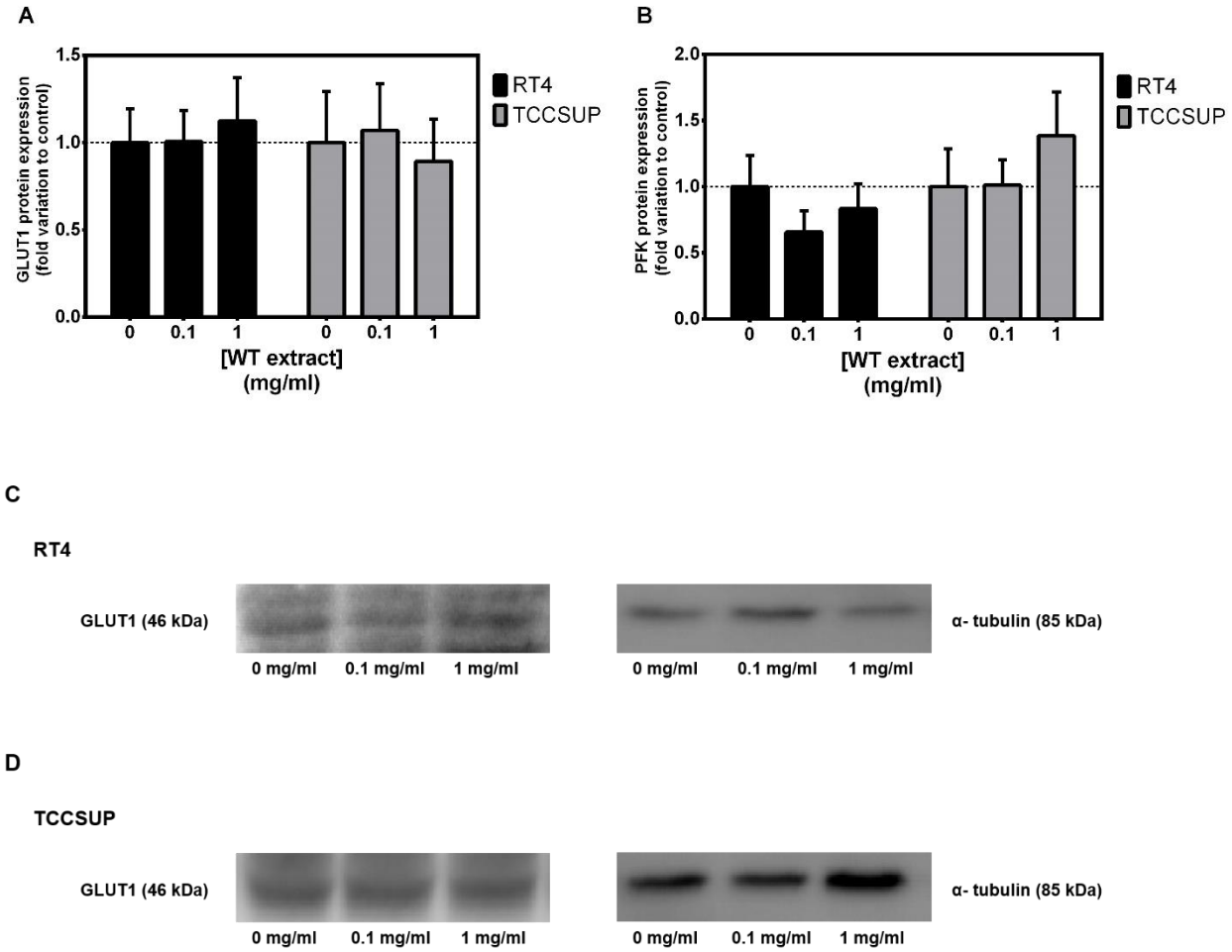


Figure 13. Glucose metabolism and uptake in bladder cancer cells representative of a primitive (RT4) and a highly invasive stage (TCCSUP) treated with different concentrations of WT extract. The protein expression levels of glucose transporter 1 (GLUT1, Panel A) and phosphofructokinase (PFK, Panel B) are presented. Representative western blot bands are presented in panels C and D, for RT4 and TCCSUP cells, respectively. Results are expressed as mean±SEM (n=3) and normalized in relation to α-tubulin.

6. Expression levels of LDH and MCT4 are not altered by WT extract in primitive and highly invasive stages of bladder cancer

As previously discussed, excessive lactate production is also a major characteristic of cancer cells and a product of the high glycolytic flux. In order to evaluate if the treatment with WT extract influenced the expression of the main enzyme responsible for its production and the main transporter responsible for its export, protein expression levels of LDH and MCT4 were determined. Our results show that, in the RT4 cell line, which represents a primitive stage of bladder cancer, LDH expression was not significantly altered by treatment with a WT extract of 0.1 mg/ml (0.7317±0.1532-fold variation to control) or 1 mg/ml (0.9365±0.1795-fold variation to control), when compared to non-treated cells (1.000±0.0747, figure 14A). In addition, in the bladder cancer line representative of a highly proliferative stage, TCCSUP, treatment with 0.1 mg/ml (1.1587±0.2917-fold variation to control) and 1 mg/ml (1.0712±0.1059-fold variation to control) of a WT extract did not significantly influence LDH expression levels relatively to non-treated cells (1.000±0.2177, figure 14A). Results regarding MCT4 protein expression on RT4 cells, treatment with 0.1 mg/ml (0.7464±0.1587-fold variation to control) and 1 mg/ml WT extract (1.0186±0.2075-fold variation to control) also did not reveal significant differences relatively to non-treated cells (1.000±0.1962, figure 14B). Similarly, TCCSUP cells treated with 0.1mg/ml (1.0690±0.1815-fold variation to control) and 1 mg/ml (0.8304±0.1274-fold variation to control) WT extract did not display significantly altered expression levels, when compared to non-treated cells (1.000±0.2973, figure 14B).

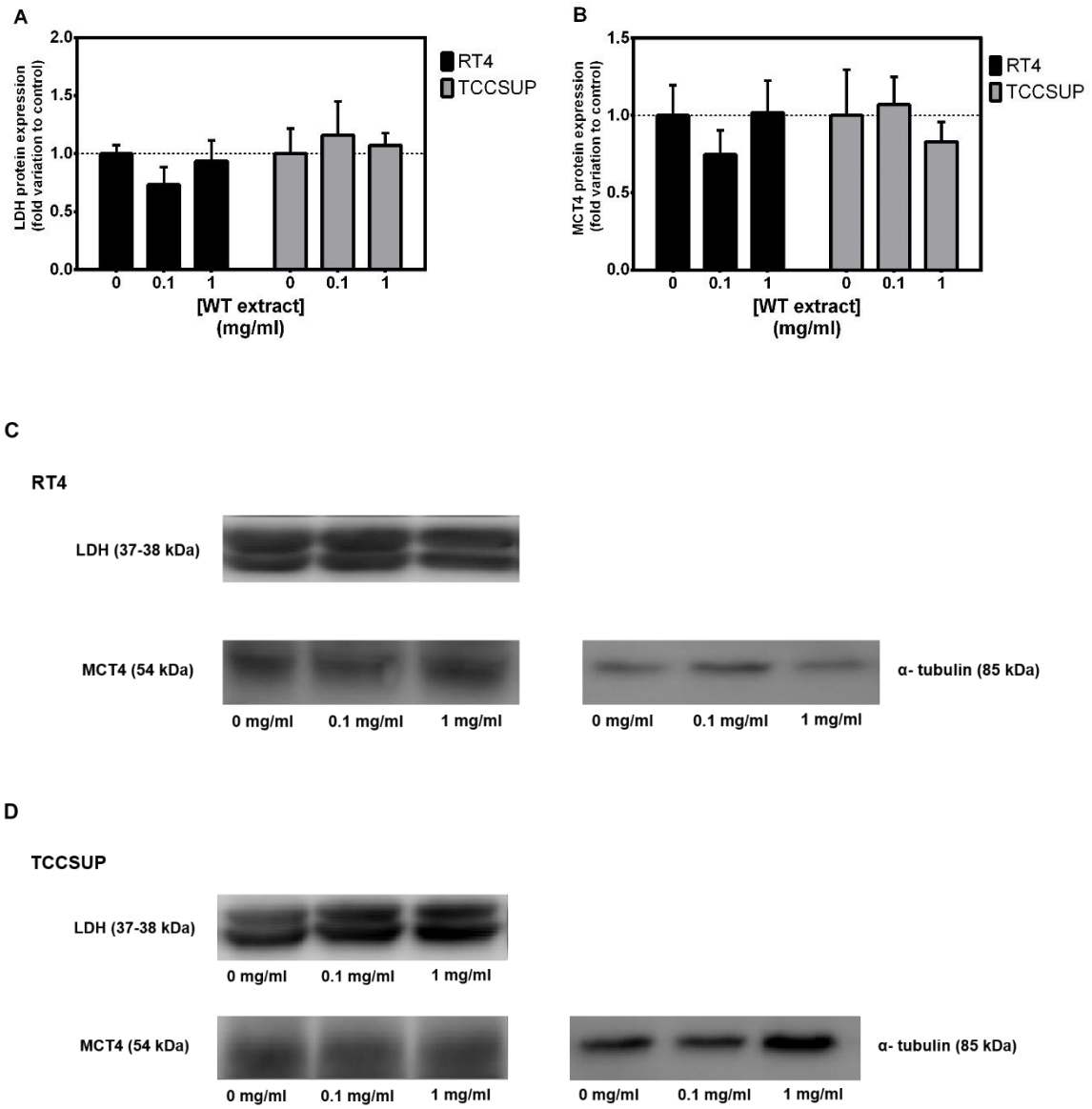


Figure 14. Expression of lactate dehydrogenase (LDH) and monocarboxylate 4 (MCT4) in bladder cancer cells representative of a primitive (RT4) and a highly invasive stage (TCCSUP) treated with different concentrations of WT extract. The protein expression levels of LDH (Panel A) and MCT4 (Panel B) are presented. Representative western blot bands are presented in panels C and D, for RT4 and TCCSUP cells, respectively. Results are expressed as mean±SEM (n=3) and normalized in relation to α-tubulin.

V. Discussion

Progression from a primitive to a highly invasive stage of bladder cancer is associated with severe alterations in glucose and pyruvate metabolism

Although the initiation and progression of bladder cancer at the genetic level are well reported, little attention has been given to the metabolic characteristics which may be involved in that process. Under normal circumstances, most cells use mitochondrial respiration to obtain the majority of the energy required for their normal functioning (Oliveira, *et al.*, 2014). However, cancer cells primarily use glucose as their primary substrate for obtaining energy (Warburg, *et al.*, 1927; Warburg, 1956; Oliveira, *et al.*, 2014). It is known that glycolysis is an inefficient way of obtaining energy, when compared to mitochondrial respiration, but it confers other advantages to cancer cells, such as the production of intermediates necessary to several other metabolic pathways (DeBerardinis *et al.*, 2007; Galluzzi *et al.*, 2013; Oliveira, *et al.*, 2014). In recent years, the study of cancer cells metabolic behavior has been linked to cell survival and uncontrolled proliferation (Elstrom *et al.*, 2004; DeBerardinis *et al.*, 2008). Moreover, several therapeutic targets associated to cancer cells metabolism have been proposed. Thus, it is imperative to disclose the alterations that occur in cancer cells metabolism during the progression from a primitive to a highly invasive stage. Herein, we studied the glycolytic metabolism of two human urinary bladder cancer cells, representative of different cancer progression stages: RT4 (primitive stage) and TCCSUP (highly invasive stage).

It has been reported that the alterations responsible for the aggressiveness of cancer cells often involve the regulation of glucose transporters or intermediates of glycolysis, illustrating that glucose consumption and uptake is of extreme relevance for cancer onset and progression (Flier *et al.*, 1987; Osthus, *et al.*, 2000). Our results show that bladder cancer cells of a primitive (RT4) and a highly invasive (TCCSUP) stage present a distinct pattern of glucose uptake and metabolism although the consumption of glucose was similar in both cell lines. GLUT1 has been reported as an intrinsic marker of hypoxia and can be used to predict survival in patients suffering with bladder cancer (Hoskin *et al.*, 2003). However, the Warburg effect and glycolytic flux stimulation is known to occur even in the presence of oxygen (for review see Oliveira *et al.*, 2014), illustrating that cancer cells prefer the glycolytic pathway before being exposed to hypoxia. Most bladder tumors are low grade papillary tumors and although progression occurs in 10-20% of the cases (for review see Cheng *et al.*, 2014), the metabolic alterations that accompanied the progression from a low grade to an invasive tumor are not characterized. Our results show that progression from a primitive to a highly invasive stage in bladder cancer, under non-hypoxia conditions, is accompanied by a decrease

in the expression of GLUT1 while GLUT3 levels remained unchanged. Nevertheless, glucose consumption remained high. These results illustrate that GLUT1 is a key control point of bladder cancer progression even in non-hypoxic conditions and suggest that although its expression is decreased, its activity may be stimulated. Cancer cells survival and proliferation has been associated to glucose uptake and also with altered expression of several metabolic intermediates of the glycolytic process, such as the enzymes PFK and PK (Pradelli *et al.*, 2014). PFK mediates the irreversible conversion of fructose 6-phosphate to fructose 1,6-biphosphate limiting the glycolytic activity of cells. Thus, it has been proposed as a target to anticancer drugs (for review see Hasawi *et al.*, 2014). Our results suggest that progression from a primitive to a highly invasive stage in bladder cancer is associated with a significant decrease in the expression levels of PFK. This is in concordance with studies performed in other cancer cell lines, whereas the cells representative of more aggressive stages also displayed lower PFK expression levels (Chehtane and Khaled, 2010; Vaz*, et al.*, 2012).

Typically, cells consume glucose and metabolize it through a series of enzymatic reactions in the glycolytic pathway. The last step of glycolysis involves the conversion of phosphoenolpyruvate to pyruvate (Feron, 2009). Thus, pyruvate is the end-product of this metabolic pathway and is usually transported to mitochondria to enhance the Krebs cycle and serve as precursor in biosynthetic pathways (Gray *et al.*, 2014). In cancer cells, given the excessive amounts of glucose consumed, there is also an excessive amount of pyruvate formed that is usually associated with the production of very high quantities of lactate (DeBerardinis*, et al.*, 2007; Oliveira*, et al.*, 2014). This shift in the glycolytic profile of cells is often reported as a hallmark of cancer onset and progression. Our results show that a shift in pyruvate metabolism follows bladder cancer progression from a primitive to a highly proliferative stage. Bladder cancer cells from a primitive stage produced a very small amount of pyruvate however, as the metabolic demands increase in the highly invasive and proliferative stage, the bladder cancer cells consume most of the available pyruvate in culture media illustrating that pyruvate alone is a primary fuel, rather than glucose, in these cells. Pyruvate can either be converted to lactate or alanine. Our results show that the production of alanine is stimulated in highly invasive bladder cancer cells when compared with bladder cancer cells from a primitive stage. However, this was followed by a significant decrease in the levels of GPT, the enzyme responsible for the conversion of pyruvate to alanine which illustrates GPT activity may be stimulated. The alanine derived from pyruvate may then be exported to the extracellular medium, a behavior that has been reported to occur in some cancer cells (DeBerardinis*, et al.*, 2007; Vaz*, et al.*, 2012).

Several studies have shown that pyruvate conversion to acetyl-CoA is inhibited in cancer cells while the conversion of pyruvate to lactate is favored (Kim *et al.*, 2006; Fan *et al.*, 2014). The lactate derived from glycolysis is then exported to the extracellular medium, by of the action of MCT4 (Feron, 2009). Our results suggest that, as bladder cancer progresses from a

primitive to a highly proliferative stage, lactate production is stimulated though the protein expression levels of LDH were found to be decreased. Of note, no alterations were detected in the expression levels of MCT4 which illustrates that the production and export of lactate is not accompanied by stimulation in the levels of this transporter.

The conversion of pyruvate to lactate is accompanied by the conversion of NADH to NAD^+, which is an essential intermediate in the glycolytic process. Therefore, the production of lactate is of extreme importance to maintain the intracellular levels of NAD^+ needed to support the high glycolytic flux (Feron, 2009). Besides, compelling evidence shows that in cancer, lactate is not a waste product of glucose metabolism. It is diffused to the extracellular space by hypoxic tumor cells and taken up by oxygenated tumor cells (Sonveaux *et al.*, 2008). This is in accordance with our results, since bladder cancer tumor stage IV (represented by TCCSUP cells) is much more aggressive and rapid proliferating than stage II (represented by RT4 cells). At a highly proliferative stage the tumors are in systematic growth and the number of hypoxic cells is expected to be much higher than in lower stages of the disease. Thus, the tumors must adapt to this new environment and ensure that all the cells are receiving the needed fuel sources. Moreover, TCCSUP cells present a significantly higher consumption of pyruvate, which is used in biosynthetic pathways but its primary action, in cancer cells, is related to the production of lactate. It was previously showed that prostate cells of different cancer stages present a distinct production of lactate. The cells representative of the most aggressive stage produced significantly higher lactate levels (Vaz, *et al.*, 2012) illustrating that this production of lactate may be a hallmark of cancer progression in several types of cancer. Overall, our results suggest that more than glycolysis and glucose uptake, pyruvate metabolism plays a pivotal role in the progression of bladder cancer from a primitive to a highly proliferative stage. Moreover, since the conversion of pyruvate to alanine or lactate is coupled with NADH and NAD^+ reactions, the lactate/alanine ratio is well known as an indicator of the redox state of the cell since it reflects the equilibrium between those two intermediates. Our results show that the lactate/alanine ratio is significantly higher in TCCSUP cells, suggesting that progression of the cancer stage is correlated with higher oxidative stress levels due to stimulation of pyruvate metabolism and lactate production. In fact, it is well known that the increased glycolytic flux presented by proliferating cells is associated with high oxidative stress (for review see Pelicano *et al.*, 2004; Pavlides *et al.*, 2012). The comparison between cell lines of the same bladder cancer type (TCC), but it yet representative of different stages of the disease, is very important and highly informative regarding the metabolic alterations that are cause or consequence of cancer progression. The differences detected in the metabolism of the cells of different cancer stages yield valuable information that can and must be used in the search for a suitable anticancer agent. Regarding bladder cancer, there is not much information available on the metabolic profiles of cells from different stages. Our results provide compelling evidence that bladder cancer progression is accompanied by several changes in the

metabolism of the cells (figure 15), particularly regarding stimulation of pyruvate consumption and lactate production. These results also suggest that these are strategies that cancer cells use to continue to proliferate and produce biomass in sufficient quantities to maintain their progression.

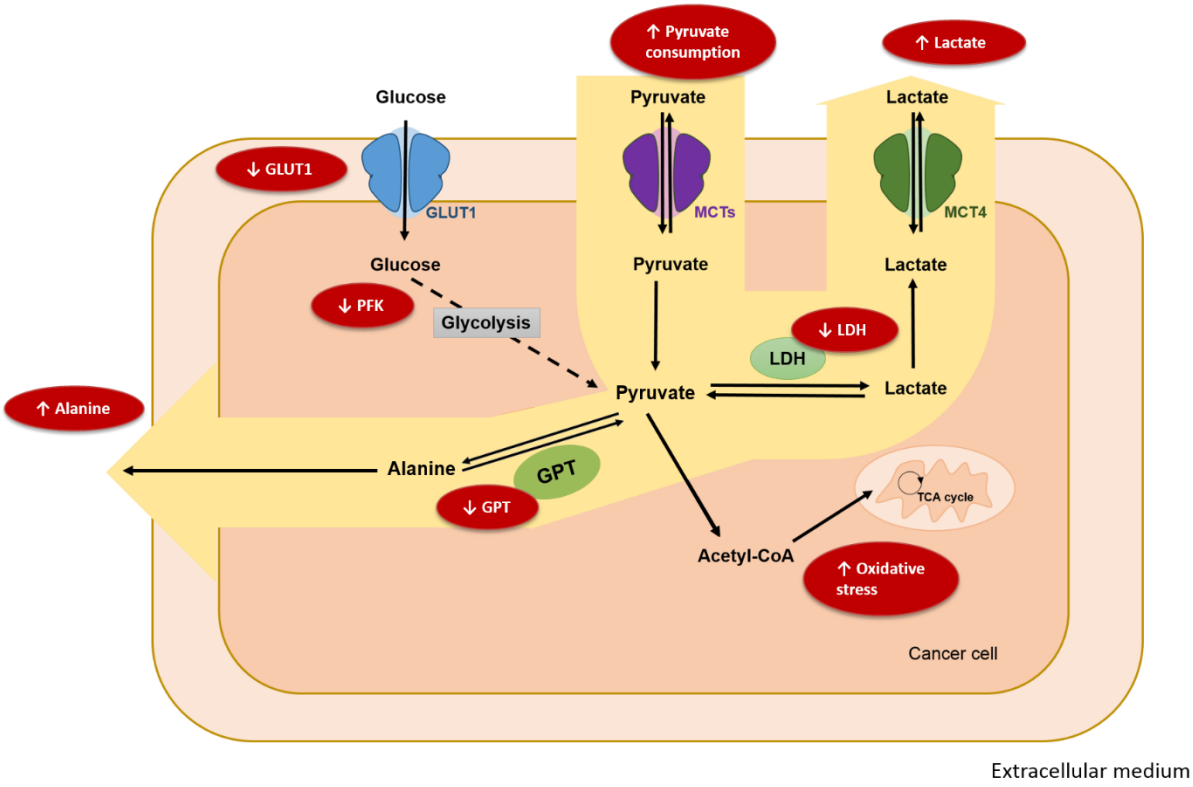


Figure 15. Summary of bladder cancer cells metabolism and the alterations induced by the progression from a primitive to a highly proliferative stage. Progression of bladder cancer is associated with a decrease in the expression of glucose transporter 1 (GLUT1), phosphofructokinase (PFK), glutamic-pyruvate transaminase (GPT) and lactate dehydrogenase (LDH). However, the consumption of pyruvate is highly stimulated as well as the production of alanine and lactate. Of note, the lactate/alanine ratio provides evidence that progression of bladder cancer is associated with increased oxidative stress.

WT extract successfully inhibits cell growth in primitive and highly invasive stages of bladder cancer cells, but preliminary results suggest that antiproliferative effects are not associated with expression of glycolysis-related enzymes and transporters

Tea presents several anticarcinogenic properties. In bladder cancer, inhibition of tumor growth, induction of apoptosis and cell cycle arrest are well reported (Kemberling, *et al.*, 2003; Chen, *et al.*, 2004; Chen, *et al.*, 2011; Hsieh, *et al.*, 2011). Thus, the anticarcinogenic properties of tea can be achieved through several approaches. For example, tea components can cause cell death and impede tumor growth by interfering in the mitochondrial apoptotic pathway (Chen, *et al.*, 2011; Hsieh, *et al.*, 2011), generating reactive species (Hou, *et al.*, 2005), and modulating gene expression or interfering in transporter functioning (Chen, *et al.*, 2004; Hou, *et al.*, 2005), among other mechanisms. Besides, targeting metabolism has been suggested as a suitable approach to avoid cancer onset and progression (Masoudi-Nejad and Asgari, 2014). Although it is the less studied type, compelling evidence suggests that WT has the most significant health promoting properties among all types of tea, from which the anticancer activity may be highlighted (Dias, *et al.*, 2013). Herein we hypothesized that a WT extract could have anticancer effects on bladder cancer cells by modulating the metabolic behavior of cells representative of a primitive (RT4) and highly proliferative (TCCSUP) stages. Preliminary results were obtained concerning the cytotoxicity and antiproliferative effects of the WT extract and its effect in the expression of selected enzymes and transporters of glycolysis.

Previous reports from our team have already determined the phytochemical profile of the WT extract. It was reported that the WT extract is very rich in flavonoids, particularly EGCG and EGC, and possesses high levels of caffeine (Martins, *et al.*, 2014). Of note, our previous reports have also shown that the levels of these phytochemicals are significantly higher in a WT extract than in a GT extract (Dias, *et al.*, 2014), illustrating that a WT extract may have a more relevant biological activity. It was demonstrated that EGCG can inhibit cell growth and provoke cell death by modulating multiple signaling pathways in cancer cells. Several mechanisms have been proposed to justify these effects. Studies reported that this catechin is capable of inhibiting the activation of epidermal growth factor receptors (EGFRs), the initial kinases in the epidermal growth factor (EGF) signaling pathway, which are tremendously important for cell proliferation (Sah *et al.*, 2004; Hou, *et al.*, 2005; Milligan *et al.*, 2009). However, the exact mechanism through which this inactivation is achieved is still unclear, and several different hypothesis have been proposed. For example, some authors hypothesized that EGCG undergoes auto oxidation and can inactivate EGFRs through action of

the formed radicals. These radicals would attack and inactivate the receptors by binding directly to the tyrosine kinase active sites or by altering the protein conformation, therefore inhibiting the receptors ability to undergo autophosphorylation as the ligands bind (Hou, *et al.*, 2005). This would alter the downstream signaling and consequently modify gene transcription. However, others verified that EGCG can inhibit the activation of the receptors, but is ineffective at inhibiting the functioning of constitutively activated receptors (independent of ligands) (Milligan, *et al.*, 2009). Compelling evidence suggests that EGCG is capable of interfering with the membrane lipid rafts, decreasing the number of receptors activated (Milligan, *et al.*, 2009). Despite a consensus was not yet achieved regarding the mechanism of action, EGFR inhibition by EGCG is well documented. Several studies reported that these effects may have significant alterations at various levels, which can be related to some of the several reported possible EGCG targets and mechanisms of action. Interference of EGCG in Akt pathway has been reported in bladder cancer cells associated with decreased cell proliferation and apoptosis (Jeong *et al.*, 2008; Chen, *et al.*, 2011; Shen *et al.*, 2014); also, inhibition of Akt may increase the levels of p53 and p27 proteins, well known for interfering in cell cycle regulation (Shin *et al.*, 2002; Sah, *et al.*, 2004; Jeong *et al.*, 2005). Cell-cycle arrest induced by EGCG in bladder cancer cells was reported in other studies (Chen, *et al.*, 2004). Similarly, EGCG-induced increase in Bad levels in bladder cancer cells were also reported (Chen, *et al.*, 2011; Hsieh, *et al.*, 2011); as result of the interference in the EGF signaling, dephosphorylated Bad protein is capable of inactivating the Bcl-XL antiapoptotic protein (Datta *et al.*, 1997; Cheng *et al.*, 2001; Sah, *et al.*, 2004), which was also reported to be decreased in EGCG treated bladder cancer cells (Sah, *et al.*, 2004). Moreover, dephosphorylated forkhead transcription factor (FKHR) can increase the expression of the proapoptotic molecules Bcl-2–interacting mediator of cell death (BIM) and Fas-ligand (Dijkers *et al.*, 2000), therefore inducing cell apoptosis, also reported in EGCG treated bladder cancer cells (Chen, *et al.*, 2011; Hsieh, *et al.*, 2011). Other studies also referred EGCG's ability to increase the activity levels of several caspases (Chen, *et al.*, 2011; Hsieh, *et al.*, 2011). These results clearly show that EGCG may modulate apoptotic pathway and successfully induce cancer cell death. Moreover, GT-induced decrease in bladder cancer growth and proliferation were also reported (Sato, 1999; Lu, *et al.*, 2005). In fact, compelling evidence suggests that tea's anticancer properties may be more effective by the treatment with tea extract or its powdered leaves, rather than its isolated components, due to the beneficial synergistic effects of all components present in tea (Sato, 1999; Sato and Matsushima, 2003). Our results are in concordance with the studies reporting cell death induced by GT and the major component of tea's extracts, EGCG. WT extract was capable of inducing cell death at the concentrations of 0.25 mg/ml and 1 mg/ml in the primitive bladder cancer stage (represented by RT4 cells). As expected, a higher concentration (1 mg/ml) was needed to inhibit the growth of the high invasive stage (represented by TCCSUP). These results suggest that, as the cancer progresses, the cells are able to develop defenses or become immune/unresponsive to some of the signals that EGCG and other WT components

may induce, thus explaining the difference in cell death induction by 0.25 mg/ml WT extract in the primitive stage that is not verified in the highly invasive stage. Therefore, it is expected that at these two different cancer stages cells depend on/develop slightly different survival and proliferative mechanisms, which could be due to modifications in cell signaling or gene expression. The first part of our study clearly showed that the progression of bladder cancer from a primitive to a highly proliferative stage was accompanied with profound alterations in the metabolism of bladder cancer cells. Thus, we performed preliminary studies to disclose alterations in selected enzymes and transporters of the glycolytic pathway after exposure to a WT extract. As discussed, cancer cell metabolism is the result of the cooperation between several pathways whose functioning is altered in relation to normal cells. Among these is elevated glycolytic flux, with the primary objective of generating energy and biomolecules in sufficient amounts to meet the high demands for cell proliferation and survival (for review see Lunt and Vander Heiden, 2011; Oliveira*, et al.*, 2014). Due to its importance, several studies suggest that this pathway may be an excellent target for anticancer agents (for review see Pelicano *et al.*, 2006). Moreover, it is known that bladder cancer can be modified by dietary factors. It was also reported that some food nutrients may affect the glycolytic metabolism in different types of cancer (for review see Keijer *et al.*, 2011), which illustrates the possibility of nutritional-based therapies. Several studies reported that tea and its components are able to interfere in bladder cancer progression and development, through many different pathways. However, interference in the glycolytic metabolism of the cells remains to be tested. The glycolytic pathway is thought to be regulated not only by the enzymes that mediate the intermediate reactions, but also by other cellular pathways (Frauwirth *et al.*, 2002; Elstrom*, et al.*, 2004). One of their major signaling components is the Akt protein. In fact, Akt activation promotes GLUT1 expression and maintains high activity of PFK, thus stimulating the glycolytic metabolism (for review see Hatzivassiliou *et al.*, 2005). Therefore, it is expected that, given the already discussed interference of tea and its components in the activity of this signaling protein, modulation of the glycolytic metabolism of bladder cancer cells could occur. Moreover, previous studies reported that WT extract is capable of significantly reduce the uptake of glucose in metabolic very active cells with a high glycolytic flux, mainly by decreasing GLUT1 mRNA and protein expression levels. Interestingly, treatment with WT extract was also able to significantly decrease PFK expression in those cells (Martins*, et al.*, 2014). Similarly, a study demonstrated an EGCG-induced decrease of GLUT1 mRNA expression in colon cancer cells (Hwang *et al.*, 2007). Also, inhibition of glucose uptake by EGCG and quercetin (both present in tea) was reported in breast cancer cells (Moreira *et al.*, 2013). Our preliminary results did not show significant alterations on the expression of GLUT1 and PFK either in the primitive stage of bladder cancer (represented by RT4 cells) or the highly invasive stage (represented by TCCSUP cells), when compared to non-treated cells. This suggests that the anticarcinogenic properties of WT may be exerted through modulation of other cellular pathways, or other glycolysis intermediates. For example, studies reported that EGCG prevents glucose uptake in

adipocytes, not by altering the expression levels of GLUT1, but by interfering in the translocation of specific glucose transporters (also maintaining its expression levels) (Ku *et al.*, 2014). Moreover, it is plausible to suggest that although the expression of GLUT1 and PFK are maintained, their activity may be altered. Therefore, further studies will be needed to consolidate these findings, particularly concerning glucose consumption and metabolism by these cells. Also, the expression levels of LDH and MCT4, which are key intervenients of lactate metabolism, were also not significantly altered in the treated cells. Despite these results, there are studies reporting EGCG and quercetin interference in lactate production (Moreira, *et al.*, 2013). Therefore, it should be highlighted that these are only preliminary results, and that analysis of other metabolic intermediates such as GLUT3 and GPT expression, and glucose, pyruvate, alanine and lactate production levels is strictly required in order to draw reliable and precise conclusions about the effects of a WT extract on bladder cancer progression. Also, the fact that WT extract did not alter the expression of the referred transporters and enzymes does not mean that they are functioning properly, since its activity may be altered. Therefore, studies focused on the activity of these transporters and enzymes should also be conducted.

VI. Conclusions

Bladder cancer is one of the most common types of cancer. Depending on the cancer stage, it presents high risk of recurrence and may be lethal. Although cancer stage progression is well studied at a genetic level, alterations in the cellular metabolism require more attention. Several studies verified that cancer cells display a high glycolytic flux with consequent production of excessive levels of lactate, a phenomena known as Warburg effect. Accompanying these alterations is the upregulation of several glycolytic intermediates such as glucose transporters and regulatory enzymes and impaired or non-utilized mitochondrial phosphorylation. This phenomena has also been observed in bladder cancer cells. Although it is expected that bladder cancer stage progression implicates several alterations at metabolic levels, as it does at genetic levels, specific studies on cell metabolism in bladder cancer progression are lacking. These studies are very important since they may allow the discovery of molecular markers or pathways implicated in cancer progression that may serve as therapeutic targets. As expected, our results support the hypothesis that bladder cancer progression from a primitive to a highly invasive stage is accompanied by several metabolic changes. Our work clearly shows that among those changes, pyruvate consumption seems to be a major factor. Further studies will be needed to evaluate the signals that mediate the stimulation of that metabolic pathways and how it relates to malignant transformation. Our work also provides compelling evidence that glucose uptake is not altered and higher levels of alanine and lactate are produced, which illustrates a more accelerated metabolism and may indicate how bladder cancer cells respond to aggressive environments such as hypoxia. The advantage of maintaining a high glycolytic flux in cancer cells is not well understood. However, it is a hallmark of cancer onset/proliferation and bladder cancer is no exception. Therefore, understanding the metabolic alterations that are associated with bladder cancer progression, and thus elaborate the cells metabolic phenotype, is essential to develop therapeutic strategies that may hamper cells proliferation or induce their death. However we must note that cancer cells exhibit a great metabolic plasticity that must be considered when testing drugs that selectively act on cancer cells based on their metabolic behavior.

Moreover, several studies reported that bladder cancer in humans may be prevented by several dietary factors. This fact is very important and it should be addressed, since food and beverage constituents are widely available and may constitute a more safe and economic approach to treat or prevent bladder cancer than synthetic drugs. Particularly tea, a beverage obtained by infusion of *Camellia sinensis*' leaves or buds, is thought to have remarkable anticancer effects. These effects are well reported in *in vivo* and *in vitro* bladder cancer studies, and include induction of apoptosis, inhibition of metastization and cell cycle arrest. However, the molecular mechanisms by which tea and/or its phytochemicals exert the possible protective effects remain unclear. A key feature of cancer cells is proliferation, which has been associated with changes in cellular metabolism. Although some studies showed the potential effect of tea and its components to change cancer cells metabolism, it remains to be tested in bladder cancer cells. WT, the rarest type of tea, presents a chemical

composition that is thought to confer superior health promoting properties, relatively to the other types of tea. Moreover, WT's ability to interfere in the glycolytic metabolism of cells has been reported. However, the effects of WT on bladder cancer cells have not yet been tested. Therefore, we proposed to conduct a preliminary study on the effects of different concentrations of WT extract on the survival of bladder cancer cells as well as in some enzymes and transporters of the glycolytic pathway of these cells. Our results clearly demonstrated WT's ability to promote cell death and thus inhibiting cancer growth. The maximum concentration of WT extract tested, 1mg/ml, has showed remarkable growth inhibition properties in both cell lines representative of a primitive and a highly invasive bladder cancer stages. Moreover, 0.25 mg/ml WT extract also showed significant cell growth inhibition in the RT4 cell line, but not on the TCCSUP cell line illustrating that the latter stage of bladder cancer, as expected, is more aggressive and difficult to inhibit its growth. Although specific mechanistic studies have not yet been performed, this may suggest that the cells in different cancer stages possess slight differences in their survival or progression mechanisms, thus responding in different ways to WT extract death-inducing properties. On the other hand, our preliminary results show that the anticancer property of the WT extract may not be directly related to alterations in GLUT1, PFK, LDH or MCT4 expression. However, these preliminary results do not exclude the possibility that WT may interfere in the glycolytic metabolism of cancer cells, since there are some studies, performed in cells from other types of cancer, reporting that tea and its components can modulate glucose uptake and metabolism. Therefore, more studies will be needed. Of note, this study demonstrates that WT may indeed possess potential as chemopreventive or chemotherapeutic agent. Its exact mechanisms of action, as well as the contribution of each of its components and their combination should be further analyzed. This may yield new insights and contribute to the development of more effective anticancer drugs or food supplements.

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Annex I

The publications arising from the work developed by Vanessa Conde during her M.Sc. in Biomedical Sciences are:

Conde, V.R., Alves, M.G., Oliveira, P.F. and Silva, B.M. Tea (*Camellia sinensis* (L.)): a putative anticancer agent in bladder carcinoma?. (submitted)

Nunes, A.R., Alves, M.G., Tomás, G.T., Conde, V.R., Cristóvão, A.C., Moreira, P.I., Oliveira, P.F. and Silva, B.M. Daily consumption of white tea by prediabetic Wistar rats improves cerebral cortex metabolic and oxidative profile. (submitted)