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IDENTIFICATION AND CHARACTERIZATION OF GLYCOBIOMARKERS IN BLADDER CANCER STEM CELLS

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“O único sitio onde o sucesso vem antes do trabalho é no dicionário”

Nota preliminar

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Resumo

O tratamento e decisão clínica no cancro de bexiga avançado permanecem críticos, principalmente devido à sua grande heterogeneidade molecular. Assim, urge identificar novos biomarcadores e criar novas ferramentas para estratificar com maior precisão o risco de progressão e o prognóstico. Adicionalmente, é importante criar um racional molecular que permita definir seletivamente as células estaminais tumorais de bexiga, que fenotipicamente se caracterizam pela grande agressividade, quimioresistência e indução de disseminação da doença.

Este trabalho descreve a sobre-expressão de *O*-glicanos sialilados de cadeia curta (sialil-Tn e (di)sialil-T), em tumores avançados de bexiga, em comparação com os seus homólogos neutros (Tn e T). O antigénio sialil-Tn (STn) foi o mais relevante sob o ponto de vista da sua associação com mau prognóstico em tumores de bexiga musculo-invasivos e não musculo-invasivos. Além disso, a sua ausência ou expressão marginal no uretélio saudável confirma a sua natureza cancro-específica. Cumulativamente, o antigénio STn foi encontrado em tumores quimioresistentes e caracterizados pela expressão de marcadores estaminais. A possível associação entre a expressão de STn, fenótipos estaminais e quimioresistência foi ainda reforçada através de estudos *in vitro* utilizando modelos celulares representativos das diferentes vias de carcinogénese da bexiga (linha celular de cancro de bexiga T24, FGFR3/CCND1; linha celular de cancro de bexiga HT1376, E2F3/RB1). De modo concordante, o antigénio STn foi encontrado em lesões basais, fenotipicamente caracterizadas pela sobre-expressão de *KRT*14 e/ou *KRT*5 em comparação com a *KRT*20, descritas como sendo maioritariamente compostas por células estaminais quimioresistentes. A presença de células STn-positivas em lesões luminais (*KRT*14^- e/ou *KRT*5^- e *KRT*20^+) menos agressivas também foi associada com pior sobrevivência dos doentes. Estes dados foram posteriormente utilizados para melhorar a capacidade preditiva de um modelo de estratificação baseado na expressão de citoqueratinas em cancro de bexiga. Verificou-se ainda que o antigénio STn estava presente nas metástases de tumores de bexiga, sugerindo um papel na dessiminação da doença. Assim, explorou-se a expressão de STn em células tumorais circulantes (CTC) através de uma plataforma de microfluídica, tendo-se demonstrado uma elevada frequência de CTCs de bexiga a expressar este antigénio com características basais, à semelhança dos tumores primários e metástases. Estes dados suportam que a expressão de STn nas células tumorais de bexiga poderá possuir um papel na disseminação da doença e no estabelecimento

de metástases. Com base nestas observações, desenvolveu-se um dispositivo de microfluídica com afinidade para glicanos para capturar seletivamente STn^{+}CTC, tendo em vista a sua aplicação num contexto de medicina de precisão.

Em conclusão, este trabalho demonstra uma relação entre a expressão de STn, mau prognóstico e fenótipos estaminais, caracterizados pela resistência à quimioterapia e, potencialmente, pela capacidade de regenerar a heterogeneidade tumoral. Além disso, a natureza disseminada das STn^{+}CTC e a sua prevalência nas metástases suporta o seu papel na propagação da doença, o que exige confirmação futura. O racional clínico está agora estabelecido para explorar o glicoproteoma associado ao STn, tendo em vista desenvolver ligandos novos e mais específicos para cancro de bexiga, com intuito terapêutico. O STn é apresentado como um biomarcador de CTC que deve suscitar interesse da comunidade médica e científica com o propósito de melhorar a abordagem dos indivíduos com doença avançada.

Abstract

Advanced bladder cancer management remains critical, due to its high molecular heterogeneity. Therefore, it is urgent to identify novel biomarkers and molecular tools to stratify patients facing worst prognosis and at higher risk of rapid disease progression. Moreover, it is important to generate the molecular rationale for targeting bladder cancer stem-cells, phenotypically characterized by aggressiveness, chemoresistance and involvement in dissemination of disease.

Here, we observed that advanced bladder tumours overexpress sialylated short-chain *O*-glycans (sialyl-Tn and (di)sialyl-T) in comparison to their neutral counterparts (Tn and T antigens). The sialyl-Tn (STn) antigen was the most relevant in terms of association with poor prognosis in both non-muscle and muscle invasive bladder tumours. Noteworthy, STn was not detected in healthy urothelium, demonstrating its cancer-specific nature. In addition, this antigen was found in chemoresistant tumours enriched for a panel of epithelial-to-mesenchymal transistion and stem-cell markers. A possible association between STn, stem-cell molecular phenotypes and chemoresistance was further reinforced using *in vitro* studies in cell models representative of different invasion pathways (T24 bladder cancer cell line, FGFR3/CCND1 pathway; HT1376 bladder cancer cell line, E2F3/RB1 pathway). Likewise, the STn antigen was found in basal-like lesions, phenotypically characterized by the overexpression of *KRT*14 and/or *KRT*5 in relation to *KRT*20, which have been described as enriched for chemoresistant cancer stem cells. Nevertheless, the presence of STn-positive cancer cells subpopulations has also been associated with worst survival of patients exhibiting less aggressive luminal lesions (*KRT*14$^-$ and/or *KRT*5$^-$ and *KRT*20$^+$ phenotype). Such findings were subsequently used to improve on the prognostication capacity of this *KRT*-based stratification model for bladder cancer. Finally, we have explored STn expression using microfluidic-isolated circulating tumour cells (CTC). Accordingly, it was demonstrated that the majority of bladder cancer CTC express the STn antigen and possess a basal-like character, mimicrying the primary tumour and metastasis. These findings support STn-expressing bladder cancer cells may have a key role in disease dissemination and metastases development. Based on these observations, we have specifically developed a glycan-affinity microfluidics device to target STn$^+$CTC, envisaging precision medicine applications.

In conclusion, this work presents a more definitive clinical link between STn expression, poor prognosis and stem-cell phenotypes, characterized by resistance to chemotherapy and capability of regenerating tumour heterogeneity. Moreover, the widespread nature of STn$^+$CTC and its prevalence in the corresponding metastasis supports a

role in disease dissemination, warranting future confirmation. The clinical rationale is now set to comprehensively address the STn-glycoproteome envisaging novel and highly specific bladder cancer ligands for novel therapeutics. STn is presented as a relevant CTC biomarker that should be carefully exploited envisaging to improve the management of advanced stage patients.

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Abbreviations

AKT Protein Kinase B

ALDH1A1 Aldehyde dehydrogenase 1 family, member A1

Asn Asparagine

B2M Beta-2-Microglobulin

BC Bladder cancer

BCG Bacillus Calmette–Guérin

BCSC Bladder cancer stem cell

BMDC Bone marrow-derived cell

C1Gal-T1 *N*-Acetylgalactosamine 3-Beta-Galactosyltransferase 1 or T synthase

C2GnT1 Beta-1,6-*N*-acetylglucosaminyltransferase

CCSC Circulating cancer stem-cell

CD133 Cluster of differentiation 133

CD147 Cluster of differentiation 147

CD15 Cluster of differentiation 15

CD44 Cluster of differentiation 44

CD47 Cluster of differentiation 47

CD49 Cluster of differentiation 49

CD60a Cluster of differentiation 60a

CD77 Cluster of differentiation 77

Cis carcinoma *in situ*

CO2 Carbon dioxide

COX-2 Cyclooxygenase-2

CSC Cancer stem cell

DNA Deoxyribonucleic acid

dST Disialyl-T antigens

EGFR Epidermal growth factor receptor

EGFR Epidermal growth factor receptors

EMT Epithelial-mesenchymal transition

ER Endoplasmic reticulum

FACS Fluorescence-activated cell sorting

FFPE Formalin-fixed paraffin-embedded

FGFR Fibroblast growth factor receptor

FGFR3 Fibroblast growth factor receptor 3

FUT Fucosyltransferase

GA Golgi apparatus

Gb4 Globoside 4

GD2 disialoganglioside 2

GDP Guanosine diphosphate

GlcNAcT-III Beta-1,4-*N*-acetylglucosaminyltransferase III

GlcNAcT-V *N*-acetylglucosaminyltransferase V

GSTM1 Glutathione S-transferase mu 1

HERV Human endogenous retroviruses

HRAS Harvey rat sarcoma vírus oncogene or transforming protein p21

KRT keratin

Le lewis blood group related antigen

LR Laminin receptor

MAPK Mitogen-activated protein kinase

MIBC muscle-invasive bladder cancer

mTOR Mechanistic target of rapamycin

NAT2 *N*-acetyltransferase 2

NEU Neuraminidase

NMIBC Non-muscle-invasive bladder cancer

PCC Premature chromosome condensation

PD1 Programmed cell death protein 1

PG Prostaglandin

PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase

ppGalNAc-T *N*-acetylgalactosamine transferases

PTEN Phosphatase and tensin homolog

PUNLMP Papillary urothelial neoplasm of low malignant potential

qPCR Quantitative polymerase chain reaction

Rb1 Retinoblastoma 1

S6T Sialyl-6-T

Ser Serine

SLe^{a} Sialyl lewis a

SLe^{x} Sialyl lewis x

ST Sialyl-T

ST3Gal.I Beta-Galactoside Alpha-2,3-Sialyltransferase 1

ST6GalNAcI *N*-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 1

STn Sialyl-Tn

TBS Tris-buffered saline

TGF-β Transforming growth factor β

Thr Threonine

Tis Tumour *in situ*

TMB Tetramethylbenzidine

TP53 Tumour protein 53

TP63 Tumour protein 63

TSC1 tuberous sclerosis complex 1

TUR Transurethral resection

VEGFR Vascular endothelial growth factor receptor

Introduction

Introduction

1. Bladder cancer epidemiology

Bladder cancer (BC) is the ninth most common cancer worldwide, being in the top fifteen causes of death(1). Bladder cancer incidence increases with age, reaching a peak between 60 and 70 years, and is three-to-four times more common in men than in woman(2). Factors involved in urothelial carcinogenesis may contribute to explain these demographic trends, including disparate activity of sex steroid hormone pathways, different metabolism of carcinogens by hepatic enzymes, resulting in differential exposure of the urothelium to carcinogens, gender- associated differences in exposures to risk factors, and delay of diagnosis in woman compared to men(2).

Environmental risk factors play a key role in BC initiation. In western world, cigarette smoking is the most relevant hazard factor, accounting for approximately 50% of BC cases and tripling BC risk compared to non-smokers(3). Occupational exposure to chemical compounds, e.g. aromatic amines, aniline dyes, nitrites, nitrates, acrolein, coal and arsenic, as well as chronic irritation, indwelling catheters, *Schistosoma haematobium* infection, and pelvic irradiation, have all been established as causal factors for BC(4-6). The inherited genetic background is also believed to play a role in BC carcinogenesis, particularly modulating the aptho physiological burden resulting from gene-environmenta interactions, such as the slow acetylator N-acetyltransferase 2(*NAT2*) genetic variant and glutathione S-transferase mu 1(*GSTM1*)–null genotypes(7). Somatic genetic alterations that associate with BC include mutations in oncogenes, such as *TP63*, the epidermal growth factor receptor (*EGFR*), and Ras p21 proteins, as well as in tumour suppressor genes *TP53* and *RB1*(8).

2. Bladder cancer pathophysiology, progression and management

Bladder cancer has a complex and multifactorial etiopathogeny, where multiple steps are involved in the development of urothelial cell carcinomas. Particularly, BC cells and their surrounding partners (tumour microenvironment) undergo several genetic and molecular alterations in order to achieve a characteristic phenotype that resembles the “hallmarks of cancer”, which include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, reprogramming energy metabolism, evading immune destruction and activating invasion and metastasis(9). These alterations further affect downstream pathways through loss of cell cycle control, telomere dysfunction, genomic instability, and growth advantages(10). Low-grade non-muscle-invasive bladder cancer (NMIBC) and high-grade muscle-

invasive bladder cancer (MIBC) are two types of bladder cancers that are divided into different oncogenic pathways **(Figure 1)**. Well-differentiated, non-invasive papillary urothelial cancers are derived from benign lesions through a process called urothelial hyperplasia, whereas poorly differentiated muscle-invasive cancers are derived from carcinoma *in situ* (Cis or Tis), urothelial dysplasia, or high-grade papillary bladder lesions. Although just a small fraction of superficial lesions progress to muscle invasive tumors, this process is highly dependent on genetic instability and accumulation of genetic alterations(11, 12) that correlate well to the clinicopathological phenotype(13, 14). Moreover, the occurrence of NMIBC and MIBC may depend on their originating site and aberrations in specific signalling pathways. As such, NMIBC exhibits frequent mutations of *FGFR3* (75%)(15-17), and *HRAS* (30-40%)(18), affecting MAPK and PI3K pathways (PI3K3CA 25-48% (19-21); PTEN 50% (22, 23); TSC1 50% (24)) in differentiated (uroplakin+ and KRT-20+) or intermediate (KRT-18+, p63+/−, cytokeratin-5+/−, and CD44+/−) urothelial cells, whereas MIBC exhibits mutations of the tumor suppressor genes *p53* (>50%) (25, 26), *Rb* (25-30%)(27), and *PTEN* in basal cells (KRT-14+, KRT-5+/−, KRT-17+, CD44+/−, and p63+)(28-30).

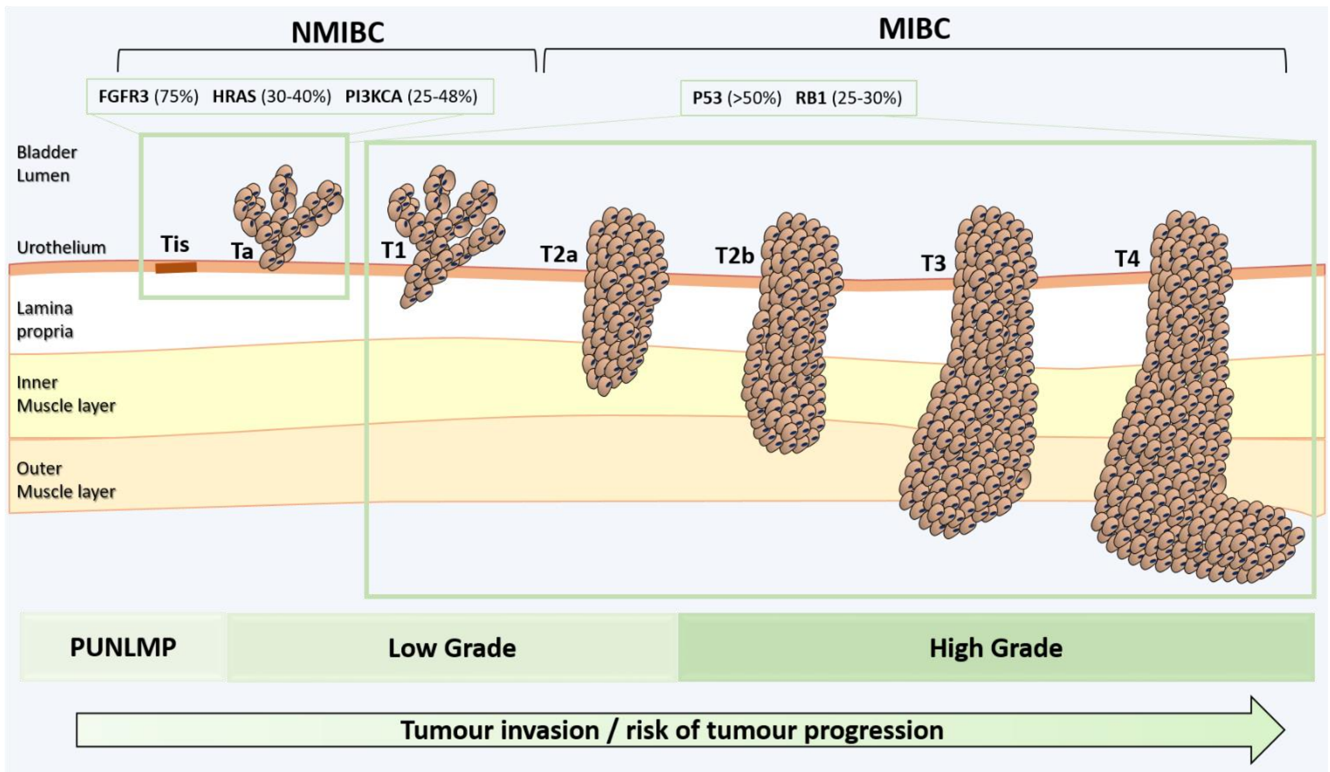

Figure 1. Schematic representation of bladder cancer stage and grade. The stage of the primary tumour (T) is based on the extent of invasion into the bladder wall. Regarding tumour grading, bladder lesions can be classified as urothelial papilloma (a benign lesion), papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade papillary urothelial carcinoma and high-grade papillary urothelial

carcinoma. Noteworthy, PUNLMP lesions do not have cytological features of malignancy and have a very low risk of progression, despite having a high tendency to recur. Tis, Tumour *in situ*: ‘‘flat tumour’’; Ta, Non-invasive papillary carcinoma; T1, Tumour invades sub-epithelial connective tissue; T2, Tumour invades muscle; T2a, Tumour invades superficial muscle (inner half); T2b, Tumour invades deep muscle (outer half); T3, Tumour invades perivesical tissue; T4, Tumour invades any of the following: prostate, uterus, vagina, pelvic or abdominal wall. The genes associated with urothelial dysplasia and CIS progression, as well as associated with invasive bladder tumors and metastasis are shown in the figure.

Histologically, more than 90% of diagnosed bladder tumours are urothelial cell carcinomas, with the remaining 5-10% comprising mesenchymal neoplasms and epithelial tumors of other types(31). At the time of diagnosis, 70-80% are NMIBC, of which 50-70% will recur and 10-20% will progress to MIBC(32). Non-muscle invasive bladder tumour patients are endoscopically treated using complete transurethral resection (TUR) and adjuvant intravesical therapies, such as Mitomicin C (MMC) or Bacillus Calmette–Guérin (BCG) depending on disease severity (33). However, only two thirds of patients respond to BCG, with one third of the responders facing recurrent disease with poor prognosis(34). Particularly, high-grade NMIBC presents strikingly high recurrence and progression to muscle invasive disease(33); as such additional therapies are needed for BCG non-responders. Conversely, 25% of newly diagnosed patients have MIBC or metastatic disease are currently treated with radical cystectomy and cisplatin-based chemotherapeutic regimens(35), which still have a high rate of tumour relapse and disease progression. In patients with disease progression following chemotherapy, some promising second line options are now available such as systemic immunotherapy with checkpoint inhibitors like atezolizumab (a programmed cell death protein 1 (PD1) inhibitor)(36, 37). Therefore, bladder cancer patients represent a significant management hurdle due to high recurrence rates, rapid progression, dissemination and poor response to chemotherapy, associated with a larger lag in the time-to-availability of novel targeted therapeutics in comparison with other solid tumours(38, 39). In addition, the high molecular heterogeneity of bladder tumours of apparently similar histology seems to be responsible for significant variations in disease course, inconsistency in therapeutic responses and prognostic uncertainty(40), thus urging for individualized disease management. Prognostication difficulties are often aggravated by the lack of efficient follow-up strategies, especially of non-invasive nature, for early detection of imagiological occult micrometastasis, real-time monitoring of therapy response and metastatic risk assessment, which would enable timely and eventually life-saving interventions. Taken together, these insights ultimately suggest that improving bladder cancer survival will require earlier detection, more effective local control and new therapies for metastatic disease.

3. Tumor Heterogeneity and Cancer Stem Cells (CSCs)

Advanced stage BC is a highly heterogeneous, recurrent metastatic disease, which results in poor prognosis(41). Despite the latest advances in diagnosis and therapeutics, both chemo and surgical, the survival rate of MIBC patients remains low at 5-year, current strategies often fail to prevent tumour relapse and/or progression. According to the CSC theory, the majority of bladder non-CSCs are eliminated in response to systemic chemotherapy, even though a small population of bladder CSCs (BCSCs) resists, ultimately resulting in tumor recapitulation and disease recurrence from BCSC clones.

The recent establishement of novel molecular targets for BC (e.g. EGFR, FGFR, VEGFR, PI3K-Akt-mTOR, PD-1, COX-2, Aurora kinase A, and several miRNAs) with corresponding investigation of targeted therapies in clinical trials(42) has yielded only modest improvement in survival, thereby supporting a role for BCSCs, contributing to explain clinical observations such as tumor heterogeneity, chemoresistance, recurrence, and metastasis(43, 44). Thus, understanding CSCs' role in BC will add significantly to our comprehension of recurrence and metastasis, and facilitate the development of novel therapeutic strategies.

The origin of cancer stem cells remains controversial(45, 46); still, the bladder possesses many types of stem cells, such as urothelial stem cells, adipose-derived stem cells, bone marrow-derived mesenchymal stromal cells, mesenchymal stem cells, and urine-derived stem cells(47, 48). Bladder tumours may develop from some of these populations or from more differentiated progenitor cells as a result of random mutations, chromatin modifications, epigenetic reprogramming, and microenvironmental cues(49). Nevertheless, generation of BCSCs may also be a late event in tumorigenesis, after transformation of early BC cells into BCSCs through epithelial-to-mesenchymal transition (EMT), de-differentiation, tumour-associated cytokines, and cancer cell fusion (45, 50). In EMT, while tumour progresses cancer cell lose its epithelial features and acquire a mesenchymal phenotype (51). Moreover, during EMT cells gain the expression of stemness markers, and the ability to form spheres(52, 53). Further data concurring to BCSCs' role, demonstrate that activation of hypoxia inducible factor (HIF) and transforming growth factor β (TGF-β) pathways, which contribute to EMT and self-renewal, are also activated in BC (54-57). As found in other tumours, the stemness of BCSCs might be regulated by cytokine networks(58). Noteworthy, circulating interleukin-6 (IL-6) levels are elevated in BC patients compared to healthy controls, and its downstream signalling pathway Jak2-Stat3 is upregulated in tumours, resulting in malignancy growth and aggressiveness(59,

60). Importantly, MIBC driven through Stat3 signaling was predominantly composed of stem cells, which were characterized by KRT14 positive staining and enhanced tumor sphere-forming ability; thereby confirming the Stat3-induced stem cell expansion model(61). Cyclooxygenase-2 (COX-2) is another protein overexpressed in BC compared to normal urothelium(62), and its major bioproduct, prostaglandins (PGs), has been reported to be involved in BC biology, including its role in inflammation and carcinogenesis(63). Nuclear COX-2 is significantly associated with the upregulation of the stemness markers Oct3/4 and CD44v6, suggesting that COX-2 activation might be involved in inflammation-mediated bladder CSC proliferation(64). Notably, a wide variety of stem cell markers is up-regulated in CSCs obtained from MIBC cell lines(65). Moreover, most BCSCs were identified in metastatic MIBC but not in NMIBC(66-71).

Pathological dedifferentiation is observed in BC, especially in MIBC. Approximately 10% of BC contains glandular differentiation, and up to 60% contains squamous differentiation(72, 73). Moreover, rarely, BC cells can dedifferentiate into other aggressive and prognostically unfavourable variants, including sarcomatoid, lymphoepithelioma-like, and small cell carcinoma(74-77). Remarkably, the gene expression profile of MIBC reveals that squamous or sarcomatoid differentiation is enriched in the basal-type of MIBC, which displays a CSC phenotype(30, 75).

Cancer cell fusion also promotes BC aggressiveness and stemness(78, 79), resulting in premature chromosome condensation (PCC)(80, 81), nuclear reprogramming(82) and CSC generation(83). This process requires fusogens such as the BCSC marker CD44(45, 84) to allow the fusion of tumour cells with stem cells, macrophages(85), or bone marrow-derived cells (BMDCs)(80, 86). Several mechanisms of cancer cell fusion have been proposed, but oncogenic viruses are the most likely candidate(87). Human endogenous retroviruses (HERVs) may have oncogenic potential, and HERVW-1 is reportedly overexpressed in 75.6% of BC, whereas it is overexpressed in only 6.1% of matched non-cancer tissues(88). Interestingly, smoking increases HERV transcription in the urothelium(89). Apoptosis is another mechanism of cancer cell fusion, resulting in hybrids with an enhanced migratory capacity that may enable dissemination and metastasis (45).

As previously described, recurrent BC is usually chemoresistant, even if initially apparently chemotherapy-sensitive(90). Besides unfavourable drug pharmacokinetics and abnormal tumor vasculature that result in suboptimal delivery of cytotoxic agents to the tumor (44, 91), emerging data supports a direct effect of BCSCs in chemoresistance(90-92). Actually, chemotherapy combined with stem cell-targeted therapy would be the ideal strategy for advanced stage BC patients. Therefore, it is important to identify BCSCs chemoresistance mechanisms and uncover ways to circumvent it. Hitherto, the mechanisms behind BCSCs chemoresistance reside in favourable alterations in drug transport and metabolism, enhanced DNA repair mechanisms, alterations in cell cycle regulation,

inhibition of apoptosis and regulation of cytokines and other immune mediators that decrease immunosurveillance (91). Studies have brought glycosylation into the spotlight by demonstrating that selective inhibition of *N*-glycosylation pathways may constitute an important therapeutic strategy to overcome cancer cells' chemoresistance. Additional and novel findings now pinpoint that the influence of glycosylation might extend beyond these events, given its pivotal role in the activation of several oncogenic pathways that sustain chemoresistance (reviewed by JA Ferreira, Peixoto A, Neves M *et al*) **(Annex A)**(91).

Increasing evidence suggests that a presumably small subset of cancer stem cells has the functional plasticity for transitioning between mesenchymal-like and epithelial-like states through EMT and cellular dedifferentiation; these cells are indeed a relevant source for metastasis at distant sites and are commonly termed circulating cancer stem-cells (CCSCs)(93, 94). As compared to non-tumorigenic/metastatic bulk circulating tumour cells (CTCs), CCSCs may not only be capable of evading from primary tumor, but also escape from immune surveillance, survive in circulation and subsequently metastasize to distant organs(95). Thus, CCSCs seem to represent a subset of exclusively tumorigenic cancer stem cells characterized by their invasive characteristics.

3.1. Bladder cancer stem cell (BCSC) markers

Bladder cancer stem cells were identified in 2009 using normal stem cells surface markers (71). Since then, additional cell surface markers, such as CD44(71), CD44v6(64, 70), laminin receptor (67LR)(66), Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1)(69), CD133(96, 97), CD47(71), CD49(98), and keratin 14 (KRT14)(99) have been used to isolate BCSCs. Cancer stem cells can also be sorted by Fluorescence Assisted Cell Sorting (FACS) based on their ability to efficiently efflux the DNA fluorescent dye Hoechst 33342 through ATP-binding cassette transporters, demonstrating enhanced colony formation, self-renewal and multi-directional differentiation, which are features typical of CSCs(67). The sequential transplantation of xenograft tumors is increasingly required for the confirmation of CSCs isolation(100-102). To rise isolation specificity, most of these markers are used with mutually exclusive indicators, such as cytokeratins (CD44+ KRT5+ KRT20−)(71). Importantly, a molecular model has been recently proposed to improve patient stratification and prognosis based on the sub-classification of bladder tumours as luminal or basal-like(75, 99, 103). Accordingly, well differentiated and prognostically favourable luminal tumours (KRT14- , KRT5- and KRT20+) were distinct from basal-like lesions (KRT14+ or KRT5+, or both and KRT20-), characterized by reduced survival and higher chemoresistance(75, 99, 103). Albeit only 50% of MIBC could be subtyped through this approach, it may provide the premise for molecular-

based clinical decision. The introduction of novel biomarkers capable of further refining patient stratification seems now warranted. Exploring cancer-associated glycans, which are often of oncofetal nature, presents a unique and highly specific opportunity to target CSCs. In fact, several studies reported alterations in the glycopatterns of CSC of tissue from different origins compared to other cancer cells. Many of these findings are related with the overexpression of glycans commonly found in glycolipids (CD60a, CD77, GD2, Gb4) and proteins (CD147 or Le^y, CD15 or Le^x)(104). More recently, data confirmed that CSC resistant to gemcitabine(105) and doxorubicin(106) present distinct glycosylation patterns when compared to drug sensitive parental cell lines. Terao *et al*. described that CSC derived from the gemcitabine chemoresistant pancreatic cancer cell line Panc1 display significant overfucosylation and upregulation of fucosyltransferases, GDP-fucose synthetic enzymes, and GDP-fucose transporters(105). However, knockdown of GDP-fucose transporters did not improve gemcitabine response, suggesting that overfucosylation is a result of CSC transformation with little influence on chemoresistance. Moreover, increased fucosylation has been associated with invasive and metastatic properties of cancer cells(107). A recent study by Desiderio *et al*. investigated the role of fucosylation in cancer stem cells and found that inhibition of fucosylation affected sphere formation and invasion ability of cancer stem cells (108), whereas in another report, inhibition of fucosylation was found to affect E-selectin binding and cell extravasation(109), all crucial features for the metastatic process(110). Thus, fucosylation may be a novel mechanism exploited by cancer stem cells to acquire invasive and metastatic features in order to generate metastatic cancer stem cells, although simultaneously amendable for therapeutic intervention. Conversely, Azuma *et al*. explored the oversialylation of doxorubicin-resistant hepatocellular CSC and identified highly specific glycoprotein species(106). Taken together, these findings contributed to underline the need for an in-depth glycome and glycoproteome assessment of CSC populations towards the development of more specific biomarkers.

4. Protein glycosylation in bladder cancer

It has long been known that advanced stage bladder tumours present severe dysregulations in glycosylation pathways, translated by the loss of terminal ABO (H) blood group determinants at the cell-surface of ABH secretor individuals(111), over- and/or *de novo* expression of short-chain *O*-GalNAc glycans(112), Lewis blood group related antigens, and their sialylated counterparts(113, 114), as well as oversialylation and fucosylation of glycan chains(115) **(Figure 2)**. Gycosylation patterns dynamically change with the course of disease (a nossa revisão do oncotarget)as a result of: i) altered glycogenes expression(116, 117); ii) impaired glycosyltransferases' chaperone function(118); iii) altered glycosidase/glycosyltransferase activity(117); iv) reorganization of glycosyltransferases

topology(119, 120); v) bioavailability of sugar nucleotide donors and cofactors(121); and vi) alterations on the conformation of peptide backbone or on the nascent glycan chain structure(121). The resultant aberrant and cancer-associated glycans seem to be implicated in the activation of oncogenic pathways(121), establishment of tumour-tolerogenic immune responses(122), and in epithelial-to-mesenchymal transition (EMT), towards invasion and metastasis(123, 124). Thus, many glycoepitopes, and their related glycosidases/glycosyltransferases, can be considered relevant tumour-associated antigens(125, 126), with possible clinical significance in bladder cancer. Those found at the cell surface are easily accessible to antibodies and lectins to selectively target specific tumour cells, while those secreted or shed into bodily fluids can be explored for non-invasive cancer detection through serological assays. As such, cancer-specific alterations in protein glycosylation might provide a unique opportunity for clinical intervention. Accordingly, the majority of glycobiology studies in bladder cancer describe alterations in terminal stuctures shared by both *N*-glycans (in Asn residues of a glycoprotein chain) and *O*-glycans (in Ser and/Thr residues) of membrane and secreted glycoproteins as well as glycolipids, mostly based in immunohistochemistry results(127). Other commonly reported alterations are related with the overexpression of short-chain *O*-glycan chains(127). Noteworthy, very few studies have undergone a comprehensive mass spectrometry-based glycomic characterization of bladder tumours(127).

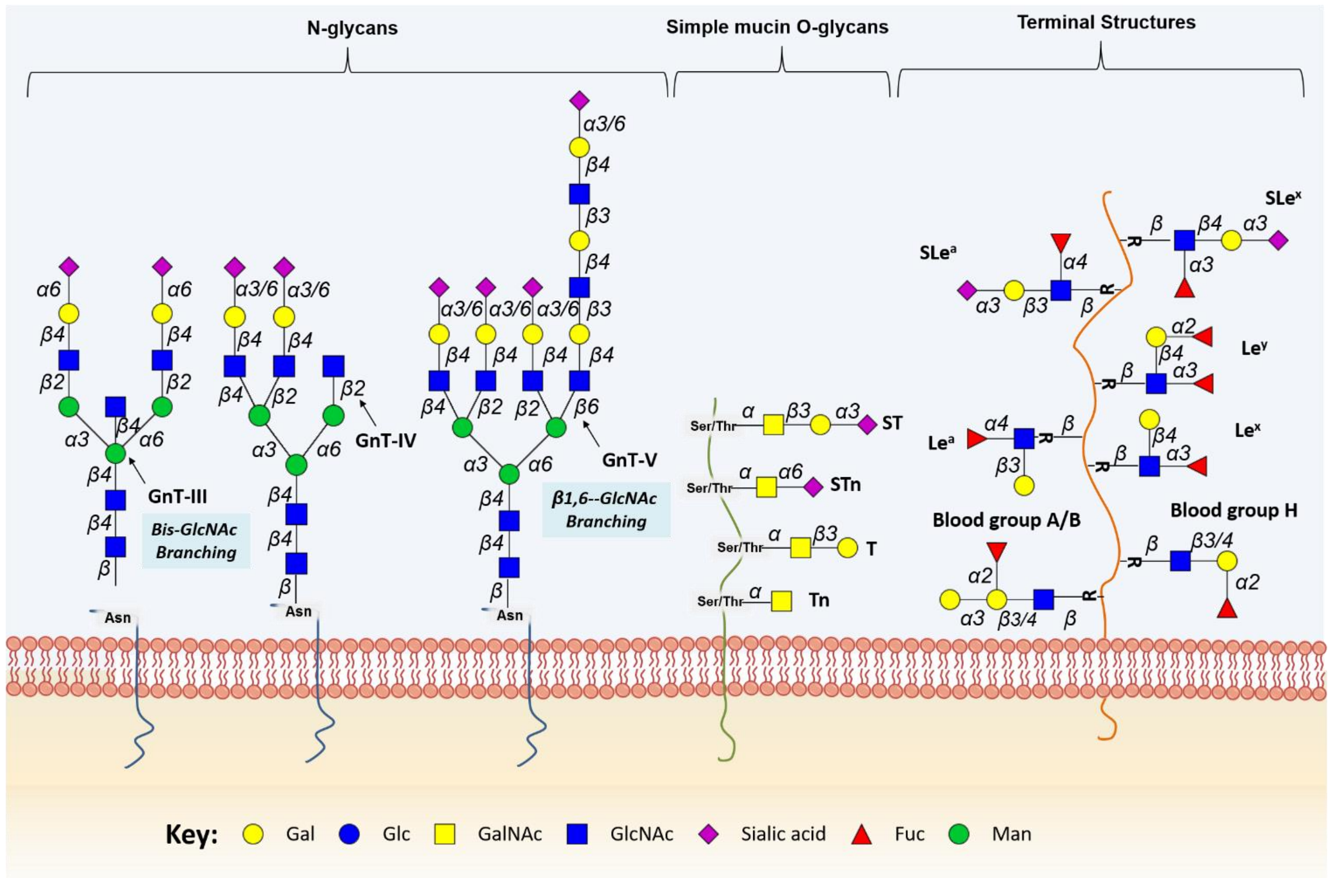

Figure 2. The figure represents specific *N*-linked and *O*-linked glycan structures, as well as terminal Lewis and sialylated Lewis structures that have biological significance in bladder cancer. Key enzymes mediating the addition of specific sugars are also shown. Protein *N*-glycan alterations include the β1-6 branching of N-glycans in result of GlcNAcT-V (GnT-V) overexpression, and the addition of bisecting GlcNAc branches by GlcNAcT-III (GnT-III) glycosyltransferases. Alterations in *O*-glycosylation pathways are also a common hallmark of malignant transformations of the bladder. Herein, we represent the overexpression of simple mucin-type *O*-glycans and their sialylated counterparts, T, sialyl T (ST), Tn and sialyl Tn (STn) antigens. Altered expression of terminal structures is also a common feature of bladder tumours. Namely, the abnormally low or absent expression of ABO(H) blood group determinants are frequently found in high grade and invasive disease. Carbohydrate terminal Lewis antigens are significantly under-expressed in healthy urothelium when compared to bladder tumours and are also highlighted here. Lewis type 1 antigens includes Lewis a (Le^{a}), and sialyl Lewis a (SLe^{a}), while the type 2 group includes Lewis x (Le^{x}) and sialyl Lewis x (Sle^{x}).

Alterations in N- and O-glycans terminal structures

The loss of ABO(H) blood group determinants in secretor individuals and the overexpression or expression *de-novo* of Lewis (Le) blood group related antigens are amongst the most common alterations in terminal epitopes of *N*- and *O*-glycans in glycoproteins and glycolipids in bladder cancer. Particularly, ABO(H) blood group antigens are present on normal bladder urothelium but not on some

low grade and early stage urothelial carcinomas(128), with its loss being associated with higher grade and recurrent bladder cancer(129-131). Moreover, the absence of ABO(H) antigens in the initial biopsy of bladder carcinomas is predictive of increasing chance of subsequent invasion compared to tumours in which these antigens are detectable(132). However, a significant number of patients whose initial tumours were reported as blood group antigen negative failed to develop an invasive tumour(132). These conflicting results may, at least in part, be explained by differences in methodology, interpretation, or both. Furthermore, the loss of activity of the A and B gene-encoded transferases(128), ABO(H) gene, and/or its promoter hypermethylation(133) have been proposed to drive the deletion of these antigens in bladder tumours.

The A, B, H antigens have biosynthetic and structural similarities with the Lewis antigens, including the type 1 lewis a (Le^a) and type 2 $Lewis^x$ (Le^x) antigens and their sialylated counterparts sialyl-lewis a (SLe^a) and sialyl-lewis x (SLe^x). Several authors associated patterns of Le^a expression with malignant transformations, reporting significantly lower expression of this antigen in healthy urothelium and non-invasive tumours compared to invasive tumours(134). As such, the expression of Le^a may be associated with worse bladder cancer phenotypes. Moreover, Le^a antigen expression patterns change at an early neoplastic stage, suggesting that Le^a determination might be useful in the diagnosis of very early premalignant changes in the urothelium(134). In addition, the employment of Le^a staining scores allows the sub-classification of histologically identical tumours into prognostically different groups, pointing to a relationship between the pathological grade and stage of the evaluated tumours and a morphological and functional dedifferentiation(134). Given this, Le^a antigen is a valuable functional marker of the malignant potential in superficial bladder cancer. In turn, the Le^x antigen is not expressed in normal urothelium, except for occasional umbrella cells, but it is demonstrated in most invasive tumours, regardless of blood type and secretor status of the individuals studied(135). Overall, despite the few reports on Le antigens expression in BC, most studies point to an association with malignant potential of cancer cells towards more invasive phenotypes(135, 136). Oversialylation of Le antigens originates selectin ligands SLe^a and SLe^x Both terminal epitopes present high affinity for E- and P-selectin, mediating cancer cells adhesion to endothelium and subsequent extravasation into the blood stream, thereby favouring the metastatic process in several malignancies(115, 137, 138), including bladder cancer(136). Besides metastasis, these glycans have been shown to mediate tumour growth, invasion and angiogenesis in BC(113, 139) and numerous other tumour types(140-142). Specifically, it has been demonstrated that loss/reduction of SLe^a expression is associated with higher atypia grade(136), while SLe^x has been closely linked to invasive and metastatic potential of primary bladder tumours(113). Nonetheless, another study demonstrated no associations between SLe^x, grade or stage in urothelial carcinoma of the renal pelvis, ureter, and

urinary bladder(136). Notwithstanding, the overall overexpression of sialoglycans ($SLe^{a/x}$) correlates with tumor aggressiveness, capacity to invade surrounding tissue and metastasis, and the EMT process is characterized by the overexpression of these sialoglycans due to ST3Gal-I, -III, and -IV expression (143). This indicates that the upregulation of sialyltransferases and subsequent expression of sialoglycans during EMT represents an important step underlying the migratory phenotype of metastasizing cancer cells. Moreover, downregulation of the lysosomal sialidases NEU1 and NEU4 favours tumor metastasis through enhanced signalling of the laminin receptor β4-integrin(144), and reduced hydrolysis of SLe^{x} antigens(145). Moreover, the overall increase in cell-surface sialic acid content was shown to reduce the attachment of metastatic tumour cells to the extracellular matrix, possibly protecting them from recognition by the alternative pathway of complement activation, favouring metastatic spread(146). Nevertheless, these processes need to be explored in the context of BC in order to understand the contribution of sialyltransferases and sialidases in cancer cell aggressiveness and ability to metastasize.

Sulfation has also been found as an important mediator of SLe epitopes functional and clinical role. Namely, sialyl-6-sulfo $Lewis^{x}$, is preferentially expressed in normal epithelial cells compared to cancer cells(147). Essentially all genes involved in the synthesis of SLe^{x} are predicted to be the same as those governing the synthesis of sialyl-6-sulfo $Lewis^{x}$, except for the genes engaged in its sulfation. This finding supports the hypothesis that reduced expression of sialyl-6-sulfo $Lewis^{x}$ induces SLe^{x} expression in cancers(148). Sialyl-6-sulfo $Lewis^{x}$ antigen is expressed in bladder urothelial carcinomas, promoting its progression through E-selectin-mediated tumour cell adhesion to vascular endothelial cells, which is potentially associated with metastasis, while aiding lymphocyte recruitment to enhance anti-tumour immune responses(149).

The *O*-6 sialylation of LeA originating disialyl-LeA also provides means to distinguish non-malignant cells from malignant urothelium, and is useful as a marker for tissue injuries occurring in benign diseases(150, 151). It has been suggested that the α-2-6 sialylation of the GlcNAc moiety is impaired in cancer cells compared to non-malignant. Moreover, this event has been associated with the epigenetic silencing of the *ST6GalNAcIV* gene, responsible for the α2-6 sialylation of the βGlcNAc moiety in cancer cells(150).

Alterations in O-GalNAc glycans

Significant amount of evidences support that bladder cancer progression and dissemination is also accompanied by profound remodelling of *O*-GalNAc glycans(127). This type of glycosylation inititates with the transfer of a GalNAc residue from UDP-GalNAc to either serine or threonine in a polypeptide chain, originating the monosaccharide Tn antigen. This reaction is catalysed by several

UDP-GalNAc:polypeptide N-acetylgalactosaminyl transferases (ppGalNAc-Ts) in the ER, in a substrate dependent manner (152). Further extension originates the T antigen (Gal-β1-3GalNAc-α1-Ser/Thr), catalysed by C1Gal-T1 and its molecular chaperone Cosmc(153). Both Tn and T antigens can be sialylated, giving rise to sialyl-Tn (STn), sialyl-T (ST) and disialyl-T antigens (dST), which generally stops further *O*-glycosylation extension **(Figure 3)**. Alternatively, the Tn antigen can be the precursor of more extended glycans (cores 2-4), which may be further extended and present terminal structures similar to those found in *N*-glycans and glycolipids despicted in the previous section **(Figure 1, Chapter 1).**

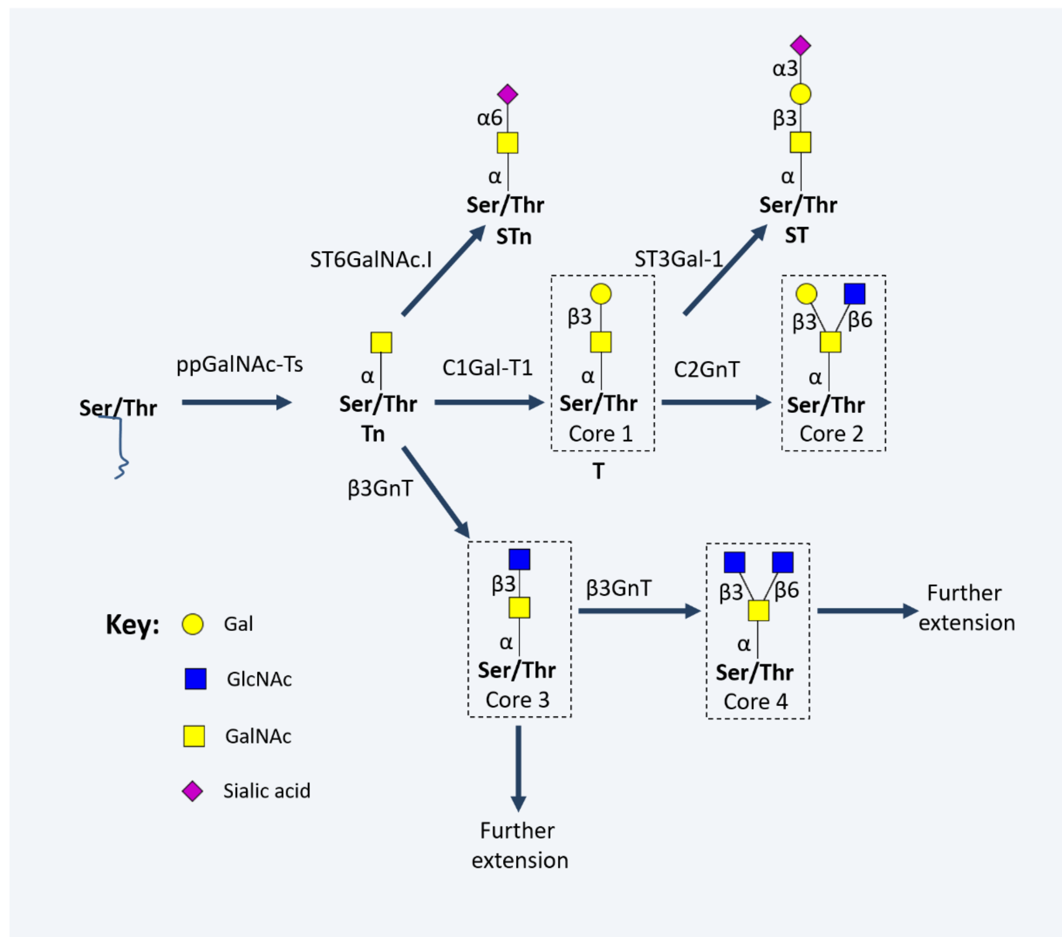

Figure 3: Schematic representation of short-chained *O*-linked glycan structures. The addition of specific sugar monomers to Ser/Thr residues of a protein backbone begins with the action of polypeptide N-acetylgalactosamine transferases (ppGalNAc-Ts; a family of 20 enzymes, including GalNAc-T1, GalNAc-T2, GalNAc-T3, GalNAc-T4, GalNAc-T5 and GalNAc-T6) given rise to the Tn antigen, which is generally extended with a Gal residue by C1Gal-T1, originating the Thomsen-Friedenreich or T antigen (core 1). Alternatively, Tn and T antigens can be sialylated by α2,3-sialyltransferases (ST3Gal-Ts) and α-GalNAc ST6Gal-I (ST6GalNAc-I), forming the sialyl-Tn (STn), and sialyl-T antigens. On the other hand, core 1 may be

extended originating cores 2-4 by the action of N-acetylglucosamine (GlcNAc) transferases (GnTs; such as GnT-III, GnT-V, core 2 GnTs (C2GnTs) and β3GnT).

Main structural alterations in *O*-GalNAc glycans include glycan chains shortening and oversialylation. The simplest *O*-GalNAc glycoforms, including the Tn and T antigens and its sialylated counterparts, are absent or marginally expressed in healthy urothelium, and their overexpression is strictly associated with higher bladder tumour stage and poor prognosis, making them ideal theragnostic targets in bladder cancer management (more extensive reading in Azevedo R and Peixoto A et al, 2017), annex B(127, 154). Several reports associated the presence of Tn and T antigens with recurrence and metastization in BC, suggesting that these antigens may be surrogate markers of profound cellular alterations(155-157). Furthermore, there are growing evidences linking the overexpression of the sialyl-T antigen and ST3Gal.I, the enzyme responsible by T antigen sialylation, with BC aggressiveness, recurrence, poor prognosis, and tumour grade(155, 158). Moreover, the expression of T antigen is significantly associated with higher risk for subsequent recurrences with deep muscle invasion and metastatic involvement of regional lymph nodes(155). In addition, there is a predominance of truncated sialoglycans over neutral glycoforms (Tn and T antigens) in advanced stage bladder tumors(159). In particular, the overexpression of ST6GalNAc-I has been found to promote the premature sialylation of the Tn antigen and consequent formation of the STn antigen in bladder cancer(160). Specifically, the STn antigen is absent in the healthy urothelium, while present in more than 70% of high-grade NMIBC and MIBC, denoting a cancer specific nature(160). This posttranslational modification of cell surface proteins is mostly expressed in non-proliferative tumour areas, known for their high resistance to cytostatic agents currently used to improve the overall survival of advanced stage BC patients(160). Recently, a novel STn-dependent mechanism for chemotherapeutic resistance of gastric cancer cells to cisplatin has been described, in which STn protects cancer cells against chemotherapeutic-induced cell death by decreasing the interaction of cell surface glycan receptors with galectin-3 and increasing its intracellular accumulation(161). The relationship between chemoresistance and STn overexpression remains to be fully explored in bladder cancer. Furthermore, STn expression is significantly higher in MIBC when compared to NMIBC, denoting its association with muscle invasion and poor prognosis(160). Furthermore, STn-expressing BC cells have shown the ability to induce a tolerogenic microenvironment by impairing dendritic cells maturation, allowing cancer cells to evade innate and adaptive immune system responses(162). Interestingly, the tolerogenic effect of short-chained O-glycans has also been correlated with bladder tumor metastasis through a mechanism in which MUC1 carrying core 2 O-glycans functions as a molecular shield against NK cells attack, thereby promoting metastization(163). In addition, STn

expression in BC tissues has been used in combination with other surrogate markers of tumour aggressiveness envisaging patient stratification regarding disease stage and therapeutic benefit. Specifically, STn and sialyl-6-T (s6T), an STn-related antigen, expression are independent predictive markers of BCG treatment response and were found useful in the identification of patients who could benefit more from this immunotherapy(164).

In agreement with their vital role in many physiologic processes, several reports imply that aberrant expression of sialic acids confers major advantages to tumor cells, such as inhibition of apoptosis(165) and anoikis(166), as well as resistance to chemotherapy(167, 168), including cisplatin-based schemes(161, 169). Overexpression and/or altered activity of sialyltransferases is a major mechanism underlying modifications in the sialome of cancer cells, partially due to the impact in proto-oncogenes overexpression (e.g. Ras and c-Myc) (143, 170), hypoxia(91, 171) and high hormone levels(172). Alternatively, increased substrate availability or overexpression of genes involved in sialic acid biosynthesis also contribute to hypersialylation in tumour cells, mostly in glycoproteins associated with extracellular matrix interactions and cell migration(173). Finally, downregulation of endogenous sialidases (NEU1, 2, and 4) can also lead to accumulation of sialoglycans in tumour cells(174). Although the molecular mechanisms responsible for the sialome modulation are starting to be unravelled, there is a lack of clarification of these processes in bladder cancer. Also, urges to elucidate if the hypersialylation of glycoproteins alters their intrinsic function, thereby contributing to tumorigenesis, or if overexpression of sialoglycans is a bystander consequence of malignant transformation. Moreover, the selective blocking of aberrant sialylation has antagonized tumor growth and metastasis formation by using sialyltransferase inhibitors(175, 176), advocating for the application of these inhibitors in preclinical models for cancer therapy development. Furthermore, these truncated structures are observed in heavily *O*-glycosylated proteins relevant in BC, such as CD44, β1-integrin, and mucins(91, 159, 177, 178). Importantly, and similarly to other malignancies, BC patients serum *O*-glycomics has uncovered that both ST and dST antigens are significantly increased in whole serum glycoproteins compared to controls, suggesting its probable non-invasive biomarker potential(179). Nevertheless, larger-scale glycomics studies are needed to confirm whether the detected glycoform changes are directly reflected from those in BC cells/tissue or are derived from other homeostatic alterations affecting glycoprotein metabolism during tumor proliferation. However, despite promising results, glycans failed implementation in clinical practice for BC non-invasive detection, mostly due to small patient cohorts, biased patient series, variations in detection and processing methods, lack of endpoint standardization and use of samples from different origins. As such, future studies should consider comprehensive functional glycomics approaches where glycans are incorporated in broader biomarker panels, envisaging highly sensitive detection methods. Moreover, current data will foster

glycobiomarkers validation in larger patient series, reinforcing their potential as novel therapeutic targets for BC therapy.

Aims and study outline

Aims and study outline

This work was grounded on previous knowledge about bladder cancer glycobiology and sought to expand on the potential of the cancer-associated *O*-GalNAc glycan sialyl-Tn (STn) as biomarker, in the context of advanced stage BC and response to chemotherapy. Additionally, it was designed to identify putative targets and scheming highly specific targeted therapeutics against bladder cancer stem cells.

This work comprehends the following specific aims:

i) Define the role of the STn antigen in the prognostication of muscle-invasive bladder cancer;

ii) Define the role of the STn antigen as a chemoresistant and stem-like cell biomarker;

iii) Include STn expression in a recently proposed molecular model for bladder cancer prognostication based on the degree of tumour differentiation, towards precision medicine settings;

iv) Explore STn expression in CTCs;

v) Development of a target-directed microfluidics device for the isolation STn^{+}CTC.

Preliminary observations from our group supported the hypothesis that STn expression may play a key role in disease outcome, thereby fostering current investigation. In addition, solid tumours often express the T antigen and its sialylated form ST, whose overexpression has been associated with poor prognosis. However, the ST antigen comprises a heterogeneous group of mono- (sialyl-6-T: S6T; sialyl-3-T: S3T) and disialylated glycoforms that remained to be individually evaluated in cancer.

Building on these insights, chapter 1 addresses the first specific aim of this work. Accordingly, bladder tumours were screened for the above-mentioned cancer associated glycans and special emphasis was given to STn, which was shown to be a key cancer-associated antigen highly linked with advanced disease and poor prognosis in bladder cancer.

Chapter 2 addresses the second specific aim of this project. Hence, two MIBC cell lines (T24 and HT1376) were exposed to an IC50 dosage of cisplatin and evaluated according to the expression of STn and CD44, a typical BCSC-associated glycoprotein usually related with drug resistant phenotypes and poor prognosis. Both, chemotherapy-challenged cell lines and muscle-invasive bladder tumours, were submitted to transcript quantification of a gene panel reflecting stem, mesenchymal, epithelial-to-mesenchymal (EMT), and epithelial markers, as well as cytokeratins (KRT) to assess cellular

differentiation states. Overall, data suggested that chemotherapy acts a selective pressure, creating tumour niches composed of undifferentiated stem-like cells characterized by the expression of CD44-STn^+ glycoforms. Moreover, results reinforce that STn is a biomarker of aggressiveness and resistance to chemotherapy in bladder tumours.

Chapter 3 addresses the third and fourth specific objectives. Therefore, a retrospective series of 80 muscle-invasive primary bladder tumors and associated metastasis were screened for *KRT14*, *5*, and *20* as well as STn by real-time polymerase chain reaction and immunohistochemistry. Concomitantly, peripheral blood was collected from a subset of patients and CTCs isolated through a size-based microfluidic chip and screened for KRTs and STn expression. Notably, basal-like lesions presented worse cancer-specific and disease-free survival compared to luminal tumor, and inclusion of STn discriminated patients with worst survival in each subgroup (P= 0.047 for luminal; P= 0.027 for basal-like tumors). In addition, STn expression in CTCs and distant metastasis was also demonstrated. Overall, this chapter reinforces the potential of the KRT-based model for bladder cancer management and the association of STn with aggressiveness. Likewise, it was described for the first time that CTCs and the metastasis present a basal phenotype and express the STn antigen.

Chapter 4 addresses the last specific aim of this work. As such, the overexpression of the cell-surface STn antigen in advanced bladder and colorectal tumours, but not in blood cells, was explored to propose a novel CTC isolation technology. Using a size-based microfluidic device, it was shown that the majority of CTC (>90%) isolated from the blood of metastasized bladder and colorectal cancers expressed the STn antigen, reinforcing its pan-carcinoma link with metastization. More importantly, STn^+CTC counts were significantly higher compared to EpCAM-based detection in colorectal cancer, providing a novel and more efficient cell-surface biomarker for CTC isolation. Exploring this concept, a glycan affinity-based microfluidic device was built for selective isolation of STn^+CTC and an enzymatic-based strategy for the recovery of viable cancer cells for downstream biomedical research was proposed. Here, clinically relevant cancer biomarkers (transcripts and mutations) in bladder and colorectal tumours were identified in microfluidic-isolated cells, confirming their malignant origin and highlighting the potential of this technology in the context of precision oncology.

Chapter 1 | Sialyl-Tn as a biomarker of bladder cancer aggressive phenotypes

Part of the data presented in this chapter has been published in Molecular Oncology (2017; Annex C) and Plos One (2015, annex D)

Sialyl-Tn as a biomarker of bladder cancer aggressiveness phenotypes

1. Abstract

Advanced stage bladder cancer (BC) is the second deadliest genitourinary cancer, being accompanied by profound deregulations on cell surface protein glycosylation. Envisaging the discovery of novel prognosis biomarkers and therapeutic targets, two series of bladder tumours with variable clinicopathological features was screened by immunohistochemistry to uncover cancer-associated short-chain *O*-glycans, which included the Tn and T antigens, as well as their sialylated counterparts sialyl-Tn (STn) and sialyl-T(ST). T antigen sialylation was also explored using enzymatic treatments. Overall, a predominance of sialoglycans over neutral glycoforms was observed, with the STn antigen being associated with high-grade disease and muscle invasion, in accordance with our previous observations. Moreover, both S3T and S6T antigens were detected for the first time in bladder tumours, but not in healthy urothelium, highlighting their cancer-specific nature. Using a dataset composed by 47 NMIBC and 49 MIBC patients, the impact of STn on cancer-specific survival (CSS) was assessed. Of note, STn was an independent prognostic marker for CSS in bladder cancer patients, irrespective of tumour stage. In summary, it was demonstrated that STn is a biomarker for aggressive BC disease, associated with poorer prognosis.

2. Introduction

Bladder cancer is the fifth most common cancer in Western society, and the second most deadly genitourinary tumour(1). Approximately 20–30% of the newly diagnosed cases are muscle invasive (MIBC; T2-T4 stage), while 50% are non-muscle invasive bladder tumours (NMIBC) with high potential to progress and invade. MIBC treatment includes cystectomy and (neo)adjuvant cisplatin-based chemotherapy regimens, which often fail to avoid tumour relapse and disease dissemination, urging the introduction of predictive biomarkers and novel therapeutics(2-4). However, significant variations in the natural disease course and treatment responses can be observed in apparently identical tumours, reflecting its high molecular heterogeneity (5, 6). Noteworthy, approximately 50% of cases develop distant metastatic disease within 5 years, urging the identification of biomarkers to assist prognostication and the development of more effective targeted therapeutics(7).

Malignant transformation is often accompanied by profound changes in glycosylation patterns, which play key roles in tumour progression and dissemination(8, 9). Moreover, alterations in glycosylation often produce protein glycoforms holding potential for targeted therapy(4, 10). In this

context, it has been long known that advanced stage bladder tumours present severe dysregulations in glycosylation pathways, translated by the loss of terminal ABO (H) blood group determinants at the cell-surface of ABH secretor individuals(11, 12), over- and/or de novo expression of short-chain *O*-GalNAc glycans(13), Lewis blood group related antigens, and their sialylated counterparts(14, 15), as well as oversialylation and fucosylation of glycan chains(16). Special emphasis has been given to the overexpression of short-chain O-GalNAc glycans, which alter cellular adhesive properties while promoting invasion, metastasis, angiogenesis, and immune scape, often through oncogenic pathways signalling activation(17-19). Moreover, it has been previously demonstrated that, unlike healthy urothelium and superficial tumours, 70% of advanced-stage bladder tumours express the cancer-associated carbohydrate antigen sialyl-Tn(19), whose expression favours cell invasion, motility, and immune tolerance(18-20). These observations support the hypothesis that STn expression may play a key role in disease outcome, therebywarranting further investigation. In agreement, also solid tumours often express the T antigen and its sialylated form ST, whose overexpression has been associated with poor prognosis(21, 22). However, the ST antigen comprises a heterogeneous group of mono- (sialyl-6-T: S6T; sialyl-3-T: S3T) and disialylated glycoforms that remain to be individually evaluated in cancer. Building on these insights, bladder tumours were screened for the above-mentioned cancer associated glycans, envisaging a molecular rationale for the development of novel non-invasive diagnostic tools and highly specific targeted therapeutics towards precision medicine. Special emphasis was given to STn which was shown to be a key cancer-associated antigen highly linked with advanced disease and poor prognosis in bladder cancer.

3. Methods

3.1. Patients and samples

For the screening of cancer-associated short-chain *O*-glycans (Tn, STn, T, S3T, S6T and dST), formalin-fixed paraffin-embedded tissue sections (FFPE) were prospectively obtained from 47 patients with urothelial bladder carcinomas who underwent bladder surgery in the Portuguese Institute of Oncology of Porto (IPO-Porto, Portugal). Tissues were collected from 37 male and 10 female patients (n=17 low-grade NMIBC, n=12 high-grade NMIBC, n=18 MIBC), mean age of 70 years (ranging 45–89 years old). Also, FFPE with healthy urothelium was obtained from six necropsies from male individuals. The assessment of the influence of the most promising glycans in the survival of BC patients was performed on FFPE from a larger retrospective cohort study composed of 96 patients (47 NMIBC and 49MIBC), 82 men and 14 women, ranging in age from 38 to 92 years (median of 69.5

years). Sixteen cases were low grade and 80 were high-grade tumours. Furthermore, carcinoma *in situ* (Cis) was concomitantly found in 20.8% of the patients. Cystectomy was performed in 64 patients (66.7%) while the other 32 (33.3%) were submitted to transurethral resection. Lymphadenectomy was performed in approximately 47% of the patients, of which 37% presented metastasis. Fifty-four cases (56.3%) were primary tumours and 42 (43.7%) were recurrent tumors. From the recurrent tumours, 38% had no prior treatment, 27% were treated with Mitomicin C, 11% were treated with Bacillus Calmette-Guérin,19% were submitted to both treatments, and 5% were treated with neoadjuvant chemotherapy prior to the cystectomy.

All tumour samples were colected between July 2011 and May 2012 and revised by a pathologist (TA) according to the 2004 World Health Organization criteria(23). All procedures were performed under the approval of the Ethics Committee of IPO-Porto after informed patient's consent. All clinicopathological information was obtained from patient´s clinical records.

3.2. Immunohistochemistry

The screening of cancer-associated short-chain *O*-glycans (Tn, STn, T, S6T, S3T and dST) was performed using the avidin/streptavidin-peroxidase method, as described by Ferreira et al.(19). The expression of the Tn, STn and T antigens was directly evaluated using in-house mouse monoclonal antibodies 1E3, TKH2 and 3C9, respectively. For more information about these antibodies consult the following database of anti-glycan reagents: https://ccr2.cancer.gov/resources/Cbl/Tools/Antibody/About.aspx. Because there are no commercially available antibodies for S3T, S6T and dST antigens, enzymatic assays were performed to indirectly analyze the expression of these epitopes in T antigen negative tumour samples. Specifically, the expression of dST antigen was determined by comparing histological sections probed for the T antigen before and after digestion with an α-neuraminidase from Clostridium perfringens (Sigma Aldrich, St. Louis, MO, USA). The S3T antigen expression was determined by comparing histological sections probed for the T antigen before and after digestion with an α-(2,3)-neuraminidase from *Streptococcus pneumonia* (Sigma-Aldrich, Missouri, USA). The S6T antigen expression was accessed by comparing histological sections probed for STn before and after digestion with a recombinant β-(1,3)-Galactosidase from *Xanthomonas campestris* (R&D systems, Minnesota, USA). The chromogen 3,3-diaminobenzidine tetrahydrochloride (ImmPACT DAB; Vector Laboratories, California, USA) was used to visualize antibody binding sites and sections were counterstained with Harris's hematoxylin. Negative controls were performed by replacing the primary antibody with 5% bovine serum albumin (BSA). Positive controls were known positive tissues for the antigens under study. The

immunoreactive tissue sections were assessed by light microscopy by two independent observers and validated by an experienced uropathologist. Although the interobserver agreement was high ($k = 0.961$, $P < 0.001$), discordant readings were re-analysed using a double-headed microscope (Olympus BX46; Olympus Corporation,Tokyo, Japan), and consensus attained. A semi-quantitative measurement was established to score immunohistochemical labelling based on the percentage of positively stained cells, and categorized as: negative (-),no staining was observed; positive (+), 1-19% of positive cells; positive (++), 20-49% of positive cells; positive (+++), 50-79% of positive cells; positive (++++), 80-100% of positive cells.

3.3. Statistical methods

Statistical analysis of data was performed with IBM Statistical Package for Social Sciences - SPSS for Windows (version 20.0; IBM, New York, USA). Chi-square analysis was used to compare categorical variables, while Student's t-test was used to compare normally distributed continuous variables. Kaplan-Meier survival curves were employed to evaluate correlations between STn expression and cancer-specific survival (CSS). Multivariate Cox regression analysis was performed to assess the individual effect of the evaluated markers on patient's survival while adjusting for covariables (variables that could affect cancer-specific survival of NMIBC and MIBC patients). Cancer-specific survival was defined as the time frame between surgery and death from cancer.

4. Results and discussion

4.1. Screening of short-chain *O*-glycans in bladder cancer tissues

Forty-seven FFPE bladder tumours and 6 healthy controls were analysed by immunohistochemistry to detect the expression of short-chain *O*-glycans (Tn, STn, T and ST). Emphasis was given to the expression of T antigen monosialylated forms S3T and S6T, which had been so far less studied due to the inexistence of commercially available specific monoclonal antibodies to these epitopes and limitations in glycomics approaches. To address the possibility of *O*-6 GalNAc sialylation (S6T) of the T antigen, bladder tumour sections were digested with a β-(1-3)-galactosidase to remove α-3 linked Gal residues from S6T antigens prior to incubation with the anti-STn monoclonal antibody **(Figure 1A)**. Similarly, incubation with an α-neuraminidase specific for cleaving *O*-3 linked sialic acids from S3T, allowed T antigen detection in previously T antigen negative tissues **(Figure 1B)**. For detecting dST, bladder tumour sections were digested with an α-

neuraminidase to remove α-3 and α-6 linked sialic acids, allowing T antigen detection in T, S3T and S6T negative tissues.

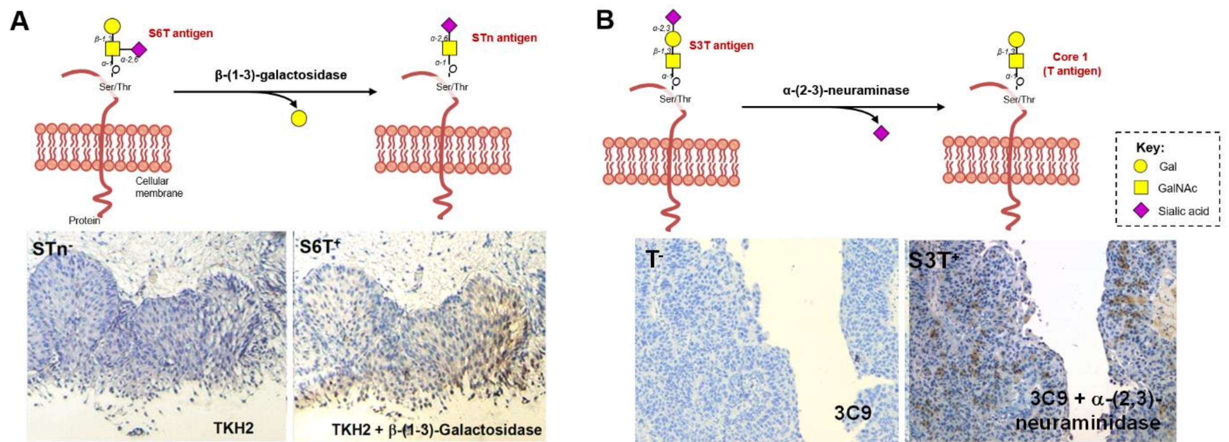

Figure 1. Schematic representation of the analytical strategy for S6T and S3T detection by immunohistochemistry. A) The S6T antigen may be considered structurally related with STn, since it presents an O-6 sialylation of its GalNAc residue. As such, STn negative tumour sections were digested by an β-(1,3)-galactosidase to remove the Gal residue of S6T; thereby exposing the STn antigen for recognition by TKH2 monoclonal antibody. In addition, after enzymatic digestion of several STn positive tumours, a significant increase in STn staining was achieved (data not shown). **B)** Envisaging to evaluate S3T antigen expression in bladder tumours, an α-(2,3)-neuraminidase was used to remove the *O*-3 Gal sialylation of this epitope to expose the T antigen for recognition by the anti-T antigen monoclonal antibody 3C9. The presence of S3T was determined by comparing T antigen expression in T antigen negative tumours sections prior and after enzymatic digestion.

Figure 2 summarizes the expression of these glycans in tissue samples representative of several BC stages and grades. Notably, we observed that none of the short-chain glycans was expressed in healthy urothelium, demonstrating the cancer specific nature of these molecular alterations, while Tn and T antigens were poorly expressed in bladder tumours (20–50% of total cases) in comparison with their sialylated counterparts STn and mono- and/or disialyl- ST (62% and 100%, respectively), irrespectiveof grade and invasion. The number of T antigen-positive cases largely exceeds the Tn-positive cases (53% vs 23%), which was particularly seen in advanced tumours when compared to low-grade superficial lesions. This could be due to overexpression of C1GalT1 (core 1 synthase, T-synthase) or downregulation of other glycosyltransferases, such as C2GnT, involved in *O*-glycan extension, as previously demonstrated in BC cells exposed to hypoxia(20). Moreover, these results reinforce previous work associating T antigen expression and tumour invasion(24). Notewithstanding,

in agreement with previous reports the STn antigen was observed in high-grade and invasive tumours (75 and 89%, respectively), whereas only 24% of low grade cases were positive.

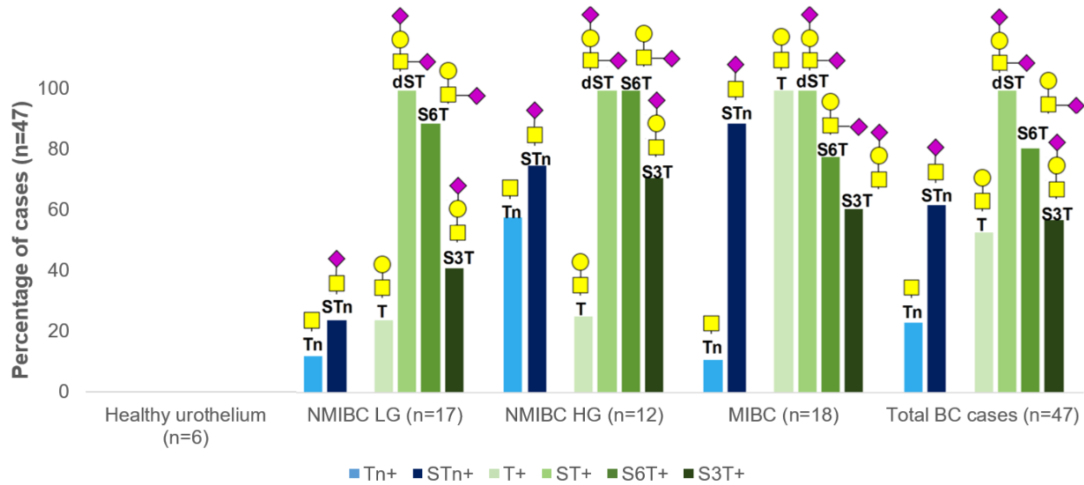

Figure 2. Expression of short-chain *O*-GalNAc glycans in bladder tumours of different clinicopathological natures and healthy urothelium determined by immunohistochemistry.

Most STn-positive cases presented low extension of expression (< 20%), focal and polydisperse, throughout the tumour. STn was mostly found in cells of the basal layer, yet it could also be detected in tumour areas presenting extensive staining (> 50%), in papillary urothelium and invasive fronts **(Figure 3A)**(18). Moreover, whenever present in the tumour, STn was also detected in the adjacent but not in the distal mucosa(18). Furthermore, the sialylated forms of the T antigen, including mono- and/or disialylated glycans, were diffusely expressed by all studied bladder tumours. The S6T antigen was found in approximately 80% of the studied tumours, with similar percentage of positive cases between NMIBC and MIBC, despiteincreased extension of expression could be observed in advanced **(Figure 1)**. In contrast with the ubiquitous nature of S6T, the S3T antigen was primarily found in high-grade NMIBC (41% low-grade NMIBC; 71% high-grade NMIBC; 61% MIBC), although we should note that many high-grade tumours co-expressed both T sialylated forms. These observations support previous associations between the overexpression of both sialyl-T and ST3Gal-I, the sialyltransferase responsible for T antigen O-3 sialylation, in high-grade tumours(22)

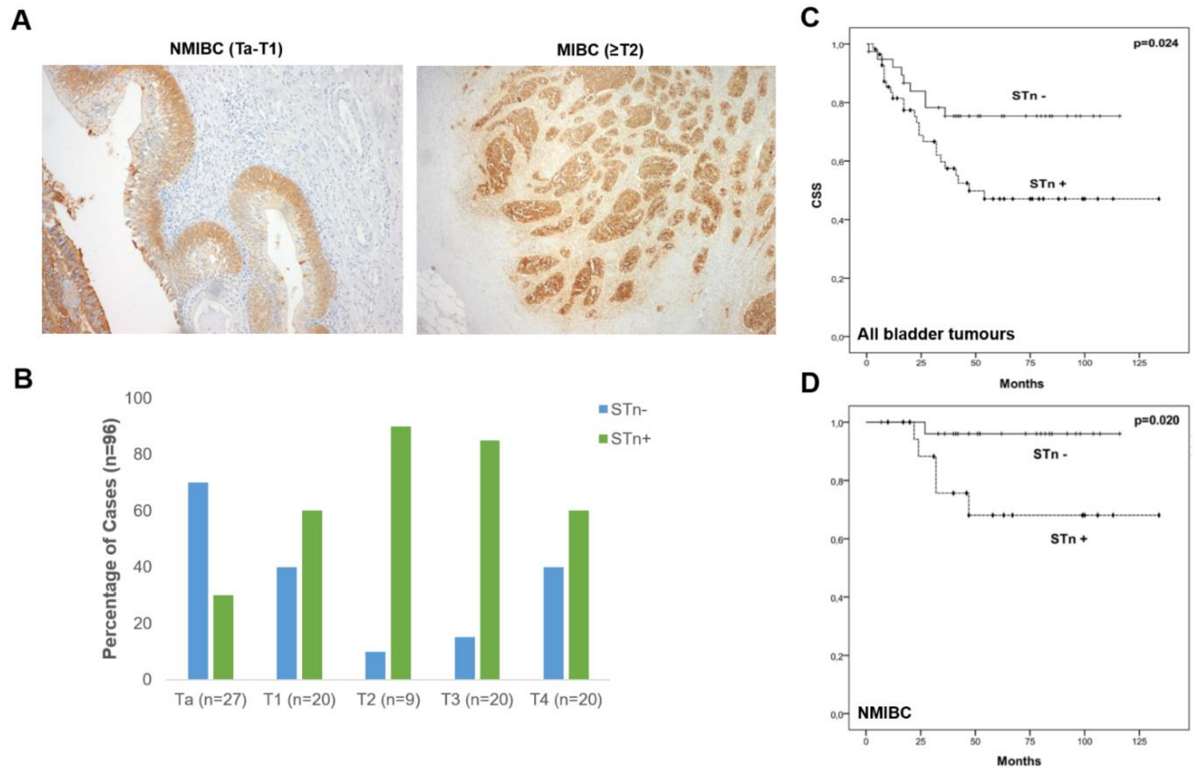

Figure 3. STn clinical significance and expression across different bladder tumor stages. A) Representative immunohistochemistry images of STn antigen patterns of expression in NMIBC (left) and MIBC (right) cases. In high-grade NMIBC cases STn positive cells are seen mostly in the basal layer, while MIBC cases present extensive staining and cells expressing STn are seen invading the muscle layer. **B)** Percentage of cases expressing STn throughout disease stages in the studied cohort of 96 patients. **C.** Effect of STn expression in cancer-specific survival of patients regardless of tumour stage. **D.** Effect of STn expression in cancer-specific survival of NMIBC patients. Kaplan–Meier analysis shows the association between STn expression and decreased CSS of BC patients. Comparison performed by log-rank test (p = 0.024); + censored STn negative tumours; ♦ censored STn positive tumours.

In summary, results herein presented reinforce the cancer-specific nature of short-chain *O*-glycans, as well as the up-until now neglected expression of T antigen sialylated glycoforms in bladder tumours. Moreover, the co-expression of non-sialylated and sialylated glycoforms was demonstrated in a minor subset of advanced-stage tumours. Finally, and given the prevalence of the STn antigen in increasingly aggressive high-grade and invasive tumours, its prognosticator role was shown in a larger patients' set, demonstrating the promising clinical value of this glycan in bladder cancer.

4.2. Biological and clinical relevance of sialyl-Tn in bladder cancer

The expression and staining patterns of STn antigen were confirmed in a larger retrospective subset of 96 BC patients. Particularly, STn was expressed in approximately 60% of all bladder tumours studied, mostly in MIBC (60-76%, $p < 0.05$), with the same staining patterns as previously observed. Figure 3B represents STn expression throughout BC stages. These findings further support STn's role in tumour invasion, as evidenced by in vitro studies(8). Accordingly, advanced stage bladder tumours were previously described to overexpress ST6GalNAc-I, an enzyme responsible for Tn antigen sialylation at the α2,6 position, originating STn(19). Notwithstanding, it is still necessary to disclose the expression of ST6GalNAc-II and possibly ST6GalNAc-IV, also known to be involved in the O-6 sialylation of the Tn antigen(25), as well as the expression of C1GALT1 and C2GnT1 in BC tissues. Moreover, the molecular mechanisms underlying glycomic alterations, including events modulating the expression, activity and distribution throughout secretory organelles of glycosyltransferases and glycosidases, as well as the mutational patterns of key enzymes involved in *O*-glycans biosynthesis and their functional impact also require further analyses.

To evaluate the association between STn antigen expression and cancer-specific survival (CSS), a Kaplan-Meier analysis of 96 BC patients was conducted. Particularly, patients with STn positive tumours had lower CSS, after adjustment for tumour stage ($p=0.024$; Figure 3C). This was also observed when evaluating NMIBC alone ($p = 0.020$; Figure 3D). Importantly, among NMIBC, STn expressing T1 tumours presented lower CSS than negative tumours ($p <0.05$). After multivariate Cox regression analysis adjusting for relevant covariates (age, gender, stage, grade, recurrence status and presence of concomitant CIS) STn remained an independent prognostic marker of worst cancer-specific survival (HR=11.836; 95%CI: 1.063–131.7; $p=0.044$).

In summary, STn constitutes a cancer-specific antigen associated with advanced disease and poor prognosis in bladder cancer.

5. Conclusions

This work highlights that advanced bladder tumours overexpress an array of short-chain *O*-glycans resulting from a premature arrest of glycosylation extension in membrane and secreted proteins, demonstrating a predominant sialylated over neutral glycoform, with emphasis on sialylated Tn and T antigens. Here, for the first time, insights on the nature of the T antigen sialylation are provided, which might be crucial for guiding future glycomics and glycoproteomics studies and for designing specific ligands against BC cells. Moreover, a significant increase in O-6 sialylation in bladder tumours is evidenced, particularly the overexpression of the STn antigen.

The altered protein glycosylation translated into STn overexpression in advanced stage tumours was reinforced by present work through *O*-glycans screening in larger patient sets that revealed STn expression association with muscle-invasive, suggesting a role for sialylation in stopping protein glycosylation in advanced stage bladder tumours. Furthermore, new insights regarding its correlation with decreased CSS, as previously observed for digestive track tumours(26-28), were provided, demonstrating that STn antigen is a biomarker of poor prognosis, particularly in MIBC.

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Chapter 2 | Cisplatin exposure as a selective pressure for Sialyl-Tn expressing stem-like cells: A proof of concept study

1. Abstract

Muscle invasive bladder cancer (MIBC) constitutes the second most deadly genitourinary cancer. Currently, the standard-of-care includes surgery and cisplatin-based chemotherapeutic schemes, which often fail to avoid tumour relapse and disease progression. Recently discovered bladder cancer stem cells (BCSCs) now help to explain observations in the clinical setting, including tumor heterogeneity, chemoresistance, recurrence, and metastasis. Therefore, novel biomarkers to aid prognostication and targeted therapeutics are warranted to improve patient management.

In this work two established MIBC cell lines (T24 and HT1376) were exposed to an IC50 dosage of cisplatin and evaluated according to the expression of the cancer-associated O-GalNAc glycan sialyl-Tn (STn) and CD44, a typical BCSC-associated glycoprotein also associated with drug resistant phenotypes and poor prognosis. Both chemotherapy challenged cell lines and muscle-invasive bladder tumours were submitted to transcriptional analyses of a panel of genes characteristic of stem, mesenchymal, epithelial-to-mesenchymal (EMT), and epithelial cell phenotype, as well as cytokeratins (KRT), to assess cellular differentiation states.

Taken together, data suggest that chemotherapy acts as a discriminatory pressure, selecting tumour niches with undifferentiated stem-like cells characterized by the expression of CD44-STn+ glycoforms. These results reinforce that STn is a biomarker of aggressiveness and resistance to chemotherapy in bladder tumours.

2. Introduction

Bladder cancer (BC) is one of the most common malignancies of the urinary tract and the second deathliest amongst genitourinary cancers(1). At diagnosis, most patients present non-muscle invasive bladder cancer (NMIBC), conservatively treated by complete transurethral resection and intravesical therapies(2). Notwithstanding, high-grade NMIBC is characterized by high recurrence rates and elevated risk of progression to muscle invasion(2). In addition, 25% of new cases display muscle-invasive bladder cancer (MIBC) or metastatic disease, currently treated by radical cystectomy and cisplatin-based chemotherapeutic regimens, which often fail to avoid tumour relapse and disease progression(3). Therefore, BC patients present a significant management hurdle due to high recurrence rates, rapid progression, dissemination and poor response to chemotherapy, accompanied by a significant lag in the introduction of novel targeted therapies when compared to other solid tumours(4, 5). Moreover, the lack of efficient follow-up strategies, especially non-invasive, for early detection of

occult micrometastasis, real-time monitoring of therapy response and metastatic risk assessment, further aggravates prognostication in bladder cancer(6). Thus, improving BC survival will require earlier detection, more effective local control and new therapies for metastatic disease. Importantly, tumour heterogeneity as been regarded to drive from minor subpopulations of cells within the tumour population endowed with distinct proliferative and differentiation capacities, termed cancer stem-cells (CSC). The CSC model supports that these cells are responsible for sustaining growth with significant clinical implications, as CSCs have been shown to be resistant to chemo and radiotherapy, especially when in quiescent state(7, 8). Particularly, recurrent BC is usually chemoresistant, even if the primary tumour is chemotherapy-sensitive(9), which might be explained either as a consequence of unfavourable drug pharmacokinetics and abnormal tumor vasculature delivering suboptimal concentrations of cytotoxic agents to the tumor (10, 11), or as a consequence of bladder cancer stem-cells (BCSCs) chemoresistance(9, 11, 12). In fact, chemotherapy's selective pressure results in enriched BCSCs populations capable of recapitulate residual tumours between chemotherapy cycles in human bladder cancer xenografts(13). Moreover, it has been shown that CSC-like cells can be isolated after chemotherapy in cancer cell lines, reinforcing the growth advantage of CSC-like cells upon therapeutic challenges(14). As such, chemotherapy in combination with CSC-targeted therapy would be ideal for advanced stage BC patients. Importantly, BCSCs were first isolated using cell-surface markers present on normal stem cells(15). Since then, superficial markers as CD44(15), CD44v6(16, 17), laminin receptor (67LR)(18), Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1)(19), CD133(20, 21), CD47(15), CD49(22), and keratin 14 (KRT14)(23) have been used, alone or in combination,to identify and isolate BCSCs. More recently, basal-like lesions presenting the *KRT*14+ and/or *KRT*5+ and *KRT*20- phenotype were found enriched for BCSCs and presented higher resistance to chemotherapy and decreased survival(25). This constrasted with well differentiated luminal lesions (*KRT*14- , *KRT*5- and *KRT*20+) with favourable prognosis (23-25). Nevertheless, despite these evidences, bladder CSC specific biomarkers for therapeutic targeting remain to be identified.

Malignant transformation is often accompanied by profound changes in glycosylation patterns, which play key roles in tumour progression and dissemination(26, 27). As such, exploring cancer-associated glycans, which are often of oncofetal nature, presents a unique and highly specific opportunity to target CSCs. Particularly, overexpression of the cancer-associated O-GalNAc glycan Sialyl-Tn (STn) has been demonstrated to be a salient feature of advanced stage bladder tumours(28, 29, chapter 2-results), being a biomarker of poor prognosis, particularly in MIBC(30; chapter 2 results). Moreover, it is also absent or marginally present in the normal urothelium, reinforcing its cancer-specific nature, while promoting cancer cell motility and invasion, as well as immune escape(29, 31, 32). Preliminary data also suggest that the STn antigen may be expressed by

chemoresistant cells in non-proliferative tumour areas (28); however its association with BCSCs phenotypes has not yet been disclosed and may provide the necessary means to improve on its indentification and targeting.

3. Material and Methods

3.1. Patients and tissue samples

A subset of 10 MIBC patients, attending the Portuguese Institute of Oncology of Porto (IPO-Porto; Portugal) and treated with neoadjuvant cisplatin-based chemotherapy, were included in this study. A total of 20 formalin-fixed paraffin-embedded (FFPE) tissue sections were analysed, 10 from prior chemotherapy transurethral resections (TUR) and 10 from post-chemotherapy cystectomies. All procedures were performed under the approval of the institution ethics committee and upon patients' informed consent. All clinicopathological information was obtained from patients' clinical records.

3.2. Cell lines and cell culture conditions

The T24 (grade III) and HT1376 (grade III) urothelial cancer cell lines used in this work were acquired from DSMZ (Düsseldorf, Germany) and recently characterized from the genetic standpoint by our group(34). Accordingly, the T24 cell line is representative of the FGFR3/CCND1 carcinogenic pathway, presenting a mutated HRAS and overexpression of CCND1, while the HT1376 cell line corresponds to the E2F3/RB1 pathway with loss of one copy of RB1 and mutation of the remaining copy. Additionally, HT1376 cells exhibit deletion of the Phosphatase and tensin homolog (PTEN) gene and no alteration of Phosphatidylinositol 3-kinase catalytic subunit alpha (PIK3CA), which in combination with the inactivation of p53, translates into a more invasive and metastatic potential. The cells were cultured in RPMI 1640+GlutaMAXTM medium (Gibco, Life Technologies) supplemented with 10% heat-inactivated FBS (Gibco, Life Technologies) and 1% penicillin-streptomycin (10,000 Units/mL P; 10,000 µg/mL S; Gibco, Life Technologies). Cell lines were cultured as a monolayer at 37˚C in a 5% CO2 humidified atmosphere, and were routinely subcultured after trypsinization.

3.3. Cell Viability Assay

To assess the chemosensitivity of the UBC cell lines to cisplatin (CDDP, cis-diamminedichloroplatinum-[II]), 1x106cells were seeded in triplicate into 6-well plates and incubated with increasing concentrations of cisplatin. Stock solutions of 1 mg/ml cisplatin in 10% NaCl were kindly provided by the Pharmaceutical Services of the Portuguese Institute of Oncology, Porto,

Portugal, from which the working solutions were prepared. The effect of cisplatin exposure on cell viability was determined at 72 h by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (M6494, ThermoFisher Scientific) according to the manufacturer's instructions. The IC50 values (cisplatin concentration that corresponds to 50% of cell growth inhibition) were estimated from at least three independent experiments, using GraphPad Prism Software. Estimated IC50 values for both cell lines were 3µg/mL.

3.4. Gene expression

Gene expression was assessed by quantitative polymerase chain reaction (qPCR). These assays included a group of genes associated with stem (*NANOG, LIN28A, POU5F1, KLF9, KLF4, SOX2*), epithelial (CDH1, DSP, EpCAM), Epithelial-to-mesenchymal transition (*SNAI1, SNAI2, TWIST1, TWIST2, ZEB1, ZEB2, RUNX1, RUNX2, FN1*), and mesenchymal (*CDH2, VIM, SPARC*) phenotypes, as well as CD44 and cytokeratins (*KRT 5, KRT14, KRT20*) described in **Table 1**. Briefly, total RNA from cultured cells was isolated using TriPure isolation Reagent (Roche) and from FFPE tissue samples using "Absolutely RNA FFPE Kit" (Stratagene, La Jolla, CA), according to the vendor's instructions. The RNA quantity and its purity were determined based on the A260/A280 using a Nanodrop Lite Spectrophotometer (Fisher Scientific). Only samples with ratios between 1.9 and 2.1 were considered for downstream molecular studies. RNA conversion and gene expression analysis was performed as previously described(35). All reactions were run in duplicates and experiments performed in triplicate. The mRNA levels were normalized to the expressions of B2M and HPRT, which were found to be the most stable genes for the cell lines(36). The relative mRNA levels were calculated using the formula $2^{-\Delta\Delta Ct}$ described by Livak et al.(37). The efficiency of each primer/probe was above 95% as determined by the manufacturer. Interpretation of biological functions translated by alterations in gene expression was done using ClueGO version 2.3.2(38) and CluePedia plugins version 1.3.2(39) for cytoscape version 3.4.0(40).

Table 1 – Taqman gene expression assays

Gene	TaqMan Gene Expression Assay ID
KRT5	Hs00934200_g1
KRT14	Hs00559328_m1
KRT20	Hs00300643_m1
CD44	Hs01075864_m1

NANOG	Hs04260366_g1
LIN28A	Hs01552405_g1
POU5F1	Hs04260367_g1
KLF9	Hs00230918_m1
KLF4	Hs00358837_g1
SOX2	Hs01053049_s1
CDH1	Hs01023894_m1
DSP	Hs00950591_m1
EpCAM	Hs00901885_m1
SNAI1	Hs00195591_m1
SNAI2	Hs00950344_m1
TWIST1	Hs01675818_s1
ZEB1	Hs01566410_m1
ZEB2	Hs00207691_m1
RUNX1	Hs01021971_m1
RUNX2	Hs01047973_m1
FN1	Hs00365052_m1
CDH2	Hs00983056_m1
VIM	Hs00185584_m1
SPARC	Hs00234160_m1
B2M	Hs00984230_m1
HPRT	Hs99999909_m1

3.5. Immunoprecipitation for CD44 and western blot conditions

CD44 was immunoprecipitated from total protein extracts (IP) with anti-CD44 (EPR1013Y; Abcam) monoclonal antibodies using Pierce Direct IP Kit (Thermo Scientific) according to the supplier's instructions. Protein samples were separated in reducing SDS/PAGE gels, transferred to 0.45-mm nitrocellulose membrane (GE Healthcare Life Sciences, Uppsala, Sweden) and blotted for the CD44 and for STn with TKH2 hybridoma-derived monoclonal antibody. Protein extracts treated with α-neuraminidase (Sigma Aldrich) were used as controls. β-actin was used as loading control.

3.6.Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (FFPE) were screened for CD44 by immunohistochemistry using the strepavidin/biotin peroxidase method. Briefly, 3 µm sections were deparaffinized with xylene, rehydrated with graded ethanol series, microwaved for 15 min in boiling citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0, Sigma-Aldrich), and exposed to 3% hydrogen peroxide for 25 min. CD44 was detected using a recombinant polyclonal antibody (anti-CD44, 1:4000 in PBS; ab157107; Abcam, Cambridge, UK) after incubation overnight at 4°C. The STn antigen was evaluated using a B72.3 monoclonal antibody (Abcam) 0.5ug/mL overnight at 4°C. The antigens were identified with UltraVision HRP Detection System Kit (Thermo Fisher Scientific, Waltham, MO, USA) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (Impact Dab, Vector Laboratories) for chromogenic development. Finally, the slides were counterstained with Harris's haematoxylin for 1 min. Negative control sections were performed by adding BSA (5% in PBS) devoided of primary antibody. The immunoassayed sections were blindly assessed using light microscopy by two independent observers and validated by an experienced pathologist. Briefly, a semi-quantitative approach was established to score immunoreactivity based on the intensity and extension of the staining. The extension of staining was rated in cut-offs of 10%, and staining intensity was rated as follows: negative-0, weak-1, moderate-2, strong-3. The tumours were then classified as a score based on the product of extension with intensity. Readings not concordant were re-analysed using a double-headed microscope and consensus was reached.

3.7. *In situ* proximity ligation assays on tissue sections

The detection of CD44-STn+ glycoforms was achieved by *in situ* proximity ligation assays (PLA), using the Duolink *in situ* detection reagent Red (Olink Bioscience, Uppsala, Sweden), according to the manufacturer's instructions. FFPE tissues were deparaffinized, rehydrated and subjected to acid- and heat-induced antigen retrieval, followed by incubation with 3% hydrogen peroxide. Then, tissue sections were incubated with blocking solution in a humidified atmosphere chamber. CD44 and STn were detected by indirect PLA using the polyclonal antibody anti-CD44 1:2000 overnight at 4°C (clone ab157107; Abcam, Cambridge, UK) and the hybridoma-derived 3F1 antibody 1:4 overnight at 4°C, respectively. The PLA probes anti-rabbit MINUS and anti-mouse PLUS were both added and sections were incubated at 37°C for 1 h. Next, ligation was performed for 30 min at 37 °C, and amplification was carried out for 120 min at 37°C to produce rolling circle products, followed by incubation with 4',6-diamidino-2-phenylindole for 10 min at room temperature and

sample mounting for fluorescence microscopy. PLA results were evaluated by two distinct observers and validated by an experienced pathologist, who independently registered colocalization of staining's.

3.8.Statistical analysis

Statistical analysis was performed using the Student's T-test for unpaired samples. Differences were considered significant when $p < 0.05$. A chi-square test was used to analyse associations between variables and clinicopathological features.

4. Results

4.1.Expression of Sialyl-Tn and CD44 in urothelial cell lines exposed to cisplatin

Muscle-invasive urothelial cell lines T24 and HT1376 were submitted to one IC50 dosage of cisplatin, a common agent in BC treatment schemes, resulting in a 50% reduction in tumour cell viability. Remain cells were maintained in culture for more than two month; however expansion in vitro was not observed under standard culture conditions presented in the material and methods. In order to evaluate the remaining clones, whole cell lysates were blotted for cancer-specific O-GalNAc glycan STn and the stem-cell marker CD44. Contrasting with the significant reduction in tumour cell viability, both STn and CD44 levels remained mostly unchanged before treatment, suggesting the selection of STn and CD44-expressing clones **(Figure 1A and 1B)**. The specificity of slot blot signals was reinforced through its decay after sialidase treatment. To validate the existence of CD44-STn+ glycoforms, we have immunoprecipitated CD44 in these samples and confirmed the expression of STn by western blot **(Figure 1C)**.

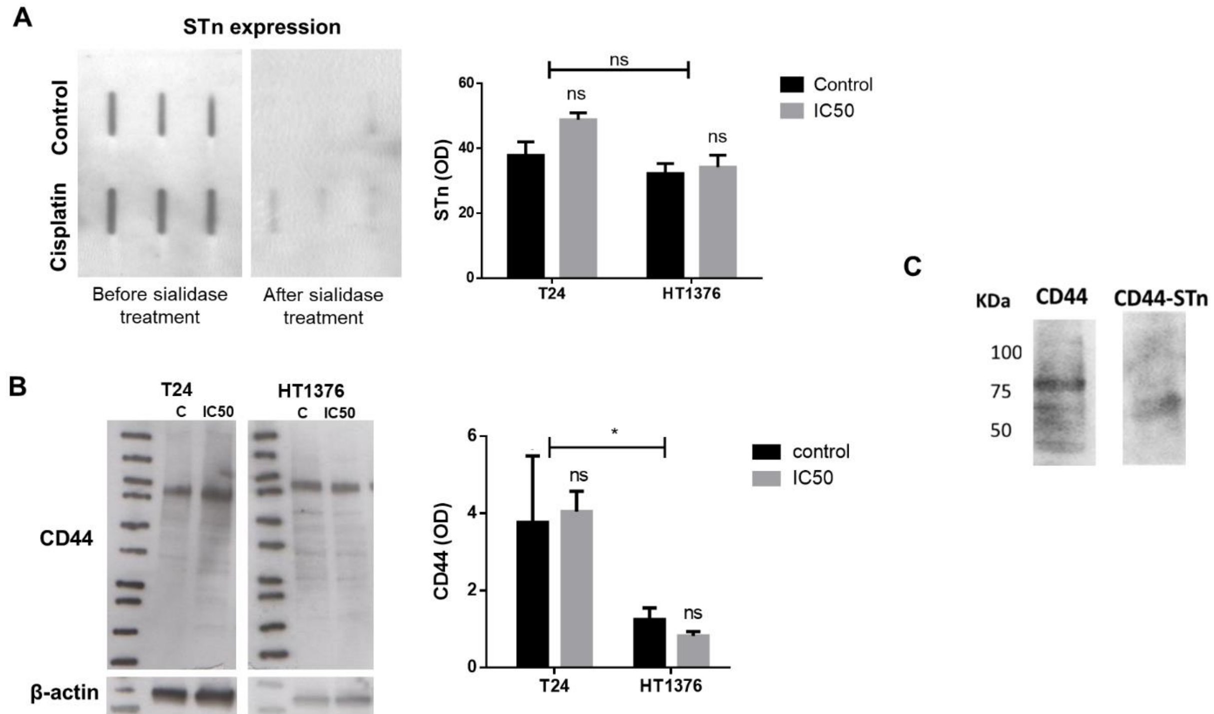
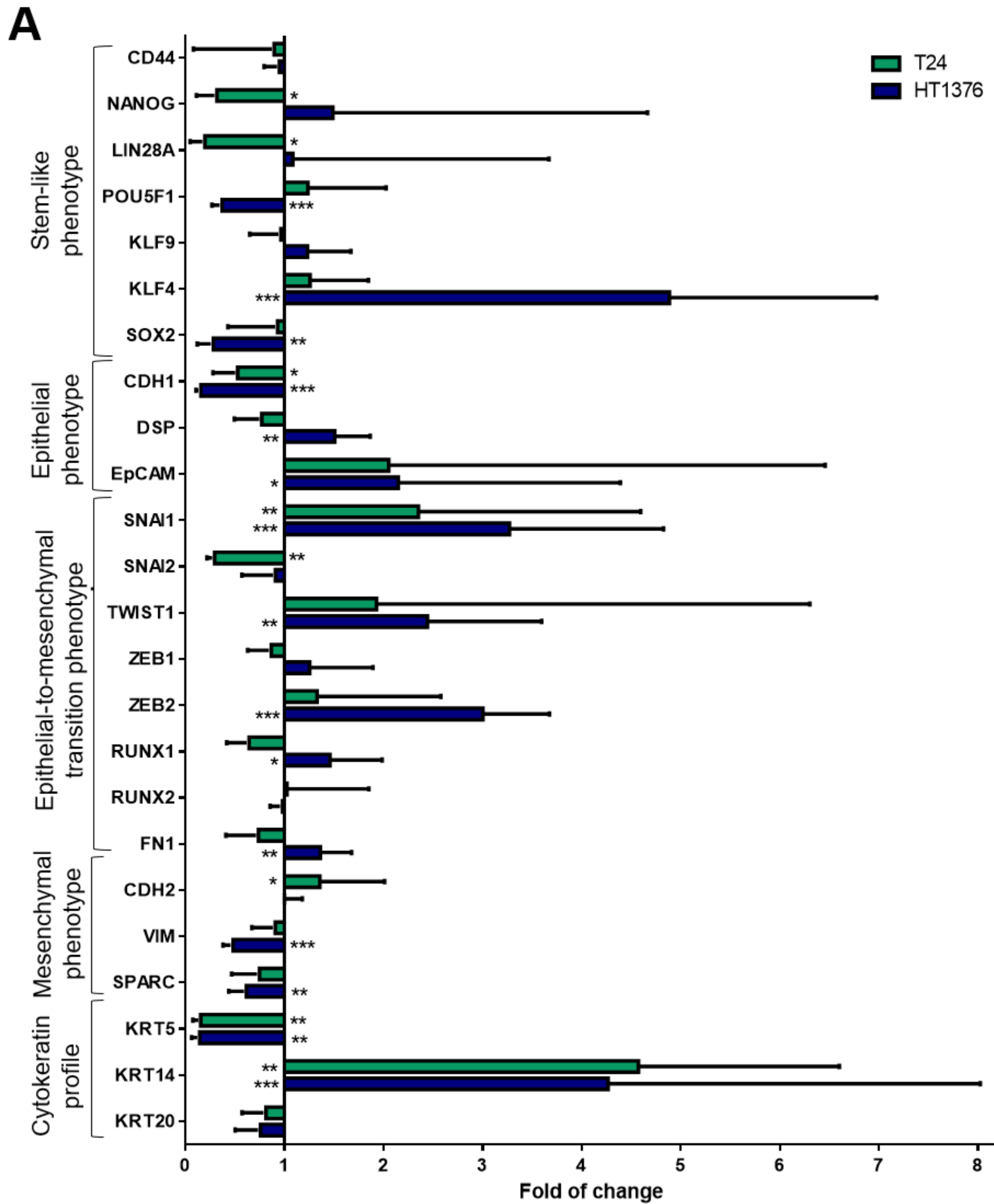


Figure 1. Expression of STn and CD44 in T24 and HT1376 cells after cisplatin exposure. A) Representative slot blot analysis of STn antigen in protein extracts from T24 and HT1376 remaining clones. This was a sialidase-sensitive signal, confirming the presence of the antigen. **B)** Western bot for the CD44 glycoprotein in T24 and HT1376 cell lines exposed to cisplatin. CD44 levels are significantly higher in the T24 cell line compared to HT1376 cell line. Nevertheless, CD44 levels did not vary for any cell line after exposure to IC50 doses of Cisplatin. **C)** Identification of CD44-STn+ glycoforms in whole cell lysates of treatment-reminiscent cells by immunoprecipitation. CD44 was immunoprecipitated from protein extracts using antibody-immobilized agarose beads and blotted for CD44 and STn thereafter.

4.2. Cisplatin modulation of gene expression in urothelial cancer cell lines

After cisplatin exposure, both T24 and HT1376 cell lines were submitted to transcripts analysis regarding a panel of genes involved in stem, epithelial, epithelial-to-mesenchymal (EMT) and mesenchymal phenotypes. Moreover, to explore the differentiation patterns of the remaining clones the cytokeratin profile was also evaluated. Overall, the HT1376 cell line overexpresses stem-associates genes, as *KLF4*, as well as EMT-related genes as *TWIST1* and *ZEB2*. In turn, T24 clones mostly overexpress EMT promoting genes such as *TWIST1* and *SNAI1*. Importantly, both cell lines strikingly overexpress *KRT14*, denoting a very pronounced undifferentiated phenotype of the residual cells. In addition, as previously described, *CD44* transcript levels remain mostly unchanged **(Figure 2A)**. The integrative analysis of transcript modulation by cisplatin in both cell lines through Cytoscape Software

has evidenced the activation of pathways promoting the maintenance of stem cell populations, as well as the negative regulation of DNA damage response through p53, and modulation of cellular adhesion **(Figure 2B)**.



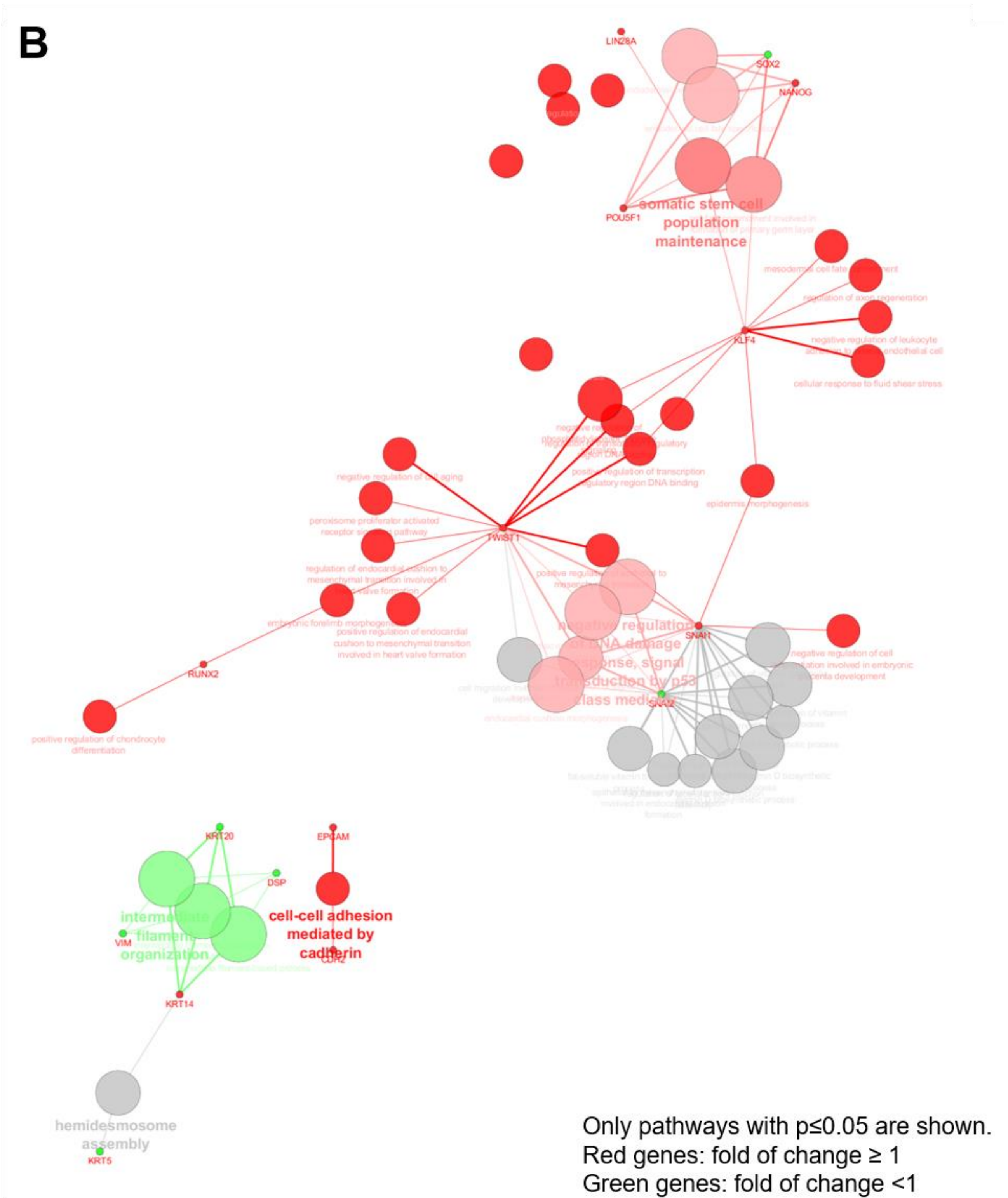


Figure 2. Cisplatin modulation of gene expression. A) cisplatin-modulated under and overexpression of cytokeratins as well as stem, EMT, epithelial, and mesenchymal phenotype genes. **B)** Functional interaction network obtained through Cytoscape Software using ClueGO and CluePedia plugins for single cluster analysis and comparison of gene clusters to explore cellular processes and their dynamics.

4.3. Chemotherapy modulation of gene expression in bladder tumours

FFPE tumours sections from a subset of 10 MIBC patients treated with neoadjuvant cisplatin-based chemotherapy were screened for STn and CD44 expression. CD44 and STn staining patterns and intensities do not significantly vary before and after chemotherapy **(Figure 3A)**. However, to validate colocalization and the existence of abnormally glycosylated CD44, the same tumour sections were screened by *in situ* proximity ligand assay (PLA), which allowed the simultaneous detection of both CD44 and STn when in close proximity **(Figure 3B)**. These findings support the existence of CD44 carrying cancer-associated STn glycoforms in bladder tumours after chemotherapy. Furthermore, the transcript levels of some stem gene, including *CD44*, and cytokeratins were also evaluated in these tumours. Of note, all evaluated stem markers were upregulated post-chemotherapy, as well as cytokeratin 14 (*KRT14*). Denoting a chemotherapy-mediated enrichment for stem and undifferentiated cells **(Figure 3C)**.

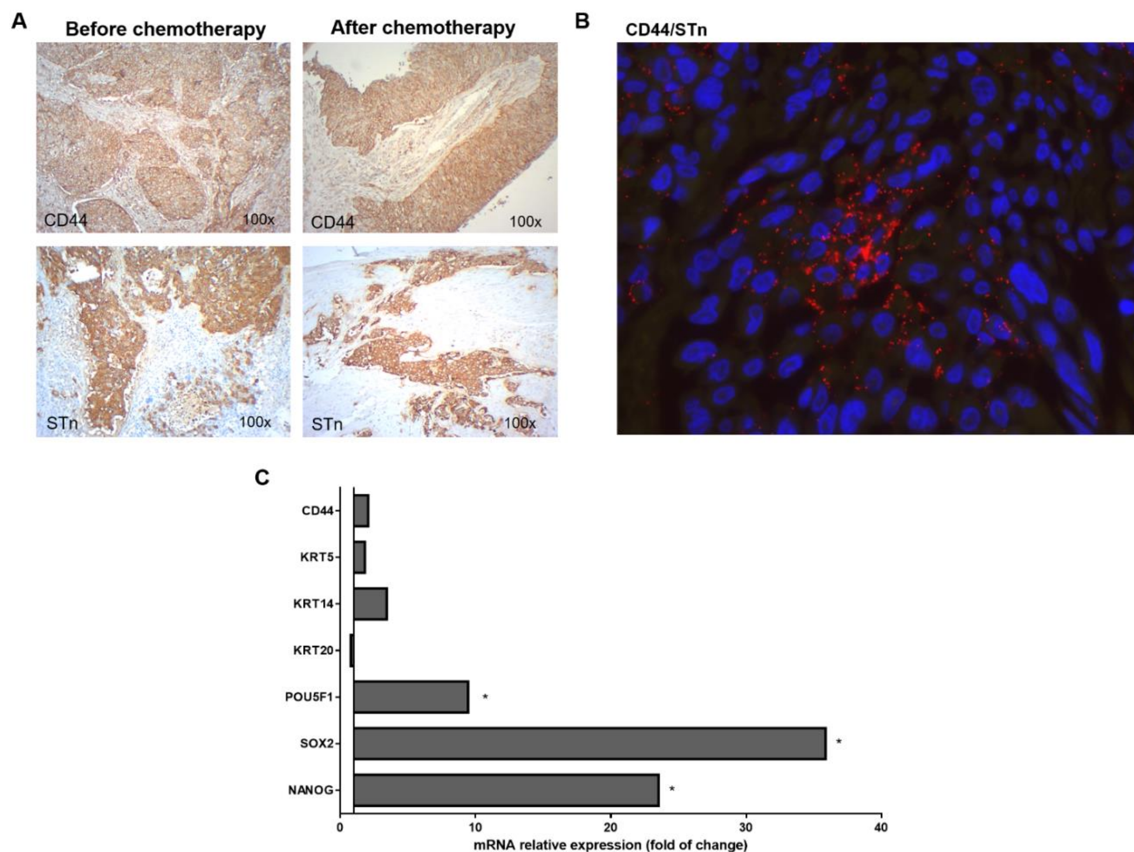


Figure 3. Chemotherapy modulation of bladder tumour phenotypes. Two complementary validation strategies were applied: A) first consecutive tumour tissues were screened for CD44 and the cancer-associated

O-glycan STn by immunohistochemistry; **B)** second, the same tumour sections were screened for CD44 and the targeted glycan STn by *in situ* proximity ligand assays, which allowed the simultaneous detection of both targets when in close proximity. Results showed the co-expression of CD44 STn in the same tumour areas and support the existence of CD44 carrying cancer-associated glycosylation in bladder tumours. **C)** Stem markers and cytokeratin transcript evaluation.

5. Discussion

In this work two MIBC cell lines (T24 and HT1376) were submitted to a dose IC50 of cisplatin and evaluated according to the expression of the cancer-associated O-GalNAc glycan sialyl-Tn (STn) and CD44, a glycoprotein commonly used in BCSCs identification panels. Interestingly, upon chemotherapeutic challenge, the remaining clones of both cell lines retain CD44 and STn expression. Both CD44 and STn have been shown to predict poorer prognosis in BC(30, 41). Moreover, while CD44 had been already associated with drug resistance in BC(42), this is the first report demonstrating the involvement of STn in the pharmacological response of BC cells. These results are strengthened by similar observations in gastric cancer where STn expression was described to induce tumor cell's resistance to chemotherapeutic drugs(43). Moreover, the reminiscent cells were submitted to transcriptional analysis of a panel of genes including stem, mesenchymal, epithelial-to-mesenchymal (EMT), and epithelial phenotypes, as well as cytokeratins (KRT) to assess cellular differentiation states. Of note, both stem- and EMT-associated were consistently overexpressed, which accompanied cytokeratin modulation toward *KRT14* overexpression leading to more undifferentiated and plastic phenotypes. In accordance with protein levels, *CD44* transcript levels were also sustained. The integration of transcriptomic data through Cytoscape Software evidenced the activation of pathways promoting the maintenance of stem cell populations, as well as the negative regulation of DNA damage response through p53, and modulation of cellular adhesion, conceptually corroborating the molecular adaptation of cells to the imposed stress.

In vitro results were validated using muscle-invasive bladder tumour sections of patients treated with neoadjuvant cisplatin-based chemotherapy. Particularly, both immunohistochemistry and PLA assays corroborate the preservation of STn and CD44 expression, as well as co-localization of CD44 and STn antigens, supporting the existence of cancer-associated CD44-STn+ glycoforms. Transcriptional analysis also demonstrated stem-cell markers and *KRT14* upregulation, suggesting a similar enrichment of cells *in vivo* towards more stem and basal-like cells.

In summary, we demonstrated in both MIBC cell lines and MIBC tumour sections that chemotherapy acts as a selective pressure for undifferentiated stem-like cells characterized by the

expression of CD44-STn+ glycoforms. Future studies should devote to confirming these preliminary findings in larger patient series. Particular emphasis should also be set on the isolation of CD44-STn+ cells and evalution of its capacity to recapitulate tumour heterogeneity and chemoresistance. Noteworthy, CD44 is expressed by most human tumour. The identifications of specific isoforms will be required to improve on cancer specificity and specificity and possibly off-target effects envisioning therapeutic development.

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Chapter 3 | Sialyl-Tn identifies muscle-invasive bladder cancer basal and luminal subtypes facing decreased survival, being expressed by circulating tumor cells and metastases

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Original article

Sialyl-Tn identifies muscle-invasive bladder cancer basal and luminal subtypes facing decreased survival, being expressed by circulating tumor cells and metastases

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Abstract

Objectives: To evaluate the potential of sialyl-Tn (STn), a cancer-associated glycan antigen present in membrane glycoproteins, to improve a recent molecular model for stratification and prognostication of advanced stage bladder tumors based on keratins (KRT14,5, and 20) expression. In addition, determine the association between STn and disease dissemination based on the evaluation of circulating tumor cells(CTCs) and the metastasis, which is a critical matter to improve patient management.

Patients and methods: A retrospective series of 80 muscle-invasive primary bladder tumors and associated metastasis were screened for KRT14, 5, and 20 and STn by real-time polymerase chain reaction and immunohistochemistry. Peripheral blood was collected in a patients' subset, CTCs were isolated through a size-based microfluidic chip and screened for KRTs and STn.

Results: Basal-like lesions presented worse cancer-specific and disease-free survival compared to luminal tumors. STn antigen inclusion discriminated patients with worst survival in each subgroup (P= 0.047 for luminal; P= 0.027 for basal-like tumors). STn expression in CTCs and distant metastasis was also demonstrated.

Conclusion: This work reinforces the potential of the KRT-based model for bladder cancer management and the association of STn with aggressiveness, supporting its inclusion in predictive molecular models toward patient-tailored precision medicine. Moreover, we describe for the first time that CTCs and the metastasis present a basal phenotype and express the STn antigen, highlighting its link with disease dissemination. Future studies should focus on determining the biological and clinical significance of these observations in the context of liquid biopsies. Given the membrane nature of STn, highly specific targeted therapeutics may also be envisaged.

Keywords: Bladder cancer subtypes; Sialyl-Tn; Predictive profile; Glycosylation; Circulating tumor cells

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1. Introduction

Muscle-invasive Bladder Cancer (MIBC, stage >T2) constitutes the second most common cause of death among genitourinary cancers. Of note, significant variations in the clinical outcome are observed for histopathologically similar tumours subjected to identical treatments, mostly due to molecular heterogeneity[1].

A molecular model has been recently introduced to improve patient stratification and prognosis based on sub-classification of bladder tumours as luminal and basal-like[2-4]. Accordingly, well differentiated and prognostically favourable luminal tumours, with *KRT14* and/or *KRT5* downregulation and *KRT20* (*KRT*14^{-}*KRT5*$^{-}$*KRT20*$^{+}$) upregulation, were distinguishable from basal-like lesions (*KRT14*$^{+}$ and/or *KRT5*$^{+}$ and *KRT20*$^{-}$), characterized by reduced survival and higher resistance to chemotherapy[2-4]. Even though only 50% of MIBC could be subtyped using this approach, the notion that it may provide the premise for molecular-based clinical decisions prevails. The introduction of novel biomarkers capable of further refining patient stratification seems now warranted.

This work is built upon our background in bladder tumour glycobiology to propose a strategy capable of further enhancing the *KRT*-based discriminatory potential in advanced stage tumours. Namely, we have described that bladder tumours overexpress membrane glycoproteins yielding the cancer-associated glycoantigen sialyl-Tn (STn)[5-7]. The STn antigen is a simple *O*-glycan (Neu5Acα2-6GalNAc-*O*-Ser/Thr residues in membrane and secreted glycoproteins) that is mainly associated with high-grade lesions and decreased survival, irrespective of grade and stage of disease[5,6,8,9]. It is also absent or marginally detectable in healthy urothelium, suggesting a malignant-specific nature. Its biosynthesis is triggered by high levels of hypoxia[8] and modulates protein functions in ways that favour motility and invasion[5,8], while promoting immune escape[10]. Such rationale supports STn as surrogate biomarker of poor prognosis, particularly in MIBC. Moreover, STn expression may be part of an array of molecular alterations favouring disease dissemination; nevertheless, its incorporation in multiplex biomarker models for improved prognostication a patient management has not yet been accomplished. Moreover, its link with metastatic progression remains to be demonstrated, which is a critical matter to improve patient management.

3. Materials and methods

3.1. Population and ethics statement

This study was performed retrospectively in a series of 80 formalin-fixed paraffin-embedded bladder tumours obtained from archived paraffin blocks at the Portuguese Institute of Oncology—Porto (IPO-Porto), Portugal. Bladder tumors were surgically removed from 68 men and 12 women, ranging from 44 to 86 years of age (median, 74 years), admitted and treated at the IPOP between 2005 and 2007. The time of follow-up was on average 31 months (1–104 months). All patients had cystectomy has primary therapy. From all cases included, 24% presented lymph node metastasis at the time of surgery. **Table 1** summarizes clinicopathological information. Cancer-specific survival (CSS) was defined as the period between the date of surgery and date of patient death by cancer or date of last clinical visit. In a subset of patients (n=6) with metastatic muscle invasive bladder tumours, blood was collected in order to analyze circulating tumour cells. Blood donors matched in age and gender without known neoplasias were included as controls. All procedures were performed under patient's informed consent to participate and after approval by the Ethics Committee at IPO-Porto. Clinicopathological information was obtained from patient's clinical records.

Table 1- Clinicopathological data of patients included in this study (*n*=80).

		n (%)
Stage	T2	22 (27.8)
	T3	36 (45.6)
	T4	21 (26.6)
Lymph-node metastasis	N_0	27 (34.2)
	N_1	19 (24.0)
	N_x	33 (41.8)
Recurrence	No	28 (49.1)
	Yes	29 (50.9)
	ND	23
Type of recurrence	Loco-regional	7 (25.9)
	Systemic	20 (74.1)
Distant metastasis	No	38 (47.5)
	Yes	42 (52.5)
Type of	Loco-regional	17 (40.5)

metastasis	Systemic	25 (59.5)

ND: not determined

3.2. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (FFPE) were screened for Keratins 5, 14 and 20 and cancer-associated *O*-glycan sialyl-Tn (STn) by immunohistochemistry using the strepavidin/biotin peroxidase method. Briefly, 3 µm sections were deparaffinized with xylene, rehydrated with graded ethanol series, microwaved for 15 min in boiling citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0), and exposed to 3% hydrogen peroxide for 20 min. STn expression was performed using clone B72.3 mouse monoclonal antibody (Abcam, Cambridge UK) at a dilution of 1:100 in PBS, after overnight incubation at 37°C. Keratin 5 expression was performed with KRT5 antibody (Monoclonal Rabbit Anti-Human Anti-Cytokeratin 5 antibody; Clone EP1601Y; Abcam) at a dilution of 1:100 in PBS, after 1h incubation at 37°C. Expression of keratin 14 was assessed with the KRT14 antibody (Monoclonal Rabbit Anti-Human Anti-Cytokeratin 14 antibody; Clone EP1612Y; Abcam) at a dilution of 1:50 in PBS, after overnight incubation at 4°C. The expression of Keratin 20 was evaluated using KRT20 antibody (Monoclonal Rabbit Anti-Human Anti-Cytokeratin 20 antibody; Clone EPR1622Y; Abcam) at a dilution of 1:100 in PBS, after 1h incubation at 37°C.After blockage with BSA (5% in PBS). The antigens were identified with UltraVision HRP Detection System Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (Impact Dab, Vector Laboratories, Burlingame, CA, USA) for color development. Finally, the slides were counterstained with haematoxylin for 1 min. Negative control sections were performed by adding BSA (5% in PBS) devoid of primary antibody. The immunostained sections were blindly assessed using light microscopy by two independent observers and validated by an experienced pathologist. Disaccording readings were re-analysed using a double-headed microscope and consensus was reached. Given the absence of STn in the healthy urothelium (160), tumors were classified as positive for these antigens when membrane and/or cytoplasmic immunoreactivity were observed in more than 5% of the tumour.

3.3. Real-time polymerase chain reaction for KRT14, 5 and 20 expressions

Total RNA was extracted from FFPE for KRT expression analysis and from cell lines for the genes indicated in **Table 2**. RNA extraction from FFPE sections was performed using AbsolutelyRNA FFPE kit (Stratagene, La Jolla, CA, USA) according to the vendor’s instructions. Total RNA from cultured cells was isolated using TriPure isolation Reagent (Roche Diagnostics GmbH, Mannheim,

Germany). The purity and quantity of RNA extracts were determined based on the A260/A280 ratio using Nanodrop ND1000 (Nano Drop Technologies Inc. Wilmington, DE, USA). Only samples with ratios between 1.9 and 2.1 were considered for downstream molecular studies. Up to 2µg total RNA from tissue sections and total RNA from cultured cells was reverse transcribed with random primers, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The amplification conditions were the following: 25ºC for 10 min, 37ºC for 120 min and RT inactivation at 85°C for 5 min. Two non-template negative controls were used. The products were amplified in a Mycycler Thermal cycler (BioRad, Hercules, CA, USA). Gene expression levels, including three reference genes (B2M, HPRT and ActB) were analysed using TaqMan assays (Applied Biosystems; assays IDs indicated in **Table 2**). Real-time PCR amplification of cDNA samples was performed in a StepOne Real-Time PCR System (Applied Biosystems) using TaqMan Gene Expression Master Mix, primers and probes provided by Applied Biosystems. Thermal cycling conditions were 10 min at 95°C followed by 45 cycles of 15 sec at 95ºC and 1 min at 60°C. All samples were run in duplicate. Relative mRNA gene expression was calculated with the $2^{-\Delta Ct}$ formula.

Table 2 – Taqman gene expression assays

Gene	TaqMan Gene Expression Assay ID
KRT5	Hs00934200_g1
KRT14	Hs00559328_m1
KRT20	Hs00300643_m1
B2M	Hs00984230_m1
HPRT	Hs99999909_m1
ActB	Hs99999903_m1

3.4. Circulating tumour cells isolation and molecular characterization

An innovative microfluidics device under patenting by our group was used for CTCs isolation based on their higher dimensions in comparison to blood cells[11]. Peripheral blood from metastatic bladder cancer patients was collected in EDTA tubes and unprocessed samples were run within two hours of collection through size-based microfluidic CTC isolation chips. These devices were fabricated in polydimethylsiloxane (PDMS) and comprise several isolation areas containing size exclusion filters which allow CTC enrichment just based on their size and deformability, irrespectively of their phenotype. After surface passivation, 5 to 8 ml of unprocessed blood samples were injected though the

system at a constant flow rate, followed by and a final washing step with PBS performed. Trapped cells were then fixed with formalin (approx. 4% paraformaldehyde) during 20 min at RT, washed again with PBS and incubated overnight at 4ºC with anti-STn clone B72.3 mouse monoclonal antibody (Abcam; 1:33 in PBS-2% BSA), followed by labelling with a secondary goat anti-mouse IgG-TRITC antibody (Thermo scientific; 1:500 in PBS-2%BSA) for 1h at RT. Cells were further stained for 1h with DAPI (1:1000), anti-pan KRT-FITC (Sigma-Aldrich, St. Louis, MO, USA; 1:50 in PBS-2% BSA) and anti-CD45-CY5 (Abcam; 1:50 in PBS-2%BSA) antibodies used as nuclear, epithelial and hematopoietic markers, respectively. Fluorescence microscopy analysis was performed using a Nikon Ti-E inverted fluorescence microscope equipped with a sCMOS camera (Andor Neo scc-01633). Cells presenting DAPI^{+}/CK^{+}/CD45^{-} our CD45^{+} were considered CTC while those presenting a DAPI^{+}/CK^{-}/CD45^{+} phenotype where regarded as blood cells[12, 13]. CTC entrapped in the microfluidics device were then lysed with Tripure (Roche) and RNA was purified with absolutely RNA FFPE kit (Agilent technologies, Santa Clara, CA, USA) and screened for *KRT*14, *KRT*5 and *KRT*20 as described in the previous section.

3.5. Statistical analysis

Statistical data analysis was performed with IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0). Chi-square analysis was used to compare categorical variables. Kaplan-Meier curves were used to analyse the influence of STn expression in the time-to-event (death). Comparison of estimates was done using Log-rank test.

4. Results

In this study, we have screened 80 MIBC tissue samples from patients treated solely with cystectomy, 24% of which presenting with metastatic disease at the time of surgery. **Table 1** shows the demographic characteristics and pathological findings. Tumours were first categorized by qPCR according with their differentiation state in either luminal (*KRT*14^{-}*KRT5*$^{-}$*KRT*20^{+}; 34%) or basal-like (*KRT*14^{+}/*KRT5*$^{+}$*KRT*20^{-}, 66%). A predominance of the basal-like tumour subtype in relation to luminal lesions could be found (data not shown), and these results were further validated by immunohistochemistry **(Figure 1A)**. Approximately 75% of tumours could be sub-classified based on this approach, while the remaining could not be addressed due to analytical constrains related with low abundance of RNA. Nevertheless, the basal-like phenotype was further associated with recurrence (p=0.045). Furthermore, patients with basal-like tumours presented a decreased cancer-specific

survival when compared with patients presenting luminal tumours (mean 29 months *vs.* 69 months; log rank, p=0.019, **Figure 1B**). The majority of the metastases presented a basal-like profile in correlation with primary tumour. These insights reinforce the association of basal-like subtypes with worst prognosis in a Southern Europe population as well as the clinical potential of this stratification.

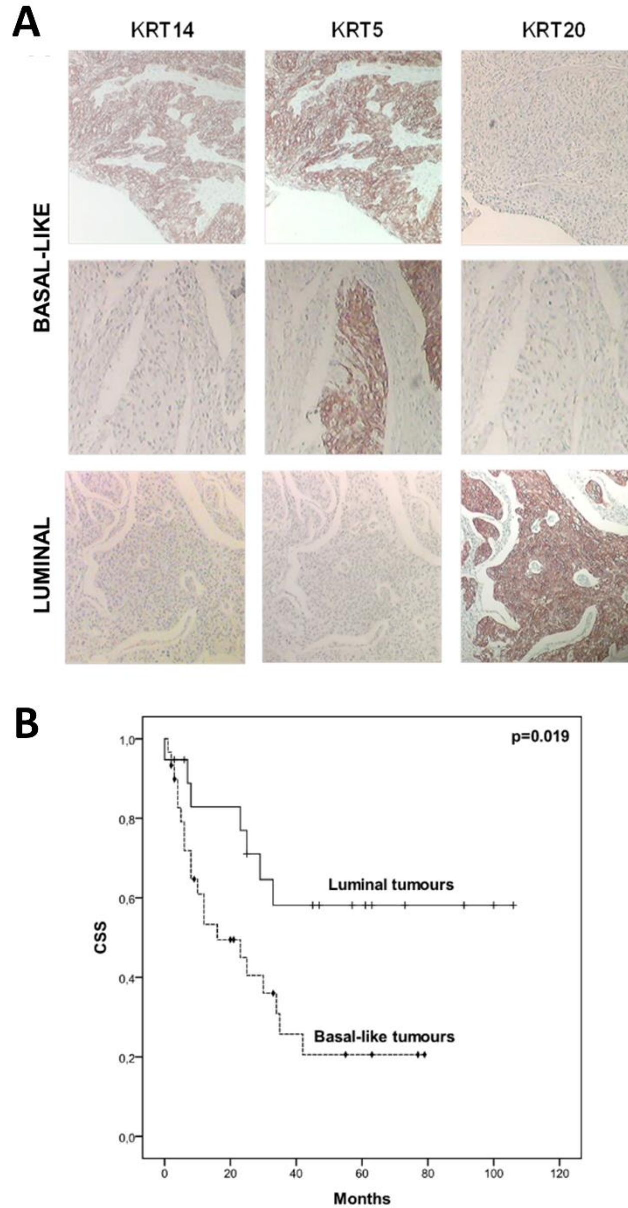

Figure 1. (A) Immunohistochemical staining showing the differential expression of keratins in bladder tumours (x100). Columns represent staining of the antigen for each keratin (KRT5, KRT14 and KRT20) and lines depict the different subtypes studied. **(B) Effect of bladder cancer subtypes in Cancer-specific survival (CSS)** Kaplan-Meier analysis to evaluate the association between CSS and the basal-like and luminal subtypes. Log rank test (p=0.019), + censored luminal tumours; ⧫ censored basal-like tumours.

In parallel, 70% of the studied bladder tumours stained positive for the STn antigen, in accordance with our previous report[5]. STn was mainly expressed at the membrane and, to some extent, in the cytoplasm of dedifferentiated cells, mostly in areas of invasion **(Figure 2A)**. STn positive cells were also observed at the invasive front and/or inside blood vessels, suggesting a possible role for this glycan in disease dissemination. Reinforcing these observations, STn overexpression was more frequent in primary tumours of patients with lymph-node metastasis when compared with tumours without metastasis (95% *vs.* 63%, p=0.013). In addition, STn was present in approximately 90% of the correspondent metastases **(Figure 2B and C)**.

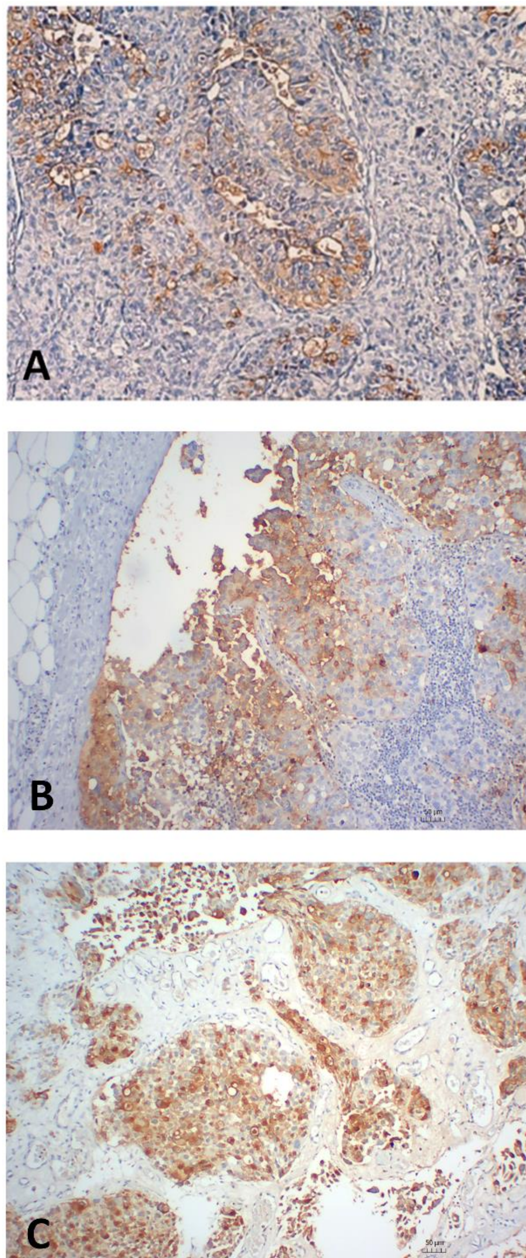

Figure 2. Expression of STn in muscle invasive primary tumours, lymph node and distant metastasis. (A) Representative image of a MIBC tumour showing STn positive cells (brown) in the tumor invasion front and

inside blood vessels, suggesting possible involvement of STn in the metastatic process. **(B)** Representative image of a STn positive lymph node metastasis of a STn positive MIBC. **(C)** Representative image of a STn positive distant metastasis (pelvic) of a STn positive MIBC. Accordingly, over 90% of the metastasis from STn positive MIBC also expresses the antigen.

We have then used an innovative microfluidic system to address the expression of the STn antigen in circulating tumour cells (CTCs) present in the blood of metastatic patients. This lab-on-a-chip device acts as a cell sorter, allowing the retention of cancer cells presenting higher dimensions, irrespectively of their molecular profile, while removing lower dimensions blood cells. Accordingly, entrapped CTCs showing a $DAPI^{+}/CK^{+}/CD45^{-}$ or $CD45^{+}$ phenotype were clearly distinguishable from residual levels of blood cells ($DAPI^{+}/CK^{-}/CD45^{+}$) also present in the device **(Figure 3)**. All cases presented CTC in concentrations that varied from 46-108 cells/mL of blood; moreover, no CTCs could be found in the blood of healthy blood donors used as controls. In addition, the majority of isolated CTC (>90%) also express STn, mimicking the primary tumour and the metastasis **(Figures 3 and 4)**.

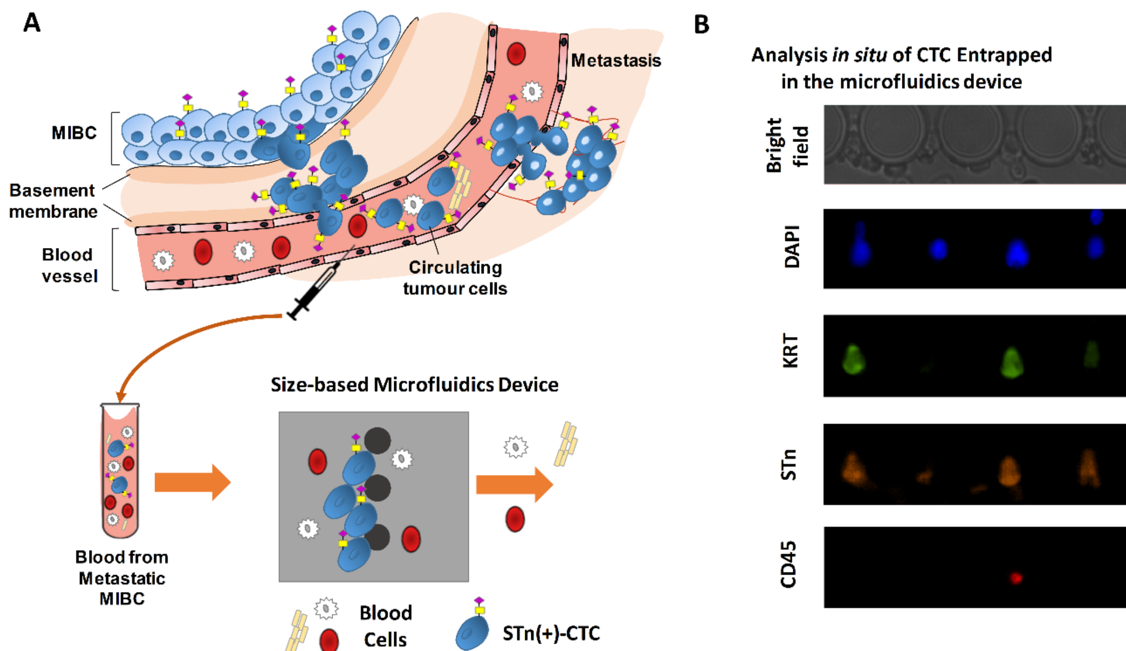

Figure 3. Expression of STn in circulating tumour cells (CTCs). (A) Schematic representation of the microfluidic device used to isolate and characterize circulating tumour cells from peripheral blood of metastatic MIBC patients. Briefly, whole blood was run through a size-based microfluidic device composed of an array of micropillars that act as a molecular sorter. CTC, which are larger than blood cells, are expected to be retained *in situ* while blood cells flow through and exit the device. **(B)** Immunofluorescence analysis of CTCs ($DAPI^{+}/KRT^{+}$ and $CD45^{-}$) trapped inside the device. Most CTCs expressing epithelial markers (pan-KRT in

green) are also positive for the STn antigen (in orange). The hematopoietic marker CD45 (in red) was used as negative control. In summary, these findings demonstrate, for the first time, that MIBC CTCs express the STn antigen.

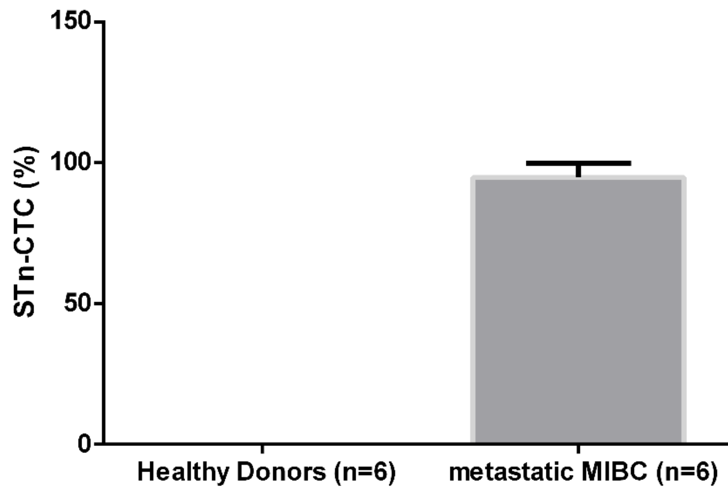

Figure 4. CTCs from metastasized MIBC patients express the STn antigen. As a proof of concept peripheral blood was collected from six metastasized MIBC patients presenting STn positive tumours. Blood from individuals without known neoplasia was used as a control. On average patients presented on average 59 CTC/mL of blood ($DAPI^+/CK^+/CD45^-$ or $CD45^+$; however significant inter-individual variations were observed (CTC counts variations: 46-108 cells/mL; data not presented in the graph). Nevertheless, STn was consistently found in approximately 94% of the isolated CTC in all studied patients. These findings suggest that STn may be a novel CTCs and metastasis marker.

We have then extracted RNA from the cells entrapped in the microfluidics device and were able to amplify *KRT*14 but not *KRT*5 and *KRT*20 in all cases. These observations demonstrate that CTCs present a basal-like phenotype and high degree of undifferentiating, consistent with their highly aggressive nature. Likewise, patients showing STn positive tumours presented a decreased CSS in comparison to STn negative tumours (mean 74 months *vs.* 47 months; log rank, p=0.034, **Figure 5**), albeit STn expression was not associated with any particular molecular subtype. Notwithstanding, presence of STn allowed to discriminate sub-groups of patients facing worst CSS within luminal and basal-like phenotypes. Accordingly, presence of STn in luminal tumours was significantly associated with decreased CSS as compared to STn-negative tumours (log rank, p=0.027, **Figure 6**). Likewise, within the group of patients with basal-like lesions presence of tumour STn was related with decreased CSS compared to those lacking STn (mean 19 months vs.53 months, respectively; log rank, p=0.027). Moreover, it was expressed in CTC as well as in metastatic tissues, irrespective of their *KRT*-phenotype.

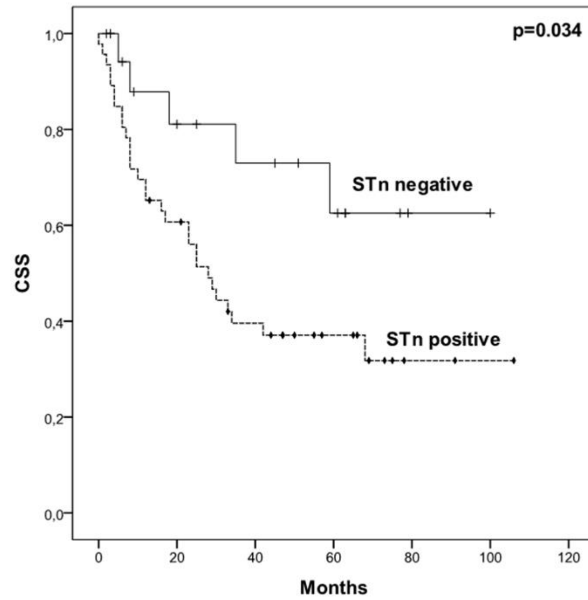

Figure 5. Kaplan–Meier analysis showing the association between STn expression and decreased CSS in MIBC patients. Log-rank test (p=0.034); + censored STn negative tumors; ⬩ censored STn positive tumors.

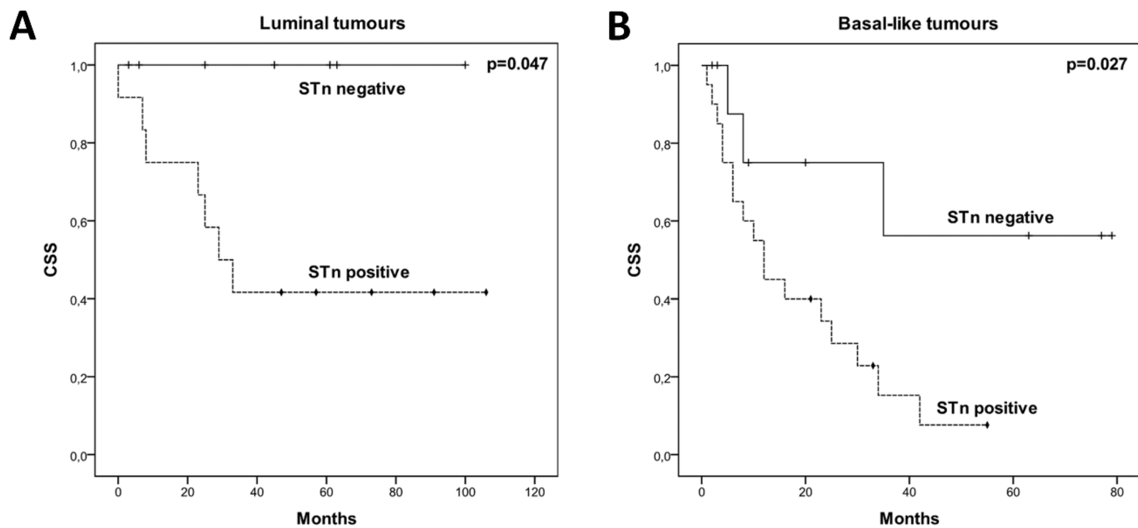

Figure 6 - Effect of STn expression in Cancer-specific survival (CSS) for luminal and basal-like subtypes. Kaplan-Meier analysis to evaluate the association between CSS and STn expression for **(A)** luminal and **(B)** basal-like subtypes. Log rank test, **(A)** p=0.047, **(B)** p=0.027. + censored STn negative tumours; ⧫ censored STn positive tumours.

5. Discussion and concluding remarks

This work supports the potential of *KRT*-based molecular classification for prognostication of bladder tumours and its introduction in clinical setting. Moreover, we report that analysis of STn expression likely improves patient stratification and holds potential for pinpointing sub-populations facing worst prognosis. More importantly, by showing for the first time STn expression in both CTC and metastatic lesions, we present a strong link between the STn antigen, tumour dissemination and metastasis, providing key molecular information to target aggressive bladder cancer cells. These results are in agreement with previous reports showing that STn expression is associated cancer cell aggressiveness features in tumours from different organs and poor prognosis of the patients[14,15].

Given its influence on cell adhesion properties[5,8] cell and immune camouflage[10], STn-positive cancer cells may be endowed with adaptive capabilities that ultimately contribute to establish metastases. However, further studies involving a higher number of patients are required to confirm these findings before application in the clinical practice. Particular attention should be devoted to exploring the molecular nature and the clinical relevance of STn-positive CTC populations, including the possibility of early detection of disseminated disease, prognosis, therapeutic monitoring and non-invasive liquid biopsies on metastatic cells. Finally, the present study highlights the importance of understanding the role of glycans in bladder cancer, which remained poorly explored in comparison to other solid tumours. A comprehensive glycomic and glycoproteomic screening of bladder tumours may provide relevant molecular information envisaging true precision medicine settings. Glycomapping heavily glycosylated glycoproteins, such as CD44, CD49f, and other cancer stem cell biomarkers[1,2] known to carry STn[5,8], may provide key insights for designing more efficient targeted therapeutics.

6. Abbreviations

BSA: bovine serum albumin; CTC: circulating tumour cell; CSS: cancer-specific survival; FFPE: formalin-fixed, paraffin-embedded tissue sections; MIBC: muscle-invasive bladder cancer; PBS: phosphate buffered saline; KRT: keratin; RNA: ribonucleic acid; STn: sialyl-Tn.

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Chapter 4 | Exploring sialyl-Tn expression in microfluidic-isolated circulating tumour cells: a novel biomarker for precision oncology applications

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Exploring sialyl-Tn expression in microfluidic-isolated circulating tumour cells: a novel biomarker for precision oncology applications

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Running head: Explore STn-CTC using microfluidics

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Number of text pages: 36, Number of tables: 1, Number of figures: 6

1. Abstract

Circulating tumour cells (CTC) detaching from the primary tumour, lymph nodes and distant metastasis hold tremendous potential for liquid biopsies by providing a molecular fingerprint for disease dissemination and its

temporal evolution through the course of disease management. Moreover, CTC enumeration, classically defined based on surface expression of epithelial cell adhesion molecule (EpCAM) and absence of pan-leukocyte marker CD45, has shown to correlate with clinical outcome. However, existing approaches introduce bias in the subsets of captured CTC, which may be excluding biologically and clinically relevant subpopulations. Here we explore the overexpression of the membrane protein *O*-glycan sialyl-Tn (STn) antigen in advanced bladder and colorectal tumours, but not in blood cells, to propose a novel CTC isolation technology. Using a size-based microfluidic device, we first show that the majority of CTC (>90%) isolated from the blood of metastasized bladder and colorectal cancers express the STn antigen, reinforcing its pancarcinomic link with metastization. More importantly, STn^{+}CTC counts were significantly higher compared to EpCAM-based detection in colorectal cancer, providing a novel and more efficient cell-surface biomarker for CTC isolation. Exploring this concept, we have then built a glycan affinity-based microfluidic device for selective isolation of STn^{+}CTC and propose an enzymatic-based strategy for the recovery of viable cancer cells for downstream biomedical research. Finally, clinically relevant cancer biomarkers (transcripts and mutations) in bladder and colorectal tumours were identified in microfluidic-isolated cells, confirming their malign origin and highlighting the potential of this technology in the context of precision oncology.

Keywords: Circulating tumour cells, glycosylation, microfluidics, cancer, liquid biopsy, lab-on-a-chip

2. Introduction

Liquid biopsies constitute the cornerstone for real-time assessment of metastasis development and its molecular nature, while overcoming the detection limitations posed by invasive procedures and imaging techniques[1]. Particularly, several reports have demonstrated that circulating tumour cells (CTC) released from the primary tumour harbour genetic and molecular features found in metastatic sites[2, 3]. Despite being one of the driving force of metastasis and cancer-related deaths, CTC are rare, accounting for less than 0.004% of all mononucleated blood cells[4, 5]. Nevertheless, CTC were proven to better predict overall survival than other cancer biomarkers[6], holding tremendous potential for aiding early intervention, therapeutic decision and predicting response to therapy[7].

The gold standard technique for CTC analysis involves affinity enrichment for cell-surface markers followed by detection by flow cytometry[5, 8-10]. Nevertheless, it requires expensive upfront investments

and maintenance, exhibits low recovery and sensitivity rates and does not guarantee the retrieval of viable populations for downstream molecular studies[5, 8-10]. Moreover, this strategy relies on the expression of epithelial proteins, as the epithelial cell adhesion molecule (EpCAM), which is often suppressed or downregulated in circulating and metastatic cancer cells[5, 10-12]. It is also estimated that more aggressive and relevant CTC populations remain uncovered by the use of current detection methods[13]. Moreover, EpCAM-expressing cells have been observed in the bloodstream of patients with benign lesions[14, 15]. As such, the introduction of alternative methods for isolation of these rare cells represents the critical step to generate advances in CTC research. The development of the first silicon-based microfluidic capture device (2007) constituted a break-through in CTC research, offering a unique opportunity to overcome the limitations of available methods, mainly related to cost, processing times and ease of use[16, 17]. Microchips allow the separation of CTC from other blood components based on their physicochemical characteristics, sometimes even without the need of prior processing, but also based on their cell-surface protein expression. In this case, the inner surfaces of the devices are functionalized with antibodies and other ligands, thereby improving binding specificity and CTC purity[4, 18, 19]. Microchips are also a versatile technology, endowing either *in situ* analysis[20, 21] or the isolation of viable cells for downstream molecular studies[22-24]. Several studies, with different chip designs, demonstrated the potential of this technology for basic research and as a clinical tool, allowing to improve CTC counts while providing phenotypic analysis[25-27]. Nevertheless, positive selection of CTC in these devices continues to rely on EpCAM expression[28, 29], and confirmation mostly depend on the DAPI+/CK+/CD45- molecular profile that contrasts with the DAPI+/CK-/CD45+ phenotype of white blood cells28. This significantly diminishes the spectra of captured CTC[5, 11, 12], urging the development of more specific tools. Moreover, CTC identification relies mostly on CKs expression, which are not cancer specific biomarkers, and most studies do not present complementary molecular methods confirming the malignant nature of isolated cells. Similar approaches exploring other cell-surface markers (PSMA30, HER2[31]) have been attempted but significant contamination with blood cells is still common in microfluidic devices[32], hampering their potential clinical application. More recently, the spectra of captured CTC has improved with the introduction of multitarget microchips[33-36]; however the development of microchips targeting more metastasis-specific biomarkers is warranted.

Glycosylation, the most common post-translational modification of cell surface proteins, plays a key role in the modulation of protein functions[37, 38]. Altered protein glycosylation is a hallmark of cancer[37, 39] and, over the last years, the detailed mapping of protein glycosylation by mass spectrometry has increased the specificity and sensitivity of cancer biomarkers[40, 41]. Our group has demonstrated the biological and clinical significance of the cancer-associated glycan sialyl-Tn (STn), which results from

a premature stop in protein *O*-glycosylation in different cancer models[40, 42-44]. Accordingly, STn is expressed by most advanced stage bladder[43, 44], gastrointestinal[40, 42] and colorectal tumours[40], being often related with metastasis[45], while absent or marginally expressed on corresponding healthy tissues[43]. Moreover, the antigen affects cell adhesion, migration, invasion, metastasis and chemoresistance, while contributing to immune escape[40, 43, 46, 47]. Despite these evidences, the expression of STn by CTC, which constitute the driving force of metastasis, remains mostly unaddressed. Based on these insights, we aim to evaluate the potential of STn antigen as a novel cell-surface biomarker for selective CTC detection and enrichment, as well as to propose the first glycan-based microfluidic device for CTC discovery and downstream biomedical applications.

3. Material and Methods

3.1 Patient samples and processing

CTC were isolated from whole blood collected from 17 cancer patients (5 bladder and 12 colorectal). Bladder cancer patients were enrolled at Urology Department, Porto Hospital Centre, St António Hospital (CHP-HSA) in Porto, Portugal, whereas colorectal cancer patients originated from the Portuguese Institute of Oncology—Porto (IPO-Porto) also in Portugal. All patients were under follow-up after surgery at these hospitals at the time of blood collection (2017). Amongst colorectal patients (4 colon and 8 rectum), 50% presented lymph node metastasis and 42% distant metastasis at the time of surgery. Regarding bladder cancer, all patients presented metastasis at the time of blood collection for CTC analysis. **Table 1** summarizes available clinicopathological information at the time of diagnosis. Formalin fixed paraffin embedded (FFPE) tumour tissues from colorectal patients were also included. Blood donors matched in age and gender, and without known neoplasias, were included as controls. Peripheral blood samples were collected following the standard venipuncture technique in EDTA-coated tubes (BD® biosciences, San Jose, CA, USA) for analysis by size-based microfluidic devices. Alternatively, peripheral blood mononuclear cells (PBMC) were separated from the sample, using Ficoll-Paque™ (GE Healthcare, Little Chalfont, UK), density gradient centrifugation, and analysed in the glycan-affinity microfluidic device. All procedures were performed under patient's informed consent, and after approval by the Ethics Committee at CHP-HSA and IPO-Porto. Clinicopathological information was obtained from patient's clinical records.

Table 1. Summarized clinicopathological data for patients enrolled in this study.

Patient	Gender	Age	TNM
Bladder cancer			
#1	M		
#2	M	82	T4N2M1
#3	M	51	T1G3N0M0
#4	M	41	TaG1N0M0
#5	M	62	T3N0M0
Colorectal cancer			
#1	M	70	T3N0M0
#2	M	77	T3N0M1
#3	F	73	T3N0M0
#4	F	72	T3N2bM0
#5	M	68	T3N1cM0
#6	M	69	T3N1M1
#7	F	89	T4N2aM0
#8	M	74	T3N1M0
#9	M	59	T2N0M0
#10	M	69	T2N0M1
#11	F	83	T3N1aM1
#12	M	63	T3N0M1

3.2. Cell models and culture

Metastatic human bladder cancer cell models MCRSTn, overexpressing the STn antigen, and MCRmock, expressing residual levels of the glycan[43], were used for this study. Cell lines were cultured in HyClone Dulbecco's High Glucose Modified Eagles Medium (DMEM; GE Healthcare) with 4,0 mM L-glutamine, and 1,0 mM sodium pyruvate, supplemented with 10% heat-inactivated FBS (Gibco, Life Technologies, Co Dublin, Ireland) and 1% penicillin-streptomycin (10,000 Units/mL penicillin; 10,000 mg/mL streptomycin; Gibco, Life Technologies). Cell lines were cultured as a

monolayer at 37°C in a 5% CO2 humidified atmosphere, and were routinely subcultured after trypsinization (Trypsin, Promega Corporation, Madison, WI, USA).

3.3.Non-targeted microfluidic devices design and fabrication

The non-targeted microfluidic devices used for screening patients CTCs for STn expression were designed to split the sample equally in different analysis areas, containing each a set of filters that isolate CTCs according to their size and deformability, as previously described[48]. Silicon moulds were fabricated using photolithography and deep reactive ion etching to achieve highly anisotropic features, as well as hydrophobized with a vapor phase treatment in trichloro(1H,1H,2H,2H perfluoro octyl)silane (Sigma Aldrich). Replicas were fabricated in Polydimethylsiloxane (PDMS, Ellsworth Adhesives Iberica, Madrid, Spain) and bonded to glass slides using oxygen plasma. Once primed with ethanol, to enhance the wettability, devices were passivated to avoid unspecific attachment. More details on fabrication procedures may be found in a recent patent application[48]

3.4. Glycan-affinity microfluidic devices design and fabrication

An innovative glycan-affinity microfluidic device was specially designed to isolate STn-expressing cells. These devices were fabricated in PDMS (Ellsworth Adhesives Iberica) and comprise an inlet, a cell enrichment area composed of micropillars functionalized with an anti-STn antibody, and an outlet. Masters for PDMS moulding were fabricated using standard photolithography techniques. Cylindrical features with diameters of 100 µm and interspacing of 100 µm were used in a geometric arrangement with a 100 µm shift in every row to maximise mixing. Silicon wafers were cleaned by subsequent sonication steps in acetone and isopropanol for 5 minutes each, dried with nitrogen and activated in oxygen plasma for 5 minutes. Upon activation, the wafers were immersed in 5% GPTMS in ethanol for 1h, rinsed in ethanol and cured at 80ºC for 1 hour. SU8-50 was dispensed on the wafer and spun to achieve a 50 µm thick layer. The wafer was soft baked in 2 steps, first at 65ºC for 10 minutes and secondly at 95ºC for 30 minutes. The wafer was then exposed through a transparency mask (JD tools) to a UV lamp with a dose of 250 mJ/cm2, and postbaked at 55ºC overnight. The sample was developed for 20 minutes and hardbaked ramping from 65ºC to 200ºC. Prior to master replication, the wafer was hydrophobized with a vapour-phase treatment in trichloro 1,1,2,2-perfluorooctyl-silane for 1 hour in a desiccator, and cured for another hour at 80ºC. PDMS was mixed at 1:10 ratio, degassed, poured over the master, degassed again and cured at 80ºC for 2 hours. After curing, the PDMS was unmoulded and the inlet and outlet were punched. Finally, clean glass slides and PDMS replicas were treated with oxygen plasma at low power for 15 seconds and

subsequently brought in contact to produce irreversible bonding.

Upon activation with oxygen plasma, the microfluidic devices were connected to a syringe pump and filled with ethanol. All the functionalization steps were carried out at 100 µL.min−1. After stabilization in ethanol, 2% (3-Aminopropyl)triethoxysilane (APTES) in ethanol was withdrawn into the device for 30 minutes and rinsed in ethanol for 10 minutes. Then, the buffer was changed to MilliQ water and stabilized for 10 minutes prior to withdrawing 1% glutaraldehyde in water for another 30 minutes and rinsing in water for 10 minutes. Sterile PBS was then withdrawn into the device and equilibrated for 10 minutes just before introducing 200 µL of anti-STn monoclonal antibody B72.3 (1:33 in PBS-2% BSA, Abcam, Cambridge, UK) in PBS and left to react overnight at 4 °C. For optimization procedures, devices functionalized with different amount of antibody were produced (0, 5, 15, 33, 50 µg). Unbound antibody was then rinsed with PBS for 10 minutes and the surface blocked with 2% bovine serum albumin (BSA) in PBS and 1% human serum albumin for 1 hour. All reagents were purchased to Sigma Aldrich unless indicated otherwise.

The functionalization process was monitored using a Quartz Crystal Microbalance (OpenQCM, Novaetech, Italy) by measuring variations in frequencies caused by mass variations. A gold sensor was coated with a thin layer of SiO2 for this purpose and used to study the functionalization process. After exposure for 15 seconds in the Plasma cleaner, in low power, the sensor was placed in a flow chamber and subjected to a 100 µL.min-1 flow. After stabilizing with ethanol, 2% APTES in ethanol was pumped until a stability in the measurement was reached for at least 1 minute. After rinsing in ethanol, the buffer was changed to MilliQ water and 1% glutaraldehyde was pumped until stability was reached, them the system was rinsed with MilliQ water. In the final step, PBS was used as buffer and monoclonal antibody B72.3 was inserted in the system and then rinsed.

3.5. CTC analysis using size-based microfluidics devices

An innovative microfluidic device48 was used for CTC isolation based on their higher dimensions a low deformability compared to blood cells. Peripheral blood from metastatic bladder and colorectal cancer patients (3.5 mL) was collected in EDTA tubes (BD® biosciences) and unprocessed samples were run within two hours of collection. After surface passivation, 3.5 mL of unprocessed blood samples were injected though the system at a constant flow rate of 80 µL.min-1, according to previous optimization[48], followed by a washing step with PBS at the same flow rate. Trapped cells were then fixed with formalin (approx. 4% paraformaldehyde, Sigma Aldrich) during 20 min at RT, washed again with PBS and incubated overnight at 4°C with anti-STn clone B72.3 mouse monoclonal antibody (Abcam; 1:33 in PBS-2% BSA), followed by labelling with a secondary goat anti-mouse

IgG-TRITC antibody (Thermo Fisher Scientific, Waltham, MA, USA; 1:500 in PBS-2%BSA) for 1h at RT. Cells were further stained for 1h with DAPI (1:1000, Sigma Aldrich), anti-pan CK-FITC (Sigma Aldrich; 1:50 in PBS-2% BSA) and anti-CD45-CY5 (Abcam; 1:50 in PBS-2%BSA) antibodies, used as nuclear, epithelial and hematopoietic markers, respectively. When appropriate, trapped cells were also probed with Alexa Fluor® 488 anti-human CD326 (EpCAM) antibody clone 9C4 (Biolegend; 1:50 in PBS-2%BSA). Fluorescence microscopy analysis was performed using a Nikon Ti-E inverted fluorescence microscope equipped with a sCMOS camera (Andor Technology Ltd, Belfast, Ireland). Cells presenting DAPI+/CK+/CD45- profile were considered CTC while those presenting a DAPI+/CK-/CD45+ phenotype where identified as normal blood cells28. In addition, cells isolated from blood of a subgroup of colorectal cancer patients (#6-10) were also evaluated for DAPI, STn, EpCAM and CD45, and analysed by fluorescence microscopy as previously described. CTC were then lysed by injection of 300 µL TriPure isolation Reagent (Roche Diagnostics GmbH, Mannheim, Germany) at 80 µl.min-1 and incubated for 30 minutes at 4ºC envisaging nucleic acid isolation. Another 300 µL of TriPure were injected into the devices at higher flow rate (250 µl.min-1) and incubated for 10 min. Chloroform (Sigma Aldrich) was then added to Tripure, and the aqueous phase containing the nucleic acids was isolated for further processing.

3.6.CTC analysis using glycan-affinity microfluidics devices

Glycan-based microfluidic devices, described here for the first time, were validated using model cell lines, prior to the analysis of patient samples. Validation steps included: i) definition of optimal amounts of immobilized antibody; ii) identification of optimal solvent flow rates; iii) estimation of capture yield for STn-expressing cells in model conditions (PBS) and spiked blood. Validation assays were also conducted using peripheral blood from metastatic colorectal cancer patients. Prior to blood samples (3.5 mL) analysis, CTC were pre-enriched together with PBMCs.

For optimization experiments, calcein (Thermo Fisher Scientific) labelled MCRSTn cells were used to determine the ideal antibody amounts for downstream studies. Cell suspensions were injected at a flow rate of 30 µL.min-1, previously described as optimal for affinity microfluidic devices with similar architecture but targeting different antigens[49]. Microfluidic devices functionalized with different amounts of immobilized antibodies were tested (5, 15, 33, 50 µg). Non-functionalized devices were used as controls. Solvent flow rate was also varied to maximize cell isolation yields during washing steps (15, 30, 45 µL.min-1). The affinity of the microchips for STn-expressing cells was determined using MCRSTn and MCRmock cells. Briefly, 10^3 cells were dispersed in 200 µL PBS and injected into the devices. Human PBMCs (10^6 cells) that do not express STn were used as controls.

Cell entrapment was estimated by fluorescence microscopy. Then, PBMCs from donors without known neoplasias were spiked with different amounts of both MCRSTn and MCRmock (10^2, 10^3, 10^4) cells, in an attempt to mimic CTC found in metastatic cancer patients. Cancer cells were isolated together with PBMCs from whole blood and processed as described above. Finally, the blood of a subset of colorectal cancer patients (#10-12) was also analysed according with the optimized conditions. Entrapped cells were probed for DAPI, panCK, STn and CD45, as previously described for non-functionalized microfluidic devices. Colorectal CTC were then released from the affinity device with a α-sialidase from Clostridium perfringens (10 U/mL, Sigma Aldrich) for 30 and 120 minutes at 37°C, under stationary conditions or by continuous injection of the enzyme at a flow rate of 10 μL.min-1. Released cells were lysed for DNA isolation for downstream mutation analysis by Droplet Digital™ PCR (ddPCR™) Technology (BioRad, Hercules, CA, USA). All experiments were performed in triplicate and presented results correspond to the average of at least two concordant replicates.

3.7.Immunohistochemistry for the STn antigen

Formalin-fixed, paraffin-embedded tissue sections (FFPE) were screened for STn by immunohistochemistry using the strepavidin/biotin peroxidase method, as described by Ferreira et al.43. Briefly, 3 μm sections were deparaffinized with xylene (VWR, Radnor, PA, USA), rehydrated with graded ethanol series (Merck Millipore, Burlington, MA, EUA), microwaved for 15 min in boiling citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0; Vector Laboratories, Burlingame, CA, USA), and exposed to 3% hydrogen peroxide (Merck Millipore) for 20 minutes. STn expression was performed using clone B72.3 mouse monoclonal antibody (Abcam) at a dilution of 1:100 in PBS, after overnight incubation at 4°C. After blockage with BSA (5% in PBS), the antigens were identified with UltraVision HRP Detection System Kit (Thermo Fisher Scientific) followed by incubation with 3,3-diaminobenzidine tetrahydrochloride (Impact Dab, Vector Laboratories) for colour development. Finally, the slides were counterstained with haematoxylin for 1 minute. Negative control sections were performed by adding BSA (5% in PBS) devoided of primary antibody. The immunoassayed sections were blindly assessed using light microscopy by two independent observers and validated by an experienced pathologist. Disaccording readings were re-analysed using a double-headed microscope and consensus was reached. Given the absence of STn in the healthy urothelium43, tumours were classified as positive for these antigens when membrane and/or cytoplasmic immunoreactivity were observed in more than 5% of the tumour.

3.8.Flow Cytometry for the STn antigen

MCRSTn, MCRmock and blood cells were screened for STn expression by flow cytometry with anti-STn mouse monoclonal antibody clone B72.3, as described by Peixoto et al.[47]. Approximately 10^6 adherent MCRSTn and MCRmock cells were converted into single cell suspensions using Versene (Gibco, Life Technologies) at 4ºC, followed by filtration using a 70 µm Nylon cell strainer (BD Falcon, BD® biosciences). A minimum of 10^5 cells were analysed by flow cytometry. The cell population in this study was gated based in FSC and SSC features to avoid background and debris (corresponding to > 95% of the measured cells in each sample). Cells digested with α-sialidase from Clostridium perfringens (10 U/mL, Sigma Aldrich) for 90 min at 37°C, were used as negative controls. This procedure was responsible by hydrolysing the sialic acid in STn, impairing recognitions by B72.3 monoclonal antibody50. Experiments with mouse IgG1 [ICIGG1] Isotype Control (Abcam) were further included as negative controls. Polyclonal rabbit anti-mouse immunoglobulins/FITC (DAKO, Agilent technologies, Santa Clara, CA, USA; F0313) was used as secondary antibody. Two independent experiments were performed in triplicate for each cell line. Whole blood samples were used and labelled with anti- STn, CD45-PO (Life Technologies), CD3-v450, CD14-APC (BD Biosciences), and CD19-PE Cy7 (Beckman Coulter) monoclonal antibodies, to assess different subpopulations of white blood cells. Samples were lysed in BD FACS Lysing solution (BD Biosciences) after staining and analysed in NAVIOS flow cytometry (Beckman Coulter). Results were analysed with the Infinicyt software (Cytognos S.L.). The percentage of STn positive cells was defined as cells with a fluorescence intensity above the Isotype Control. Blood samples from five healthy male individuals' ages between 45 and 60 (median: 52 years) were analysed.

3.9.Real-time PCR for KRT14, 5 and 20 expression in bladder CTC

Total RNA from bladder CTC entrapped in the microfluidics device was extracted from nucleic acid-enriched aqueous fractions using RNA FFPE kit (Agilent technologies). The purity and quantity of RNA extracts were determined based on the A260/A280 ratio using Nanodrop ND1000 (Nano Drop Technologies Inc., Wilmington, DE, USA). Only samples with ratios between 1.9 and 2.1 were considered for downstream molecular studies. RNA (2µg) was reverse transcribed with random primers, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The amplification conditions were the following: 25°C for 10 minutes, 37ºC for 120 minutes and RT inactivation at 85°C for 5 minutes. Two non-template negative controls were used. The products were amplified in a Mycycler Thermal cycler (BioRad). Gene expression levels of KRT5

(Hs00934200_g1), KRT14 (Hs00559328_m1) and KRT20 (Hs00300643_m1), including three reference genes (B2M: Hs00984230_m1, HPRT: Hs99999909_m1, and ActB: Hs99999903_m1) were analysed using a TaqMan Gene Expression Master Mix and TaqMan assays (Applied Biosystems). Real-time PCR amplification of cDNA samples was performed in a ABIPRISM 7500 Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were 10 minutes at 95°C followed by 45 cycles of 15 sec at 95ºC and 1 minute at 60°C. All samples were run in duplicate. Relative mRNA gene expression was calculated with the $2^{-\Delta Ct}$ formula.

3.10. ddPCR for APC mutation detection in colorectal CTC

DNA from colorectal CTC, corresponding primary tumours and metastasis, whenever available, were screened for APC wild-type (WT) and APC c.4348C>T mutation (MT) DNA. CTC DNA was isolated from nucleic acid enriched fractions isolated from microfluidics devices, whereas DNA from primary tumours and metastasis was extracted from FFPE tissue sections available at IPO-Porto biobank. DNA was isolated using QIAamp® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and quantified on a Qubit fluorometer using the dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific). DNA was diluted up to 65 ng/µl using MilliQ water. For each assay, no template controls (NTC) were used, and wild-type-only (WT-only) samples were used in order to estimate false-positive rates. Each droplet of a PCR supermix (Bio-Rad) reaction contained: 1× droplet PCR supermix, 250 nM of each probe, 900 nM primers and 65 ng of non-digested genomic and CTC DNA, in a total reaction volume of 20 µL. The following PrimePCR ddPCR™ Mutation Assay (BioRad) was used: APC WT for p. R1450* (assay ID: dHsaCP2500509) and APC p.1450* (assay ID: dHsaCP2500508). Droplets were generated and analysed using the QX100 system (Bio-Rad). Amplifications were performed using the following conditions: 1 cycle of 95 °C for 10 minutes, 40 cycles of 94 °C for 30 s and 55 °C for 1 minutes, and 1 cycle of 98 °C for 10 minutes. All samples were run in duplicates. QuantaSoft analysis software (Bio-Rad) enabled fractional abundance to be calculated for each sample. To ensure experiment quality, samples with total droplet counts lower than 10,000 were considered invalid and excluded from the analysis. Positive control samples were used to verify assay performance and facilitate the establishment of thresholds. Additionally, positive control samples were validated by comparing the fractional abundance (FA) in FFPE samples to NGS mutation frequencies. False-positive rate estimation was determined by performing 5 experiments for each assay using the WT-only samples, where total amounts of detected MT-positive droplets determined thresholds above which positive droplets in patient samples were to be considered as true positive.

3.11. Statistical Analysis

Statistical data analysis was performed with IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0) and Graphpad prism 7 (GraphPad Software, Inc.). Differences between continuous variables among the evaluated groups were accessed by Mann-Whitney non-parametric test for independent samples. Spearman’s nonparametric bivariate test was used for correlation estimate. Differences were considered significant when $p<0.05$.

4. Results and discussion

The identification of novel CTC biomarkers capable of overcoming the limitations presented by current CTC isolation technologies are warranted to pave the way for novel targeted technological and clinical applications. The STn antigen, expressed at the cell-surface of advanced stage tumours and generally associated with disease dissemination, may hold potential in this context; nevertheless, its expression by CTC has yet to be fully demonstrated. Herein, we have addressed the expression of STn in CTC from patients with distinct advanced cancers, envisaging the necessary molecular rational for precision medicine applications. A schematic representation of the experimental design used in this study is represented in **Figure 1**.

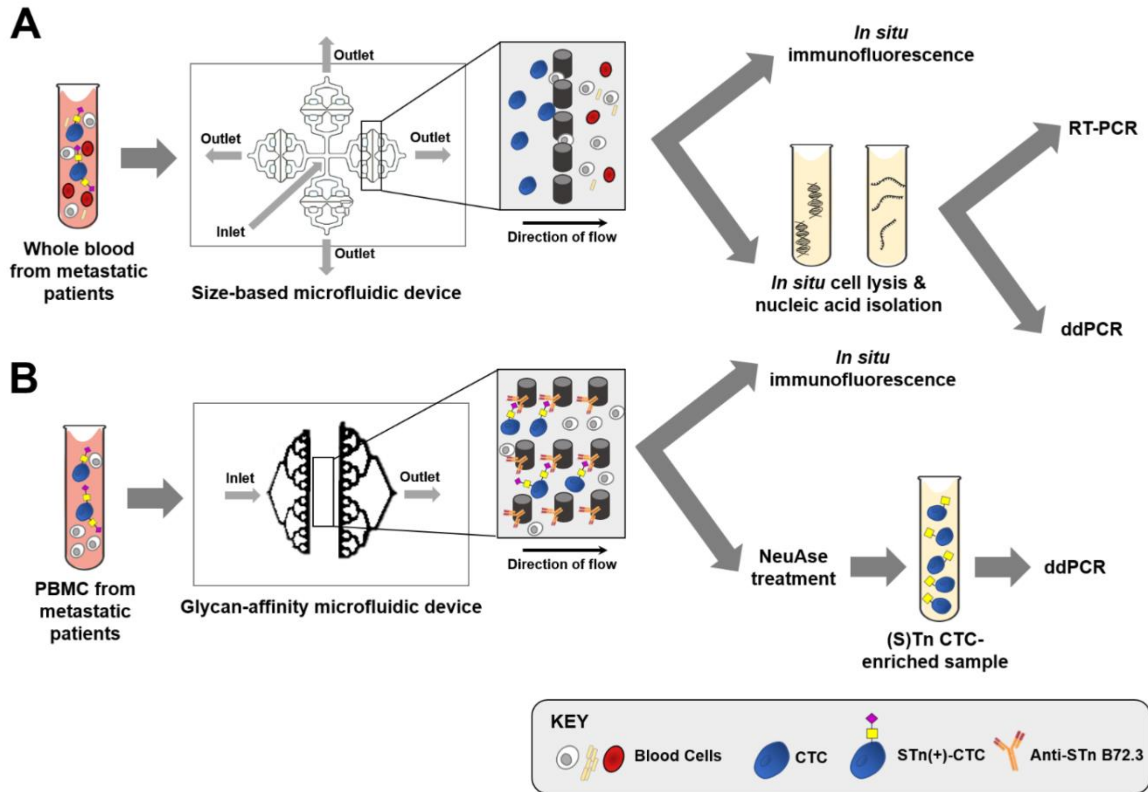

Figure 1. Schematic representation of the experimental design. **A)** Analysis of Total CTC using non-targeted size-based microfluidics devices. Whole blood from metastatic bladder and colorectal cancer patients was processed in non-targeted, size-based affinity devices. The device is composed of an inlet, a separation section presenting a micropillar architecture capable of retaining cancer cells based on their higher dimensions in comparison to blood cells, and an outlet. Cells were stained with DAPI and analyzed *in situ* by immunofluorescence for CTC biomarkers (PanCK, STn) and hematopoietic cells (CD45). Cell enumeration was performed by immunofluorescence microscopy. In addition, entrapped cells were lysed and RNA or DNA isolated for downstream transcript (RT-PCR) and mutation analysis (ddPCR™), respectively. **B)** Analysis of STn-positive CTC using glycan-affinity microfluidic devices. MCRSTn bladder cancer model, overexpressing the STn antigen, and MCRmock, showing residual level of STn, were used for validation purposes. PBMCs were recovered from whole blood of metastatic bladder and colorectal cancer patients, mixed with MCR cells and processed in glycan-affinity microfluidic devices functionalized with anti-STn B72.3 monoclonal antibody. Cells were analyzed *in situ* by immunofluorescence as described above. In addition, STn-CTC were selectively released by sialidase treatment for downstream molecular analysis. Scale bar for microscopy images is 20 µm.

Our group has previously demonstrated that all metastatic bladder tumours and corresponding metastasis overexpress the STn antigen[45]; however, such a correlation is not clear for colorectal cancer. Therefore, as a proof of concept, we have addressed STn antigen expression in colorectal (n=12) tumours and corresponding metastasis, where the prevalence of this antigen was found associated with

poor prognosis[40, 43, 44]. All histological sections presented malignant cells expressing the STn antigen at the plasma membrane **(Figure 2).** Cytoplasm staining was also observed, mostly likely derived from glycoproteins in secretory pathways, thus in accordance with previous observations[43, 51]. More importantly, all tumours presented STn positive lymph node and/or distant metastasis, irrespectively of their location (lung, liver). These observations support that STn expression in primary colorectal tumour sites are reflected in the metastasis, as previously reported for bladder cancer[45]. Moreover, it is in agreement with previous studies demonstrating that STn expression may play a key role in disease dissemination[41]. Together with previous reports[52], these observations support the pancarcinomic nature of this glycan.

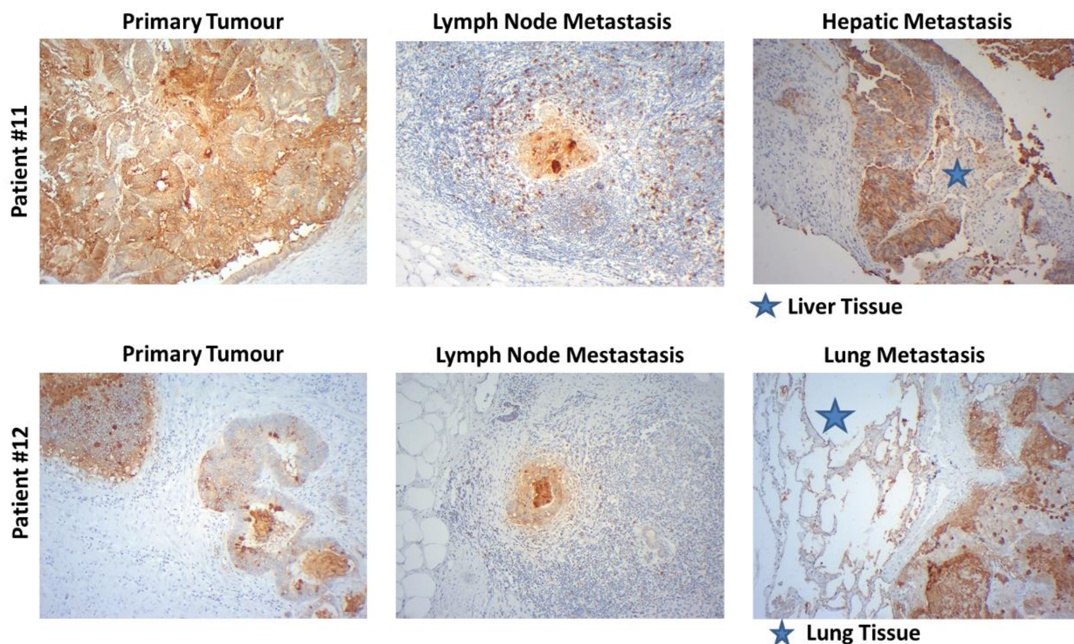

Figure 2. STn expression in colorectal primary tumours and corresponding lymph node and distant metastasis. All metastatic cancer patients enrolled in this study (#1-12) presented STn positive tumours. Moreover, the corresponding locoregional and distant metastasis (whenever available) were also positive. This Figure highlights findings for patients #11 and #12 at a 100x magnification.

In parallel, whole blood was collected from colorectal cancer patients (n=12) and the presence of STn at the membrane of isolated CTC investigated, as previously observed for bladder cancer[45]. To further validate our previous findings, peripheral blood from an independent set of bladder cancer patients (n=5) was also analysed. CTC were sorted from unprocessed blood using a label-free microfluidic device capable of retaining cells based on their size and deformability. These physical characteristics are distinct between CTC and blood cells, i.e. CTC display higher dimensions and are

less deformable[8], allowing a successful isolation and enrichment of CTC in these systems, while minimizing leukocyte contamination8. Moreover, this non-targeted approach allows isolation of CTC irrespectively of their molecular profile, thus enabling the capture of distinct, heterogeneous and clinically relevant CTC subpopulations for downstream molecular studies. Captured cells were then probed *in situ* with DAPI and screened for panCKs, CD45, and STn antigen by immunofluorescence. The absence of CK expression and the presence of the hematopoietic marker CD45 was the criteria used to distinguish CTC (DAPI+/CK+/CD45-) from normal blood cells (DAPI+/CK-/CD45+), as reported in multiple studies[14, 53, 54]. Notably, CTC were detected in all patient samples regardless of their origin **(Figure 3A)**; more importantly, CTC were not observed in the blood of controls without known neoplasia matched in age and gender, supporting the pathological nature of the cells found in cancer patient samples. Moreover, trapped CTC were found to be similar in size, displaying homogenous dimensions from 15 to 20 µm. A similar CTC size range was observed by others using a label-free microfluidic technology[55]. Occasionally, few cells presenting a maximum diameter of 10 µm could also be detected, together with residual (<0.1% of total cells) white blood cells (DAPI+/CK-/CD45+). Noteworthy, we have observed CTC in two colorectal cancer samples without imagiological evidences of metastasis, and several samples (n=5) showing just locoregional, but not systemic disease. Such findings suggest that this approach may be useful for detecting disease not yet fully disseminated, providing the necessary means for early intervention. Remarkably, the majority of CK+CTC also expressed the STn antigen (>95%; **Figure 3A and 3B**), in clear contrast to leukocytes; complementary analysis by flow-cytometry corroborated the absence of STn in blood cells **(Figure 4)**. STn signal specificity was confirmed by the loss of fluorescence upon sialidase incubation (data not shown). In summary, our results demonstrate that STn antigen is highly expressed in CTC from distinct tumour types, in mimicry of the primary tumour and corresponding metastasis. Altogether, this data highlights the potential of STn as a novel CTC biomarker for improved CTC detection and targeting.

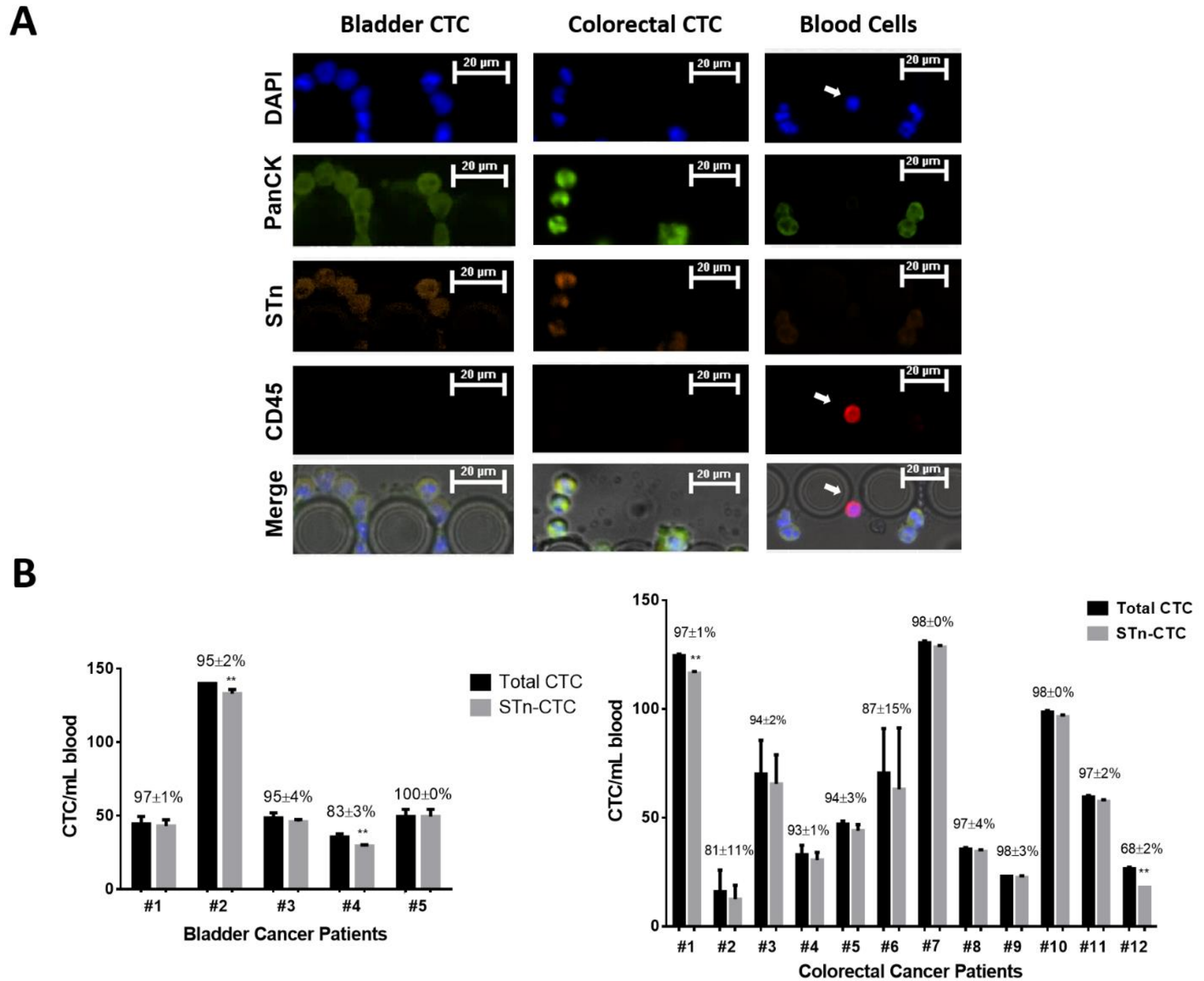

Figure 3. STn expression in bladder and colorectal CTC isolated using non-targeted size-based microfluidic devices. Whole blood from patients with metastatic bladder (n=5) and colorectal tumours (n=10) was processed using non-targeted size-based microfluidic devices, which sort CTC from blood cells based on their higher dimensions. Entrapped cells were then probed for DAPI (blue), panCK (green), STn antigen (orange), CD45 (red) and analyzed by immunofluorescence microscopy. A) Immunofluorescence staining pattern of isolated CTC for each type of tumour as well as blood cells. CTC were identified according to the following criteria: DAPI positive, panCK positive and CD45 negative. CTC were therefore distinguishable from blood cells, which presented a DAPI positive, panCK negative, CD45 positive phenotype (highlighted by a white arrow in the right panel). The left panel highlights the predominance of STn in relation to EpCAM-positive CTC. The middle panel highlights STn+/EpCAM+ CTC subpopulations, whereas the right panel highlights STn+/EpCAM- CTC, which constitute the majority of isolated CTC. **B)** Total CTC/mL vs STn-CTC/mL of blood enumeration plots for bladder and colorectal cancer patients. Total CTC for bladder cancer: 36-140 CTC/mL of blood (median: 49 CTC/mL); Total CTC for colorectal cancer: 27-131 CTC/mL of blood (median: 53 CTC/mL). The vast majority of isolated CTC in both models expressed the STn (STn-CTC/Total CTC percentage is presented on top of each column). For bladder cancer: 83-100% (median: 95%).For

colorectal cancer: 68-98% (median: 96%). The results correspond to the average of at least two independent experiments and three measurements. “**” $p < 0.01$. Scale bar for microscopy images is 20 µm.

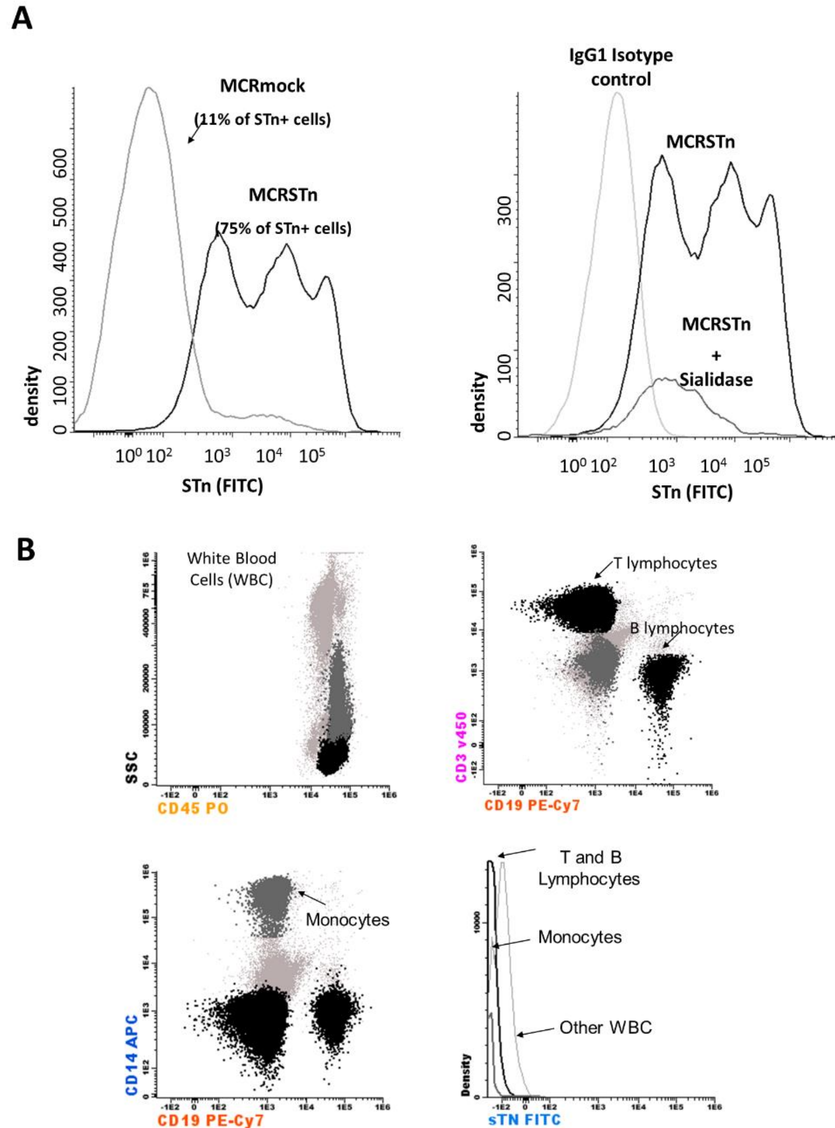

Figure 4. A) STn antigen expression in MCRCSTn, MCRmock and PBMCs isolated from the blood of healthy donors. Approximately 75% of MCRSTn cells expressed the STn antigen, contrasting with the 11% presented by MCRCmock. PBMCs did not express STn. The MCRCSTn cells were significantly sensitive to sialidase treatment, confirming the specificity of the signal. **B) STn expression in different populations of human blood cells.** All cell populations ($CD45^+$) were negative for the STn antigen using the B72.3 monoclonal antibody.

4.1. STn vs EpCAM expression in colorectal CTC

EpCAM remains the most explored biomarker of CTC and the basis for EpCAM-based CTC enrichment methods[28, 29]. This includes CellSearch, the only FDA-approved immunoaffinity method extensively explored for the use of CTC enumeration for colorectal cancer prognosis10. Prompted by our previous observations of high STn expression in CTC, we next investigated and compared the presence of STn and EpCAM at the surface of CTC on five colorectal cancer patients (#6-10). Of note, the percentage of STn^{+}CTC was, on average, 3 times higher than EpCAM+CTC for all cases analysed **(Figure 5B)**, suggesting that EpCAM^{+}CTC may be a minor subset in relation to the total CTC population present in the blood of metastatic patients. Nevertheless, CTC positive for both STn and EpCAM could also be observed **(Figure 5A, middle panel)**, which reinforces CTC plasticity and molecular heterogeneity[56, 57]. These findings further support the concept that targeting EpCAM may significantly hinder access to clinically relevant CTC subpopulations and decrease the sensitivity of current detection and isolation methods

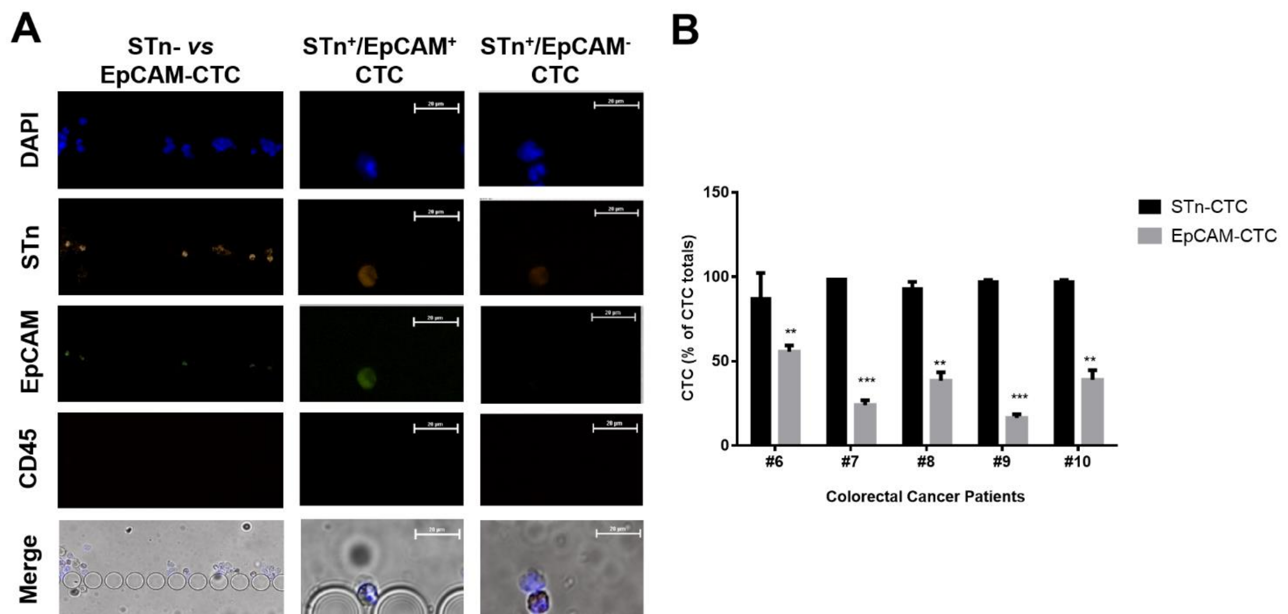

Figure 5. STn vs EpCAM expression in colorectal CTC isolated using non-targeted, size-based microfluidic devices. EpCAM has been the gold standard membrane biomarker for accessing and enriching for CTC in colorectal tumours whereas STn is showing to be widespread expressed in CTC. Accordingly, whole blood from patients with metastatic colorectal tumours (n=5; labelled #6-10) were processed using non-targeted size-based microfluidics devices, which sort CTC from blood cells based on their higher dimensions. Entrapped cells were then probed for DAPI (blue), STn antigen (orange), EpCAM (green) and CD45 (red) and analyzed by immunofluorescence microscopy. A) Immunofluorescence staining pattern of isolated CTC highlighting the predominance of STn-CTC in

relation to EpCAM-CTC. The left panel highlights the higher density of STn expressing CTC in relation to those expressing EpCAM. The middle panel shows the existence of CTC subpopulations expressing both STn and EpCAM, denoting heterogeneous populations. The right panel highlights STn+/EpCAM- CTC. **B)** Percentage of STn- vs EpCAM-CTC in relation to total CTC for colorectal cancer patients #6-10. The percentage of STn-CTC is, on average, 3 times higher than EpCAM-CTC, suggesting that EpCAM-CTC may be a minor subset in relation to total CTC present in the blood of metastatic patients. “**” $p < 0.01$; “***” $p < 0.001$

4.2. Molecular characterization of bladder and colorectal CTC

We have then devoted to confirming the malignant nature of CTC isolated in non-targeted microfluidic devices, based on complementary transcripts and genetics analysis.

For bladder cancer, the STn antigen was shown to define worst prognosis in advanced tumours presenting a basal phenotype, characterized by KRT14 and/or KRT5 overexpression and KRT20 downregulation, which was also mimicked by the metastasis[45]. This contrasted with better prognosis presented by STn- and luminal tumours (KRT14- and/or KRT5- and KRT20+ phenotype)[45]. Building on these findings, we have isolated RNA from bladder cancer CTC samples to assess KRT14, KRT5 and KRT20 genes expression by RT-PCR. We observed that bladder cancer CTC significantly upregulated KRT14 in comparison to KRT5 and KRT20 **(Figure 6A)**, similarly to CTC isolated from blood of an independent set of metastatic bladder cancer patients[45]. The KRT14+/KRT5-/KRT20- phenotype exhibited by CTC is characteristic of basal bladder cancer cells exhibiting a highly dedifferentiated, more aggressive nature, frequently associated to therapy resistance.

A different strategy was pursued to confirm the malignant origin of CTC isolated from blood of colorectal cancer patients. In this case, colorectal CTC, the corresponding primary tumours and distant metastasis (whenever available) were screened for APC mutation c.4348C>T, a frequent early stage mutation in these cells[58]. Using a cohort of 11 metastatic cancer patients (#2-12), we found that the mutation was present in 58% of the primary tumours, 67% of CTC and 75% of available metastasis **(Figure 6B)**. Moreover, there was a correlation between the mutation status in the primary tumour and CTC ($p = 0.04$). Likewise, a match between the CTC and metastasis was observed, although with no statistic correlation most likely due to the low number of samples analysed. These findings may reflect the molecular nature of STn^{+}CTC, which constitute most of the isolated CTC, highlighting the potential of microfluidic chips for further downstream molecular analysis, including evaluation of CTC mutational status.

In summary, these observations support the malignant phenotype of isolated bladder and

colorectal CTC, most likely expressing the STn antigen. Furthermore, our results reinforce the potential of exploring CTC-isolation microchips beyond cell enumeration towards molecular analysis.

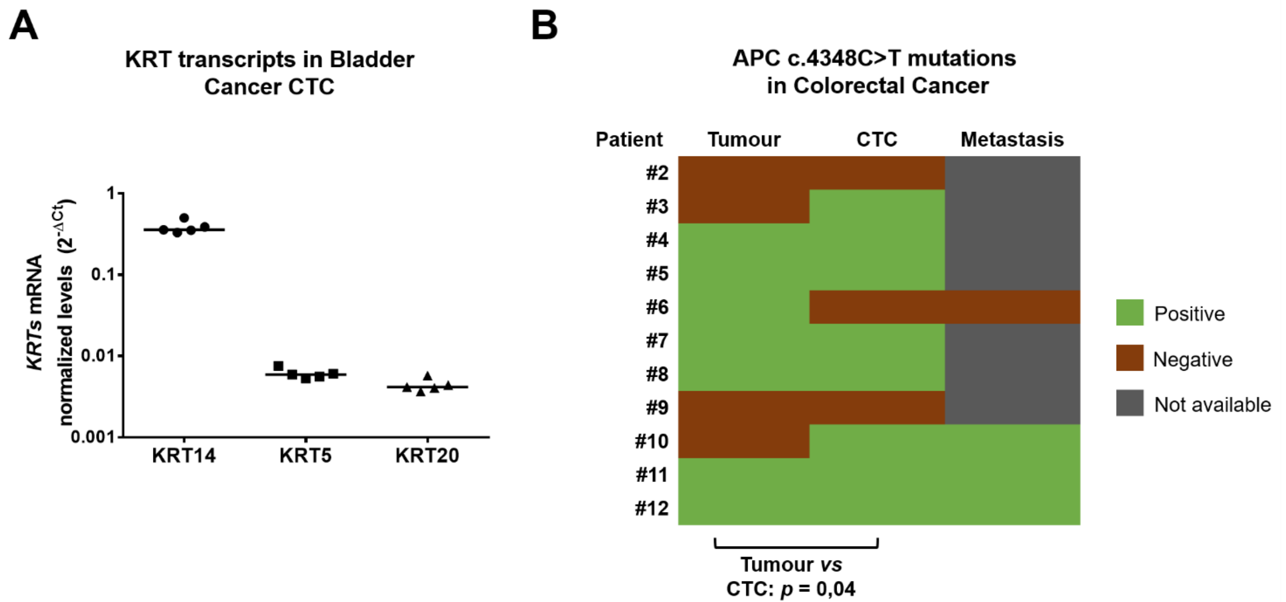

Figure 6. Transcripts and Mutation analysis in CTC isolated using non-targeted, size-based microfluidic devices. CTC isolated in microfluidic devices were hydrolyzed *in situ* for RNA (bladder CTC) and DNA (colorectal CTC) extraction and downstream molecular analysis. **A)** KRT14, KRT5 and KRT20 transcripts in bladder cancer CTC. More aggressive and potentially metastatic bladder cancer cells generally present the KRT14+ and/or KRT5+, KRT20- phenotype (basal), which contrasts with the KRT14- and/or KRT5-, KRT20- phenotype (luminal) generally present by tumour cells in lesions facing better prognosis. Here, we demonstrate that bladder cancer CTC exhibit a basal phenotype, consistent with their more aggressive nature. It is possible that this may reflect the molecular nature of STn-CTC, which constitute the majority of the isolated CTC. Moreover, it demonstrates the potential of exploring microfluidic devices for CTC transcripts analysis. **B)** Analysis of APC c.4348C>T mutations in primary tumours, colorectal CTC and corresponding metastasis, whenever available. The analysis of APC c.4348C>T, a colorectal cancer driver mutation, in a cohort of 11 metastatic cancer patients (#2-12), demonstrates that the mutation is present in 58% of the primary tumours, 67% of CTC and 75% of available metastasis. There is a correlation between the mutation status in the primary tumour and CTC (p = 0.04; Spearman's nonparametric bivariate correlation). There was also a match between the CTC and the metastasis; however, the low number of sample did not allow to determine a correlation. These findings may reflect the molecular nature of STn-CTC, which constitute the majority of the isolated CTC and the potential of exploring

microfluidic devices for CTC mutation analysis.

4.3. Glycan-based immunoaffinity microfluidics for STn-CTC analysis

Having established the STn antigen as a CTC biomarker in bladder and colorectal cancers, we have further devoted to the development of a glycan-affinity microfluidics device for specific enrichment of STn^{+}CTC. Accordingly, we have prepared a high-surface area microfluidic device, containing thousands of microposts closely arranged to maximize cell-surface interactions, and functionalized its inner surfaces with anti-STn B72.3 monoclonal antibody. Based on the surface area of the device and the availability of binding sites created in our functionalization strategy, the minimum amount of antibody required to produce a monolayer was estimated to be 5 µg. Therefore, microchips were functionalized with 5, 15, 33, and 50 µg of antibody for subsequent optimization experiments aiming to maximize cell entrapment. Functionalization efficiency was confirmed using a quartz crystal Microbalance (**Figure 7**).

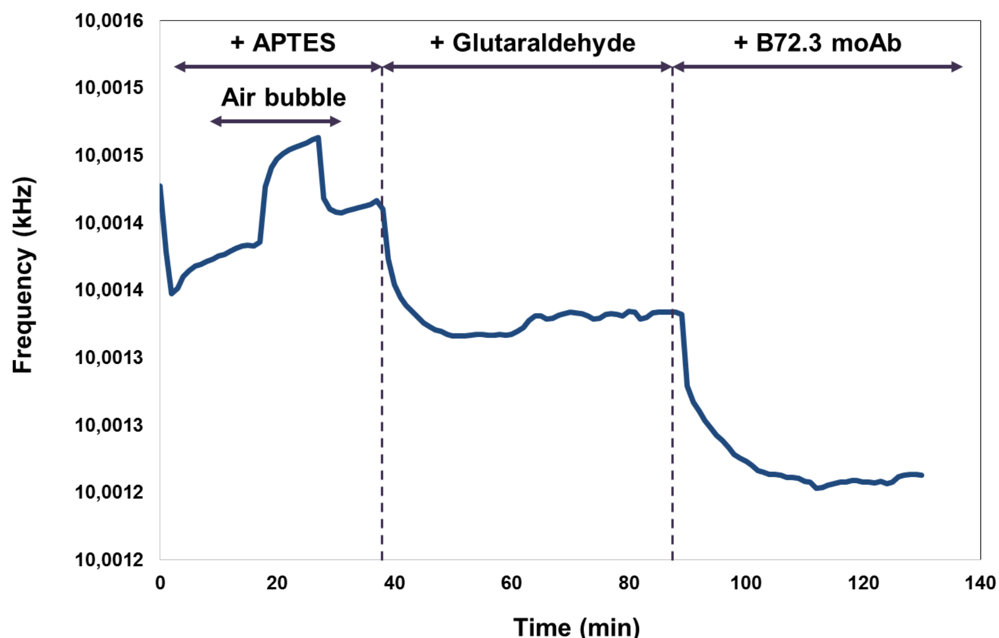

Figure 7. Evaluation of the functionalization of Glycan-affinity microchips using Quartz Crystal Microbalance. After addition of each of the functionalization reagents, the frequency decreased, and remained in the surface upon washing steps, confirming the success of the functionalization strategy.

Antibody-free devices were used as controls to provide insight on unspecific cell binding to the surface of the chips. Optimization tests were conducted using the bladder cancer cell model MCRSTn, transduced to overexpress the STn antigen **(Figure 4)**. MCRSTn cells (10^3 cells) were first labelled with calcein for better visualization and injected into the device at a flow-rate of 30 µl min^{-1}, which had been previously reported to maximize the number of entrapped cells in similar applications[59]. Accordingly, we have concluded that 15 µg of antibody was the minimum amount required for achieving maximum cell yields **(Figure 8)**.

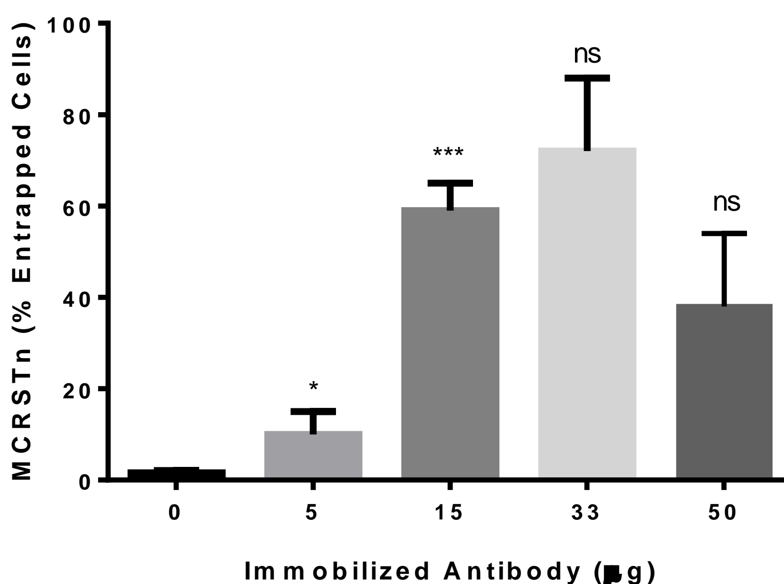

Figure 8. Optimization of the amount of antibody for glycan-affinity microfluidic devices functionalization. Briefly, glycan-affinity devices were functionalized with different amounts anti-STn monoclonal antibody B72.3 (0, 5, 15, 33, 50 □g). Maximum MCRSTn cells entrapment (approximately 59%) was observed for 15 mg of immobilized antibody. "ns": not significant; "*" $p < 0.05$; "***" $p < 0.001$.

Moreover, we have observed significantly low percentages of cell entrapment in non-functionalized devices (<2% of injected cells), suggesting the high specificity of cell to antibody binding. Similar results were found for systems functionalized with an IgG1 isotype control (data not shown), further reinforcing the chips specificity for STn-expressing cells. Therefore, devices functionalized with 15 µg of antibody were elected for subsequent experiments. Flow rates of 15, 30 and 45 µL.min^{-1}, were also tested for the washing step. Based on these experiments, the optimal flow for maximizing MCRSTn cells entrapment was 30 µL.min^{-1} **(Figure 9).**

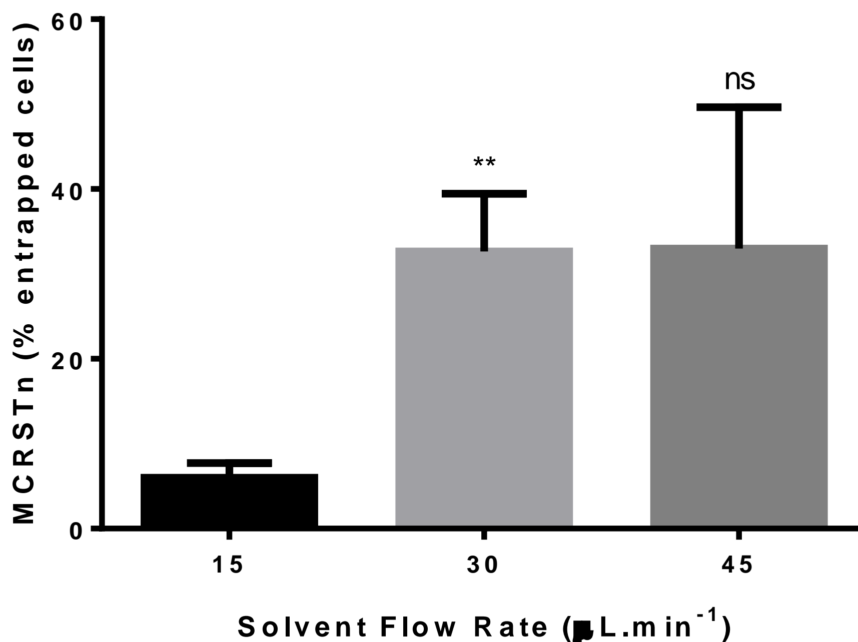

Figure 9. Flow rate optimization for glycan-affinity microfluidic devices. Briefly, different flow rates (15, 30, 45 μL.min^{-1}) were tested for the washing step in order to maximize MCRSTn cell yield. Maximum yield was observed for 30 mL.min^{-1} flow rate. "ns": not significant; "**" $p < 0.01$

We have then explored both MCRSTn and MCRmock (expressing low levels of STn antigen) cell models to assess the affinity of functionalized devices for cells expressing different levels of STn. **Figure 10A** illustrates that functionalized microchips primed with MCRSTn showed twice the number of captured cells in comparison to those with MCRmock, translating their higher STn levels. In addition, we observed that glycan-affinity devices were capable of capturing MCRSTn cells displaying a wide variation in size (<5-20 μm) **(Figure 11)**. Such observations highlight the potential of this tool for accessing CTC subpopulations spanning considerably different dimensions, in contrast to size-based microchips which frequently have underrepresented smaller CTC. In addition, we have isolated and injected 500,000 PBMC previously isolated from blood of healthy donors into glycan-affinity devices to evaluate possible unspecific binding of blood cells to the chip surface, as PBMC lack STn expression **(Figure 4)**. Accordingly, we observed residual PBMC (<0.5%) inside the devices, supporting the higher affinity and specificity of these functionalized microfluidic chips for STn expressing cells. In an attempt to mimic conditions found in patients, PBMC were next spiked with different amounts of calcein-labelled MCRSTn and MCRmock cancer cells (10^2,10^3,10^4 cells) and subsequently run through glycan-affinity microfluidic devices. The obtained results highlighted a significant increase in MCR cell entrapment with cell concentration **(Figure 10B)**, and further confirm

the affinity of these chips for capturing malignant STn expressing cells.

Finally, we have analysed in parallel the blood of three colorectal cancer patients (#10-12) with size-based and glycan-affinity microfluidics devices. Glycan-affinity microfluidics devices provided higher CTC counts in comparison to size-based devices for all cases **(Figure 12)**, suggesting a higher sensitivity of glycan-affinity devices in comparison to size-based solutions. Moreover, we observed that captured STn+CTC on all studied cases presented highly heterogeneous dimensions, 10-20 μm diameter **(Figure 12)**, in agreement with the observations for model **solutions (Figure 11)**. This clearly contrasted with the higher and more homogenous range of diameters presented by CTC entrapped in size-based microfluidics devices (15-20 μm), and may account for the higher cell counts exhibited by glycan-affinity devices. Moreover, it is in agreement with previous reports demonstrating that the same patient may exhibit CTC spanning from 4-30 μm diameter[60]. These observations suggest that glycan-based microfluidic devices may further broaden the spectra of captured CTC by enabling capture of cells presenting smaller dimensions, which may ultimately be excluded by size-based microchips.

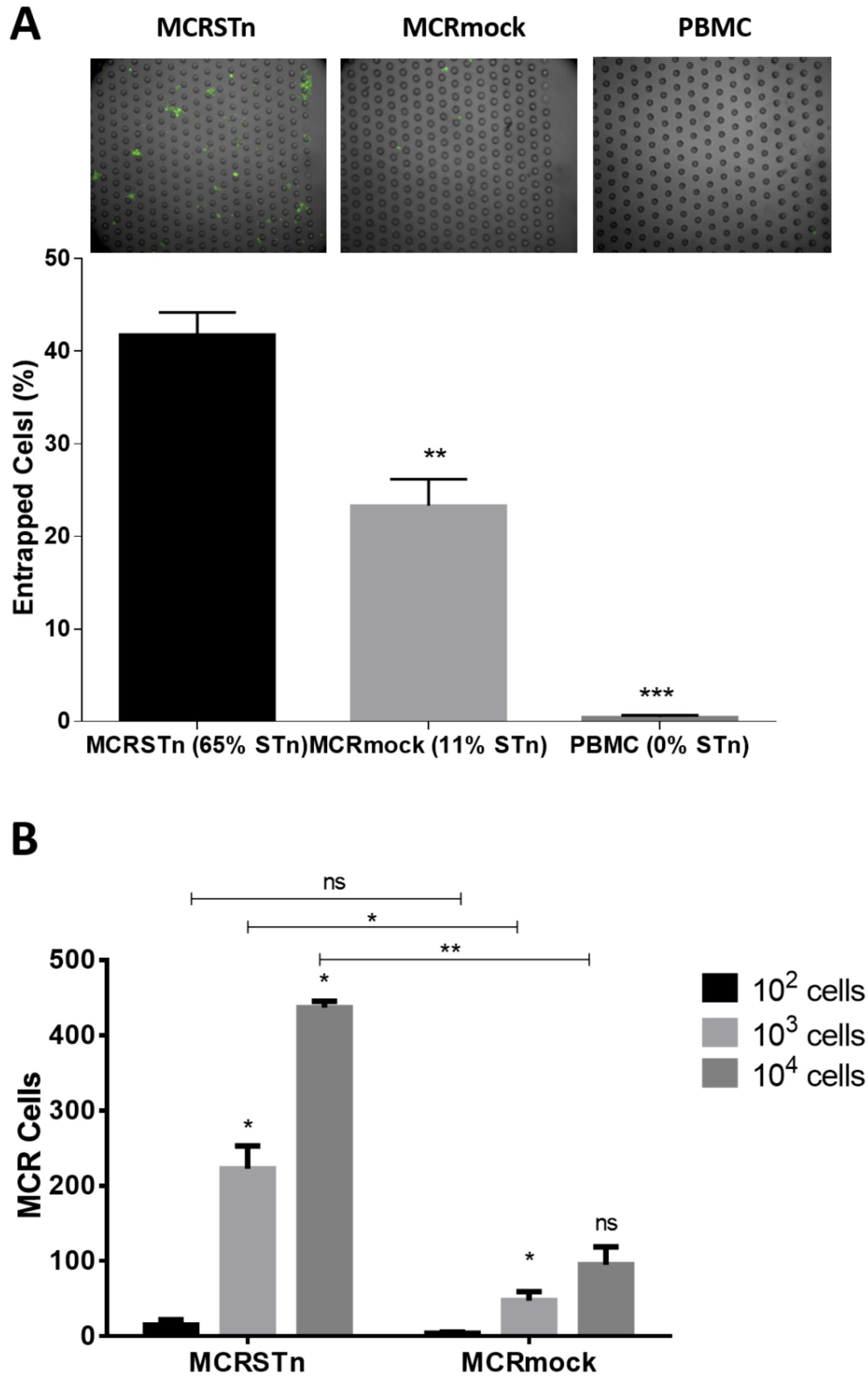

Figure 10. Percentage of MCR bladder cancer cells expressing different STn levels analyzed by glycan-affinity devices. A) Percentage of MCRSTn (75% STn expression) and MCRmock (11% STn) bladder cancer cells and human PBMC (0% STn) retained in glycan-affinity devices. Briefly, MCRSTn (75% STn expression) and MCRmock (11% STn) calcein labelled cells were analyzed using microfluidic devices. Accordingly, a higher number of cells were recovered for MCRSTn cells in comparison to MCRmock, in accordance with STn

expression. Moreover, only residual PBMCs were observed, highlighting low levels of non-specific binding. **B)** Bladder cancer cells recovery from human blood spiked with different amounts of MCRSTn and MCRmock(102, 103, 104). Briefly, PBMC isolated from the blood of healthy donors was spiked with different amounts of MCRSTn and MCRmock bladder cancer cells, in an attempt to mimic CTC in the blood of metastatic cancer patients. Bladder cancer cells were then isolated together with PBMCs and injected into the devices. Higher cells yields could be observed for MCRSTn in comparison to MCRmock, irrespectively of the initial blood cells concentration. Nevertheless, the amount of captured cancer cells increased with blood cell concentration, but it was more pronounced from 102 to 103 cells (15 times increase) than the observed for 104 (2 times increase), suggesting device saturation beyond that point. "ns": not significant; "*":$p < 0.05$; "**" $p < 0.01$; "***" $p < 0.001$.

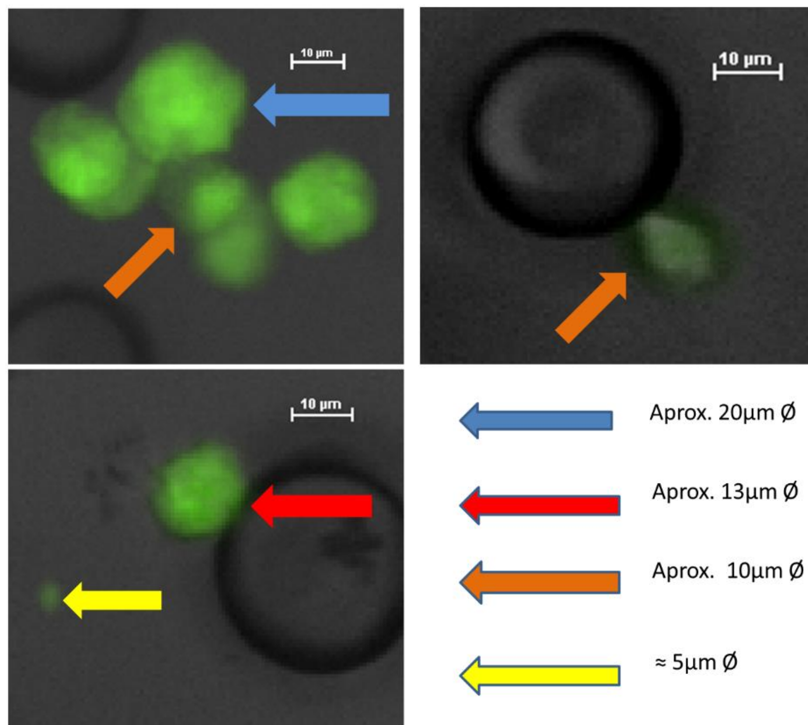

Figure 11. Glycan-affinity devices are capable of capturing MCRSTn cells of different dimensions. Briefly, glycan-affinity devices demonstrate the existence of a heterogeneous MCRSTn population in terms of dimensions. Accordingly, captured MCRSTn cells presented diameters spanning 5-20 µm.

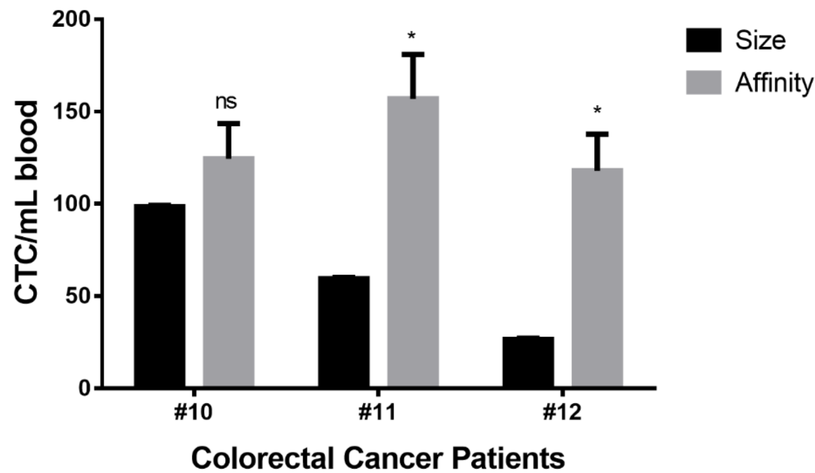

Figure 12. CTC counts for colorectal cancer patients (#10-12) in size-based vs glycan-affinity microfluidic devices. Blood collected from metastatic colorectal cancer patients was analyzed in parallel by size-based and glycan-affinity microfluidic devices. Glycan-affinity microfluidic devices provided higher CTC counts in comparison to size-based devices for all cases. ns: not significant (yet showing a trend increase); "*" $p < 0.05$.

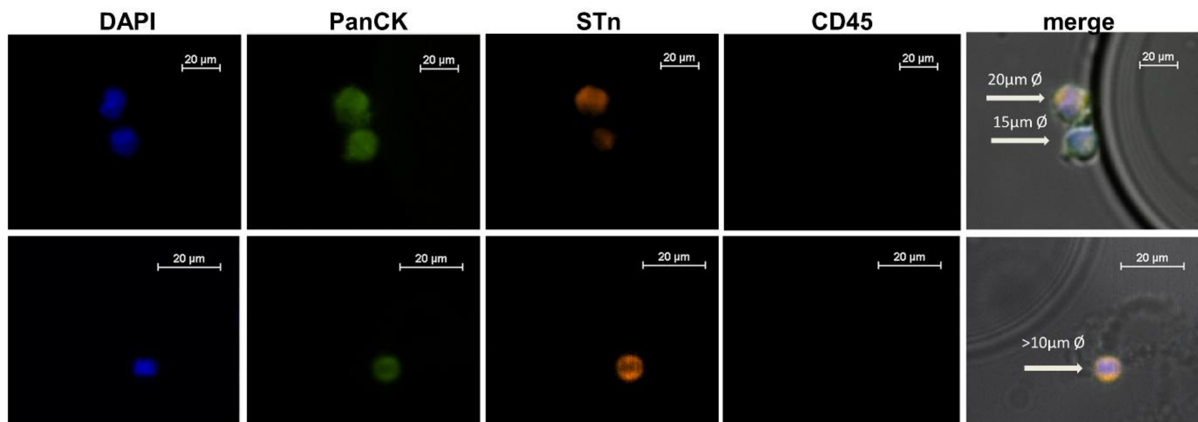

Figure 13. STn-CTC subpopulations from colorectal cancer patient #11 presenting different dimensions (>10 µm - approximately 8 µm), 15 µm, 20 µm) captured by glycan-microfluidic devices. Similar observations were made for cases #10 and #12.

4.4. Glycan-affinity microfluidics in precision oncology

While non-targeted microfluidics devices constitute an important starting point for accessing the CTC molecular landscape, glycan-affinity microfluidics devices provide the necessary platform for more specific downstream studies. This may be particularly important for cases were STn does not

constitute the majority of the entrapped CTC population, such as for bladder cancer patient #4 (83%) and colorectal cancer patient #2 (81%) and, specially, patient #12 (68%). Nevertheless, the specific recovery of STn-expressing cancer cells from the microchips is a critical matter. Envisaging this goal, a sialidase was injected into microfluidics devices with already entrapped MCRSTn and MCRmock cells and allowed to incubate under both stationary and dynamic conditions at a constant flow rate (10 µL.min-1), for 30 and 120 minutes. This enzymatic treatment cleaves the glycosidic linkage between the sialic acid and the GalNAc residue of the STn antigen, releasing the cells expressing the Tn antigen (STn desialylated counterpart), without impacting on cell membrane integrity. As highlighted **in Figure 14**, minimum cell recovery was 15% for stationary incubation whereas a maximum recovery of approximately 35% of entrapped cells was observed for 120 minutes of dynamic incubation. Such yields may, in part, be related with the formation of extracellular matrix by cancer cells in contact with the device coating, leading to unspecific binding. Stearic hindrance provided by antibody binding may also affect enzymatic cleavage and account for these findings. Nevertheless, sialidase treatment provides an elegant strategy to specifically recover STn expressing cells for analysis, with minor impact on cell molecular nature.

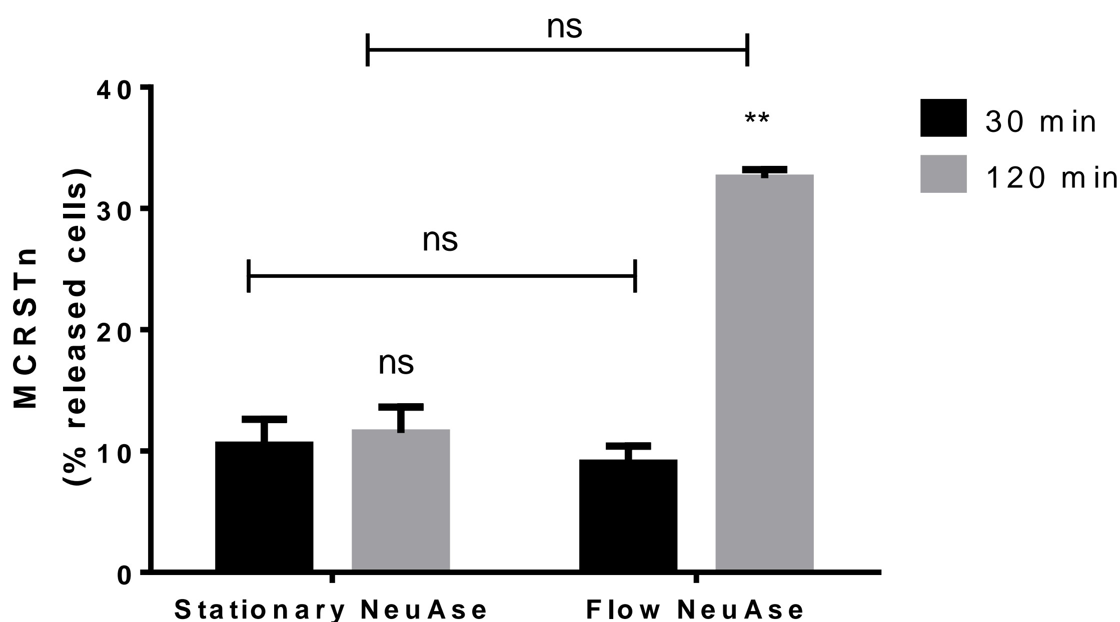

Figure 14. Percentage of MCRSTn and MCRSmock released from glycan-affinity devices after *in situ* sialidase treatment. Briefly, 10^3 MCRSTn were injected into microfluidic devices, showing a retention profile similar to Figure 5 (main manuscript; 40% entrapment). Entrapped cells were released by sialidase treatment at 37ºC under stationary conditions or by constant flow of the enzyme (dynamic flow), for 30 and 120 min. Under stationary flow, 10% of entrapped cells were recovered, which did not vary over time. Similar results were

observed under dynamic flow for 30 min. However, a 3 times higher statistically significant increase was observed after 120 min.

We have then applied this strategy to selectively recover STn^{+}CTC from colorectal case #10-12, which were subsequently screened for APC mutation c.4348C>T. Sialidase released cells from all cases were positive for the mutation, in accordance with size-based microchips analysis (data not shown). Noteworthy, while matched results to be expected for cases #10 and #11 presenting STn^{+}CTC counts that exceed 90% of the entrapped CTC, similar extrapolation could not be made for case#12, presenting only 68% STn^{+}CTC. Nevertheless, based on this strategy, we could confirm that STn-expressing cells on patient #12 mimicked the overall population. In summary, these observations set the necessary basis for a comprehensive study on STn^{+}CTC, envisaging to understand their biological and clinical relevance.

5. Concluding remarks

The identification of CTC cell-surface biomarkers remains a challenging topic in the context of liquid biopsies due to the scarce molecular information about these cells. In fact, the majority of studies presented to date explore the expression of epithelial marker EpCAM for CTC identification and isolation[28, 29], which has been suggested to significantly underestimate the number of cancer cells in peripheral circulation[5, 11, 12]. Particularly, EpCAM expression is frequently downregulated in cells undergoing epithelial-to-mesenchymal transition, a crucial milestone for metastasis development11. Using a label-free microfluidics device, we demonstrated that metastasized colorectal cancer patients present significantly higher CTC counts than first expected through EpCAM, targeting, thus reinforcing these observations. More importantly, most isolated CTC also expressed the STn antigen, confirming preliminary findings for bladder cancer[45]. Nevertheless, to the best of our knowledge, this was the first report concerning the glycosylation of bladder and colorectal CTC. Interestingly, CTC derived from different tumours of distinct aetiologies share similar cell surface glycosylation features, suggesting a common molecular ground in metastasis development that warrants careful evaluation in future studies. Accordingly, the cell-surface overexpression of STn has been previously found to endow cancer cells with a more motile phenotype and drive invasion in both models[40, 43, 47]. In addition, STn expression is part of an array of molecular mechanisms enabling cancer cells to escape immune responses46, which is critical for survival and successful colonization of distant niches. Supporting its association with more malignant features, STn expression has been frequently associated with poor prognosis and metastatic risk in bladder[43, 44, 47] and colorectal cancers[40]. Our

observations now set the necessary molecular rationale for exploiting the biological and functional significance of this antigen in metastasis development. Moreover, this study demonstrates that targeting the STn antigen may allow access to yet unknown CTC subpopulations, frequently overlooked by EpCAM-based detection methods. In addition, the membrane nature of this antigen also holds tremendous potential for selectively targeting CTC for downstream molecular characterization and ultimately the development of targeted therapies.

Envisaging this goal, we have developed and optimized a glycan-affinity microfluidics device targeting the STn antigen. This analytical tool allowed selective enrichment and further release of rare STn-expressing CTC, following PBMC extraction from whole blood of cancer patients. Isolated CTC may be directly screened *in situ* for different antigens by immunofluorescence, lysed *in situ* or selectively released from the device by sialidase treatment. This not only enables more specific downstream molecular characterization but also allows the collection of intact and viable cells, which is crucial for subsequent functional studies. Moreover, preliminary findings support that this device may provide access to a broader population of CTC in comparison to size-based solutions, most probably by enabling the capture of smaller cells. By exploring CTC recovery by sialidase release, we have further demonstrated that bladder CTC exhibit a KRT14+/KRT5-/KRT20- phenotype, previously associated to highly dedifferentiated and more aggressive basal cells implicated in metastasis45. Regarding the isolated colorectal cancer CTC, which were found to express STn at high levels, we demonstrated that it harboured a frequent APC mutation c.4348C>T, in mimicry of the primary tumour and the corresponding metastasis. Overall, such findings support the importance of targeting STn+ CTC for molecular insights on the primary tumour and the metastasis.

In summary, we have identified a novel biomarker, which is not expressed by human blood cells but is widely present in CTC, for selectively capturing and/or targeting these cells. Moreover, our data supports that targeting STn may significantly improve on CTC detection sensitivity in comparison to conventional EpCAM-based methods, holding tremendous potential for improving on prognostication using CTC-based models. In addition, we have designed a glycan-affinity microfluidics device and a method to specifically recover STn^{+}CTC from patients' blood for downstream molecular analysis and precision medicine applications. Nevertheless, while this is a proof-of-concept study, involving a small set of well characterized metastatic patients, showing consistent data, it now warrants validation in larger patient series. Particular emphasis should be devoted to exploiting the higher sensitivity provided by STn targeting, for early identification of patients with residual disseminated disease and/or exhibiting radiologically occult micrometastasis. Based on these preliminary findings, a clinical study is being setup to disclose the clinical relevance of STn^{+}CTC and gain more insights on the molecular nature of these cells. Moreover, sialidase treatment

has allowed recovering viable CTC from microfluidics devices (preliminary data, not shown), which may now be expanded *in vitro* and/or xenotransplanted into relevant animal models enabling more in depth molecular analysis, personalization of therapeutic schemes and/or testing novel treatments. Therefore, we envisage that targeting STn may allow improving on CTC-based liquid biopsies and foster molecular based precision medicine. Our findings further support the potential of a comprehensive interrogation of the glycome and glycoproteome of both primary tumours, CTC and metastasis envisaging highly specific biomarkers for improving advanced patient's management. These findings will likely pave the way for novel and improved microfluidics platforms for liquid biopsies applications.

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Concluding remarks & future perspectives

Concluding remarks and future perspectives

The management of advanced bladder cancer remains a gray zone for clinicians, in part due to its molecular heterogeneity, thereby calling for novel molecular tools that help to identify patients facing worst prognosis and at higher risk of disease progression. Moreover, it is imperative to identify biomarkers for targeting aggressive and chemoresistant cancer cells, often responsible by disease relapse and dissemination. Within this context, many glycoproteins, including CD44, integrins (αv integrins), and integrin-associated proteins (CD47) have been suggested to hold potential for targeting bladder cancer stem cells with the above-mentioned characteristics. However, these proteins lack organ-specificity, thus hampering the development of effective therapeutics.

We hypothesized that the identification of specific cancer-associated glycoforms might help narrowing these glycoproteins to clinically relevant species and, as we highlighted in a recent review **(Annex A)**, chemoresistant stem-cells often present distinct glycosylation patterns in comparison to other cancer cells. The identification of these glycoforms may be used to improve on sensitivity and specificity of cancer-associated glycoproteins-targeted therapeutics design. Building on these observations and previous reports(160), herein we disclosed that advanced bladder tumours significantly overexpress sialylated short-chain *O*-glycans compared to superficial lesions. Amongst these, STn was found to be the most relevant antigen, due to its cancer-specific nature and association with poor prognosis in both NMIBC and MIBC. Such observations are in accordance with previous reports supporting its influence on cell motility and as a promoter of invasion in bladder cancer(160). In addition, the STn antigen was found in basal-like lesions, phenotypically characterized by the overexpression of *KRT*14 and/or *KRT*5 in relation to *KRT*20 and enriched for chemoresistant cancer stem cells(180). Moreover, the presence of STn-positive cancer cell subpopulations has been associated with a significant reduction in the survival of patients exhibiting luminal tumours (*KRT*14^- and/or *KRT*5^- and *KRT*20^+ phenotype), which generally present better survival than basal lesions(180). Noteworthy, tumours relapsing after chemotherapy have also been found to be enriched for cells expressing this glycan as well as for stem-cell molecular characteristics suggesting an association between both events, as previously reported for other tumours(181). Interestingly, chemoresistant lesions have also been found to overexpress the stem-cell marker CD44 and STn^+CD44 glycoforms, as well as a basal phenotype, strongly suggesting that this glycan could be used to specifically target cancer stem cells. These findings were further reinforced by studies *in vitro* using relevant cell models representative of different invasion pathways (T24: FGFR3/CCND1 pathway; HT1376: E2F3/RB1 pathway) challenged with cisplatin. Cumulatively and notably, we described for the first time that most tumour cells found in peripheral blood circulation of metastatic bladder cancer patients also express

the STn antigen, mimicrying the primary tumour and the metastasis. Additionally, bladder STn^{+}CTC presented a *KRT*14^{+}, *KRT5*$^{-}$, *KRT*20^{-} phenotype consistent with the most undifferentiated basal state and stem-cell ((180). While the functional role of STn in metastasis remains incompletely elucidated, these findings support a potential selective advantage for cells carrying this posttranslational modification. In agreement with these observations, STn expression has been described to endow cancer cells with the capacity to circumvent immune responses(162), which is a crucial step for cancer cells survival in circulation and to successfully colonize distant locations.

Here, a reasonable amount of evidence is depicted supporting that bladder cancer stem cells, possibly endowed with chemoresistant traits, express the STn antigen. As such, the STn antigen is suggested as novel biomarker of bladder cancer aggressiveness that should be explored in the context of targeted therapies. Accordingly, future studies should focus on the validation of these preliminary findings, including a more comprehensive functional and molecular characterization of the STn-glycoproteome, with emphasis also on *O*-glycosites mapping. Emphasis should also be given to CD44, which has been consensually reported as clinically relevant in bladder cancer and considered a cancer stem-cell marker(70, 182). Nevertheless, the identification of CD44 iso- and glycoforms expressed by more aggressive cancer cells compared to healthy cells, will be particularly crucial to increase specificity and avoid potential off-target effects of novel therapeutics based on this glycoprotein, as well as theragnostic applications. These ligands may be ultimately used to specifically guide drug loaded nanoparticles, genetically modified T cells expressing chimeric antigen receptors, or other cytotoxic/cytostatic agents to tumour sites and more aggressive cancer cells; thereby, boosting specific immune responses against cancer cells or inhibiting oncogenic pathways(183).

The exploitation of STn-expression by circulating tumour cells is also demonstrated. In fact, this work not only supports STn as a novel and potentially widespread CTC biomarker, but also provides an innovative glycan-based microfluidics platform that may pave the way for molecular-based precision medicine. It has also been demonstrated that STn antigen is expressed by colorectal cancer, suggesting a pan-carcinoma nature warranting future evaluation. Moreover, preliminary studies support that targeting the STn antigen may significantly improve on the sensitivity of current methods for CTC analysis. Clinical studies should now be undertaken to determine its potential for early detection of disease dissemination, therapy selection, follow-up and development of novel therapeutic strategies. In addition, these devices may be used for accessing the molecular nature of STn^{+}CTC and may constitute important liquid biopsies platforms capable of improving advanced patient's management. Finally, the possibility of recovering CTC from microfluidics devices by means of a simple sialidase treatment may allow expansion and xenografting in relevant animal models, enabling drug testing and development.

This work presents a closer clinical link between STn expression, poor prognosis, and stem-cell phenotypes, characterized by chemoresistance and capability of regenerating tumour heterogeneity. Moreover, the widespread nature of STn^{+}CTC and its prevalence in the corresponding metastasis supports a role in disease dissemination that warrants confirmation in future studies. The clinical rationale is now set to comprehensively address the STn-glycoproteome envisaging novel and highly specific bladder cancer ligands. Moreover, STn is presented as a novel CTC biomarker that should be carefully exploited envisaging to improve on advanced stage patient’s management. In addition, a novel glycan-affinity microfluidics tool is presented, which may now help paving the way for improved patient care.

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Annexes

Annexes

Annex A. Mechanisms of cisplatin resistance and targeting of cancer stem cells: Adding glycosylation to the equation.

Annex B. Over forty years of bladder cancer glycobiology: Where do glycans stand facing precision oncology?

Annex C. Targeted O-glycoproteomics explored increased sialylation and identified MUC16 as a poor prognosis biomarker in advanced-stage bladder tumours.

Annex D. Abnormal Protein Glycosylation and Activated PI3K/Akt/mTOR Pathway: Role in Bladder Cancer Prognosis and Targeted Therapeutics.

ANEXO A

Mechanisms of cisplatin resistance and targeting of cancer stem cells: Adding glycosylation to the equation

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ACCEPTED MANUSCRIPT

Mechanisms of cisplatin resistance and targeting of cancer stem cells: Adding glycosylation to the equation

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Abstract

Cisplatin-based chemotherapeutic regimens are the most frequently used (neo)adjuvant treatments for the majority of solid tumors. While platinum-based chemotherapeutic regimens have proven effective against highly proliferative malignant tumors, significant relapse and progression rates as well as decreased overall survival are still observed. Currently, it is known that sub-populations of chemoresistant cells share biological properties with cancer stem cells (CSC), which are believed to be responsible for tumor relapse, invasion and ultimately disease dissemination through acquisition of mesenchymal cell traits. In spite of concentrated efforts devoted to decipher the mechanisms underlying CSC chemoresistance and to design targeted therapeutics to these cells, proteomics has failed to unveil molecular signatures capable of distinguishing between malignant and non-malignant stem cells. This has hampered substantial developments in this complex field. Envisaging a novel rationale for an effective therapy, the current review summarizes the main cellular and molecular mechanisms underlying cisplatin resistance and the impact of chemotherapy challenge in CSC selection and clinical outcome. It further emphasizes the growing amount of data supporting a role for protein glycosylation in drug resistance. The dynamic and context-dependent nature of protein glycosylation is also comprehensively discussed, hence highlighting its potentially important role as a biomarker of CSC. As the paradigm of cancer therapeutics shifts towards precision medicine and patient-tailored therapeutics, we bring into focus the need to introduce glycomics and glycoproteomics in holistic pan-omics models, in order to integrate diverse, multimodal and clinically relevant information towards more effective cancer therapeutics.

1. Introduction

Cisplatin (cis-diamminedichloridoplatinum(II); cis-[Pt(NH_3)$_2$(Cl)$_2$]) was first described by Michele Peyrone in 1845, but its structure was only determined in 1893 (Trzaska S, 2005). After several years of investigation, Rosenberg realized its potential to induce tumor cells death (Rosenberg B, 1973) and finally in 1978 the drug was

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approved by the FDA for the treatment of testicular and ovarian cancer (Trzaska S, 2005). Nowadays, cisplatin-based regimens are widely used as (neo)adjuvant chemotherapy against a spectrum of solid tumors including gastric, non-small cell lung (NSCLC), head and neck, gallbladder, and urinary bladder cancer. However, cisplatin treatment exhibits severe side effects including immunosuppression, renal toxicity, gastrointestinal disorders and ototoxicity (Boussios S et al., 2012; Karasawa T et al., 2015). It may also cause gonadal suppression resulting in amenorrhea or azoospermia, partial or irreversible infertility and embryo-toxicity (Brennemann W et al., 1997; Meistrich ML, 2009).

Cisplatin is an alkylating agent capable of forming adducts with macromolecules, particularly with N7 atoms of purine nucleobases. This results in inter- and intra-strand DNA cross-links that bring induce cell cycle arrest mainly in the G_2/M checkpoint (Yuan L et al., 2003). The inability to repair this DNA damage ultimately leads to programmed cell death. However, experimental evidence revealed that other mechanisms such as the production of reactive oxygen species (ROS) and the activation of inflammatory pathways, may also contribute to the induction of apoptosis (Casares C et al., 2012). Cisplatin has shown significant efficacy against rapidly proliferating tumor cells. However, despite a fairly acceptable intrinsic drug response rate, there is a 95% risk of tumor relapse in NSCLC patients. The 5-year survival rate is approximately 50% for muscle-invasive bladder cancer, (Nadal R et al., 2014) and 15-20% for ovarian cancer patients (Siddik ZH, 2003); similar survival rates have been reported for other solid tumors. It has been hypothesized that chemotherapy may either act as a selective pressure for more aggressive cell phenotypes (Freitas DP et al., 2014), or that tumor cells which are less drug sensitive may acquire mutations during the course of treatment, that enable them to evade drug-induced cell death (Crea F et al., 2011). The failure of cisplatin-based regimens is considered both life-threatening and a major burden to health care systems, as it requires the introduction of more expensive second line treatments. Therefore, deciphering the mechanisms underlying this treatment failure has been a primary goal of cancer research and, in the past two decades, some of the modalities underlying anticancer drug resistance have been identified; however the implications for improving drug therapy have been limited.

Chemotherapy resistance results from a synergism of events that include tumor cell extrinsic factors (pharmacokinetic resistance and tumor microenvironment) as well

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as intrinsic factors, namely alterations in drug transport and metabolism, relative dormancy/slow cell cycle kinetics, efficient DNA repair systems and inhibition of apoptosis (Martin LP et al.; Pommier Y et al., 2004; Raguz S and Yague E, 2008). In addition, some chemoresistant tumor cell clones may present self-renewal and pluri/multipotent differentiation capabilities, which are characteristics associated with cancer-stem cells (CSC) (Visvader JE and Lindeman GJ, 2008). Therefore, these cells constitute a small pool of CSC capable of generating more differentiated sub-populations that, during subsequent divisions, form the vast majority of the tumor bulk. The remarkable longevity of CSC also renders them more susceptible to the accumulation of genetic damage and epigenetic alterations that may ultimately promote the proliferation of heterogeneous and aggressive cell phenotypes (Muñoz P et al., 2012). Some subsets of CSC can be found in poorly vascularized hypoxic tumor niches, which favour the maintenance of stem-cell characteristics, and are consequently exposed to suboptimal drug concentrations (Lin Q and Yun Z, 2010). Furthermore, these cells may undergo epithelial-to-mesenchymal transition (EMT) in response to microenvironmental stimuli, namely prolonged exposure to low oxygen levels, and may acquire the capability to invade and metastasize to regional lymph nodes and distant organs (Jiang J et al., 2011). In summary, it became evident that cisplatin and other conventional chemotherapeutic drugs may ultimately contribute to the selection of a pool of slow dividing or quiescent CSC (Wang W et al., 2014). These cells are endowed with the capability of recapitulating tumor heterogeneity and undergo EMT, considered as one of the driving forces of cancer dissemination (Frank NY et al., 2010). As such, patients would greatly benefit from combined therapies including agents capable of selectively eliminating CSC. The ideal therapy should specifically recognize these cells from the tumor bulk, include means to inhibit resistance mechanisms, as well as include CSC-killing agents. However, the majority of membrane-bound CSC biomarkers known to date can also be found in normal stem- and non-malignant cells (Cojoc M et al., 2015), which hampers the development of specific targeted therapeutics.

More recently, several studies have demonstrated that profound alterations in protein glycosylation that often accompany malignant transformation may also influence resistance to chemotherapy. This rather neglected mechanism of drug resistance has been often associated with impaired function of membrane-bound glycoproteins, such as ATP-binding cassette efflux transporters, due to specific

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alterations in their glycosylation patterns (Beers MF et al., 2013; Nakagawa H et al, 2009). However, alterations in cell-surface protein glycosylation have also been shown to favor oncogenic signaling pathways associated with chemoresistance and CSC-like phenotypes (Dall'Olio F et al., 2014; Häuselmann I and Borsig L, 2014; Ju T et al., 2008; Pinho SS et al., 2012). Therefore, cancer-associated glycans constitute markers of chemoresistance and bear potential promise for the identification and therapeutic targeting of CSC.

Envisaging a rationale for an effective therapy, the present review discusses the main mechanisms of cisplatin resistance known to date, integrating key insights about the role of cancer-associated glycans. Although the current review focuses mainly on cisplatin, it is proposed here that many of these strategies mediate resistance to other drugs as well. The present paper also provides a comprehensive overview on the impact of the chemotherapeutic challenge in tumor biology, CSC selection and clinical outcome. Moreover, it aims to raise awareness for the fact that CSC harbor distinct glycosylation patterns that should be carefully explored towards the development of highly specific targeted therapeutics.

2. Overview on drug resistance mechanisms and CSC selection

Drug resistance is a multifactorial process which is based on both extrinsic and intrinsic factors in tumor cells (Raguz S and Yague E, 2008). Extrinsic factors such as unfavorable drug pharmacokinetics and abnormal tumor vasculature result in the delivery of suboptimal concentrations of cytotoxic agents to tumor sites (Rohwer N et al, 2011). Defective tumor vasculature also results in hypoxic and acidic niches that significantly modulate cell function in manners that favor chemoresistance (Wilson WR and Hay MP, 2011). Similarly, alterations in the extracellular matrix architecture and stromal cell paracrine signals have been found to influence chemotherapy outcome (Sherman-Baust CA et al, 2003; Tripathi M et al., 2012). In addition, tumor cells may either present, or develop during the course of treatment, various mechanisms to withstand and overcome chemotherapeutic challenges (Shen D et al., 2012). These mechanisms include for example: i) Alterations in drug transport and metabolism; ii) Enhanced DNA repair mechanisms; iii) Alterations in cell cycle regulation; and iv) Inhibition of apoptosis. Emerging evidences support the notion that chemoresistance,

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driven by the above mentioned factors, is associated with CSC-like properties as well as the acquisition of EMT capability, thereby explaining the high relapse and progression rates presented by first line chemotherapy agents (Cojoc M et al., 2015). Based on these considerations, the following sections aim to illustrate the influence of the main tumor-associated extrinsic and intrinsic properties in chemoresistance.

2.1. The impact of the microenvironment on drug resistance

2.1.1 Tumor vasculature and hypoxia

Solid tumors often present tortuous, poorly differentiated and truncated vasculature, resulting in the delivery of suboptimal concentrations of cytotoxic drugs to certain niches (Minchinton AI and Tannock IF, 2006). This also accounts for the formation of a hypoxic environment that significantly influences cell metabolism and modulates gene expression, ultimately enhancing chemoresistance and maintenance of CSC (Rohwer N et al., 2011); discussed in detail in the following sections) (Figure 1). Furthermore, hypoxia modulates the expression of genes linked to EMT – (addressed in detail in subsequent sections) and drug resistance phenotypes (Adamaki M et al., 2012; Jiang J et al., 2011; Polyak K and Weinberg RA, 2009; Ruan K et al., 2009; Shannon AM et al., 2003).

The primary transcription factor mediating the response to hypoxic challenge is hypoxia-inducible factor-1 (HIF-1) (Semenza GL, 2001). HIF-1 consists of a constitutively expressed subunit HIF-1β and a tightly oxygen-regulated subunit HIF-1α (or its paralogs HIF-2α and HIF-3α) (Brocato J et al., 2014). Under normoxia conditions, the HIF-1α protein is constitutively expressed but rapidly marked for proteosomal degradation, resulting in a very short cytoplasmic half-life (5-8 min). Under hypoxic conditions, HIF-1α is translocated to the nucleus where it binds to the co-activators HIF-1β and p300/CBP (Semenza GL, 2001), inducing an array of responses such as overexpression of angiogenic genes (i.e. VEGF, PDGF-BB and NOS) and growth factors (i.e. IGF-II) (Brocato J et al., 2014; Denko NC et al., 2003; Harris AL, 2002), decreases mitotic and metabolic rates and adapts energy requirements to the hypoxic challenge (Hockel M et al., 2001; Shannon AM et al., 2003). In particular, HIF-1α contributes to the dramatic shift of intracellular glucose metabolism from aerobic cellular respiration to anaerobic glycolysis through the transactivation of genes

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encoding glucose transporters (i.e. GLUT-1) and several rate-limiting enzymes of glycolysis (Jose C et al., 2011; Mucaj V et al., 2012). It also suppresses the tricarboxylic cycle (TCA) via the *PDK1* gene, encoding pyruvate dehydrogenase kinase 1, which inactivates pyruvate dehydrogenase, resulting in fueling the TCA cycle with acetyl-CoA (Shirato K et al., 2011). Moreover, HIF-1α has been shown to regulate the expression of cytochrome C oxidase (COX) allowing cancer cells to optimize the efficiency of respiration at different oxygen levels (Mucaj V et al., 2012). Ultimately, HIF-1α contributes to cellular adaptation to hypoxic stress which can culminate in mitochondrial autophagy (Zhang H et al., 2008). By modulating mitochondria activity, HIF-1α influences cell death mechanisms mainly by interfering with apoptotic and necrotic signaling (Greijer AE, Van der Wall E, 2004). Furthermore, HIF-1α has also been shown to act as apoptosis suppressor in cancer cells through the regulation of anti-apoptotic target genes and additional molecular mechanisms that still remain largely elusive (Adamaki M et al., 2012; Harris AL, 2002). For instance, hypoxic regulation of p53 has been proposed to be HIF-dependent; however, controversy remains over this topic. Nevertheless, most reports point to the fact that hypoxia acts as a positive selective pressure for the positive selection of p53 mutant cells and hence inducing diminished apoptotic potential (Ruan K et al., 2009), thereby compromising the response to chemotherapy (Gogna R et al., 2012; Weisz L et al., 2007). HIF-1α also acts as a regulator of drug efflux through the activation of the *MDR1* gene encoding the multidrug resistance efflux transporter P-glycoprotein (P-gp; ABCB1) (Comerford KM et al., 2002; Rohwer N et al., 2011; Shannon AM et al., 2003). P-gp belongs to the ATP-binding cassette (ABC) superfamily of transporters, however it does not recognize cisplatin as a transport substrate (Lockhart AC et al., 2003; Rohwer N et al., 2011), but is capable of markedly decreasing the intracellular concentration of a wide range of structurally and functionally distinct hydrophobic chemotherapeutic agents (Lockhart AC et al., 2003; Shapira A et al., 2011).

Under hypoxic conditions an association between high levels of HIF-1α and cisplatin resistance has been widely reported for several representative tumor cell lines, including ovarian cancer (Su W et al., 2011), NSCLC (Fischer C et al., 2015), hepatocellular carcinoma and hepatic progenitor cell lines (Jiao M and Nan K, 2012). Supporting a role for HIF-1α in chemoresistance, elevated levels of this key transcription factor have been observed in tumors of different tissue origin and linked to

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more resilient tumor cells, poor prognosis and resistance to radiotherapy and chemotherapy (Huang LA et al., 2007; Shannon AM et al., 2003; Wilson WR and Hay MP, 2011). Nevertheless, it should be noticed that, despite the pivotal role of HIF-1α, other independent mechanisms have been found to mediate hypoxia-related chemoresistance (Adamaki M et al., 2012; Scholten DJ et al., 2014). More detailed insights about the role of HIF in chemoresistance may be found in previous reviews by Rowner and Cramer (Rohwer N et al., 2011) and Raguz and Yagüe (Raguz S and Yague E, 2008).

2.1.2. The tumor stroma

The tumor stroma, mainly formed by the basement membrane, extracellular matrix, cancer-associated fibroblasts (CAFs), immune cells, and vasculature, is a complex structure whose interactions markedly affect tumor growth, invasion, and metastasis during the course of disease (Bremnes RM et al., 2011; Pietras K et al., 2010) (Figure 1). Over the recent years, the crosstalk between cancer cells and the tumor stroma has been progressively unveiled (see review Bremnes RM et al., 2011; Tripathi M et al., 2012). However, the dynamics of cancer cell death in response to cisplatin and the tumor microenvironment has yet to be fully characterized. Noteworthy, it has been shown that the presence of stroma in 574 breast cancer specimens from patients who underwent surgery combined or not with adjuvant radiotherapy, adjuvant chemotherapy or adjuvant endocrine therapy, was an independent prognostic factor for relapse-free period, particularly in the triple-negative subpopulation (de Kruijf EM, 2011). However, extracellular matrix components mediating resistance to chemotherapy are still poorly understood. Several *in vitro* studies have demonstrated that the extracellular matrix not only constitutes a physical barrier to drug dissemination (Choi I et al., 2013), but may also exert a protective effect against apoptosis induced by various anticancer drugs (Chen Y et al., 2010; Kouniavsky G et al., 2002; Sethi T et al., 1999). Miyamoto H and colleagues have further found that the grade of differentiation of pancreatic tumor cells affects the interaction with different ECM macromolecules (fibronectin, collagen I, and collagen IV) as well as the matrix-driven sensitivity to chemotherapeutic drugs, including cisplatin (Miyamoto H et al., 2004). The presence of fibronectin, type IV collagen, and laminin have also been linked to cisplatin-resistance and local recurrence

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of uveal melanomas (Bérubé M et al., 2005). The authors observed that apoptosis was less frequent after cisplatin administration in the presence of ECM when compared with cells cultured on a non-permissive matrix (Bérubé M et al., 2005). Several studies also found that the overexpression of ECM-associated genes correlates with increased cisplatin resistance (Januchowski R et al., 2014; Sherman-Baust CA et al., 2011; Sherman-Baust CA et al., 2003). In this respect, the gene expression profile of a subpopulation of the human ovarian carcinoma cell line A2780 displaying cisplatin resistance revealed a significant upregulation of the *COL6A3* gene, encoding for collagen VI (Varma RR et al., 2005). These findings suggest that chemoresistance may modulate ECM composition through altered regulation of gene expression in cancer cells. Moreover, cisplatin-sensitive cells cultured in the presence of collagen VI showed enhanced resistance *in vitro* and immunohistochemistry studies revealed an association between collagen VI overexpression, tumor grade and resistance to chemotherapy (Sherman-Baust CA et al., 2003). Furthermore, ovarian tumors with pronounced stromal/mesenchymal gene signatures exhibited the worst outcome when compared to groups with non-stroma-associated gene signatures (Verhaak RGW et al., 2012). In addition, ECM gene signatures were associated with chemotherapy resistance (Mintz MB et al., 2005). Altogether, these findings suggest that tumor cells may remodel their microenvironment in order to increase survival to chemotherapeutic challenges (Sherman-Baust CA, et al., 2003). Recent studies point towards the pivotal role of β1-integrins in ECM-cancer cells interactions and chemoresistance (Aoudjit F and Vuori K, 2012). According to Hodkinson PS et al., β1-integrin-mediated PI3K activation prevents caspase-3 activation, thereby protecting small cell lung cancer cell lines against chemotherapy-induced cell cycle arrest and apoptosis (Hodkinson PS et al., 2007). A more recent report on oral carcinoma cell lines has shown that adhesions within the carcinoma matrix create an environment in which exposure to cisplatin induces proliferation through the function of β1-integrin, talin and FAK pathways that regulate nuclear activity of NF-kB (Eberle KE et al., 2011). Based on these observations, it has been suggested that the inhibition of ECM-integrin interactions in combination with chemotherapy could have positive therapeutic implications (Mahadevan D and Von Hoff DD, 2007). Impaired binding of hyaluronan to transmembrane receptor CD44 has also been shown to influence multiple cell signaling pathways that stimulate tumor cell proliferation, migration, and matrix metalloproteinase secretion, and to promote CSC properties as well as cisplatin resistance (Bourguignon LY et al., 2013; Ohashi R et al.,

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2007; Torre C et al., 2010). Several metalloproteinases that are responsible for the proteolysis of ECM components during biological processes such as carcinogenesis, differentiation, apoptosis, migration and invasion, tumor angiogenesis and immune surveillance may also contribute to modulate response to treatment (Blons H et al., 2004; Ertan E et al., 2011; Mahadevan D and Von Hoff DD, 2007). The balance between matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMPs) is crucial for ECM stability (Nagase H et al., 2006) and acts in a coordinated manner to drive cancer progression and metastasis (Hara I, 2001; Rodriguez FO et al., 2012). Overexpression of TIMP-2, an inhibitor of MMP-2, in ovarian cancer stromal areas was found to be associated with better response to chemotherapy (Hałoń A et al., 2012). Furthermore, *in vitro* studies of squamous cell carcinoma of the head and neck have shown significant correlations between resistance to cisplatin and the expression of TIMP-2, suggesting that TIMP-2-mediated chemoresistance is dependent on the physiological environment (Akervall J et al., 2004). However, studies using ovarian cancer cell lines failed to show any association between this MMP-2 inhibitor and cisplatin resistance (Hałoń A et al., 2012). Also, expression of MMP-7 and -13 was found to be associated with cisplatin resistance in head and neck cancer cell lines (Ansell A et al., 2009). Again, these observations suggest that the crosstalk between the microenvironment and cancer cells is essential in order to influence intrinsic cell characteristics and chemotherapy resistance. These findings imply that disruption of cell-matrix interactions may provide ways to block ECM-mediated upstream or downstream intracellular signaling cascades, which could result in the overcoming of cisplatin resistance. Taking into account the encouraging results from studies using matrix modulating agents in combination with chemotherapy (Hussain A et al., 2012; Kamaraj S et al., 2010; Pisano C et al., 2014), it is now important to fully clarify the role of ECM components in platinum resistance in order to design rational therapeutic approaches.

Stromal changes also include the development of carcinoma-associated fibroblasts (CAFs) that play a crucial role in disease (Tripathi M et al., 2012). In the recent years, the crosstalk between cancer cells and CAFs has been unveiled and comprehensively reviewed by several groups (Brennen WN et al., 2012; Liu R et al, 2012; Puré E, 2009). CAFs have been found to directly drive cancer development through multiple mechanisms, which include the promotion of angiogenesis, cellular

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proliferation, invasion and inhibition of apoptosis in cancer cells (Brennen WN et al., 2012; Hale MD et al., 2013). Numerous growth factors, cytokines, proteases, and extracellular matrix proteins, such as SDF-1, FGF2, VEGF, TGF-β, HGF, tenascin-c, LOX, and MMPs are among the key molecules mediating these processes. Growth factors and other proteins produced by activated fibroblasts not only act on cancer cells but also on other components of the stroma, including adipocytes, inflammatory and immune cells as well as in the remodeling of the ECM, hence creating intricate feedback loops mediated by paracrine and autocrine signaling that drives CAFs development, disease progression and dissemination (Brennen WN, 2012; Hale MD et al., 2013). Nevertheless, only limited data on the response of CAFs to chemotherapy and their potential impact on therapy outcome are available. Namely, Sonnenberg et al. addressed the influence of CAFs in lung and breast tumors as well as in primary cultures hence revealing that, similarly to cancer cells, the sensitivity of CAFs to cisplatin is highly variable and dependent on the cancer type and its microenvironment (Sonnenberg M et al., 2008). Moreover, studies have demonstrated that the presence of CAFs contributes to the decreased response to chemotherapy in prostate (Franco OE et al., 2012), breast (Rong G et al., 2013) and lung (Sonnenberg M et al., 2008) cancers. Furthermore, strategies targeting CAFs components have proven capable of improving the response to chemotherapy (Elenbaas B et al., 2001; Loeffler M et al., 2006), suggesting a role for these cells in treatment outcome. Also, a recent systematic review by Hale et al., has highlighted the fact that stroma-derived biomarkers, with emphasis on molecules produced by CAFs, are useful biomarkers to predict response to therapy (Hale MD et al., 2013). Several studies also support the notion that CSC, known for their intrinsic resistance to chemotherapy, also receive critical maintenance cues from supportive stromal elements, including CAFs (Hasegawa T et al., 2014; Li L et al., 2013; Liao CP et al., 2010). In this respect, the interplay between CSC and CAFs towards increased chemoresistance and CSC self-renewal and invasion, have been recently demonstrated for colorectal cancer; according to this study, chemotherapy induces the remodeling of the tumor microenvironment to support cellular hierarchy through secreted factors that include IL-17A (Lotti F et al., 2013).

Overall, there is a growing amount of evidence supporting the notion that stromal components promote chemoresistance; hence, the mechanisms underlying this stromal based chemoresistance should be critically evaluated hence paving the way

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towards the overcoming of this modality of drug resistance. Furthermore, the incorporation of therapeutics which target the tumor microenvironment may be a determinant for disrupting CSC maintenance mechanisms.

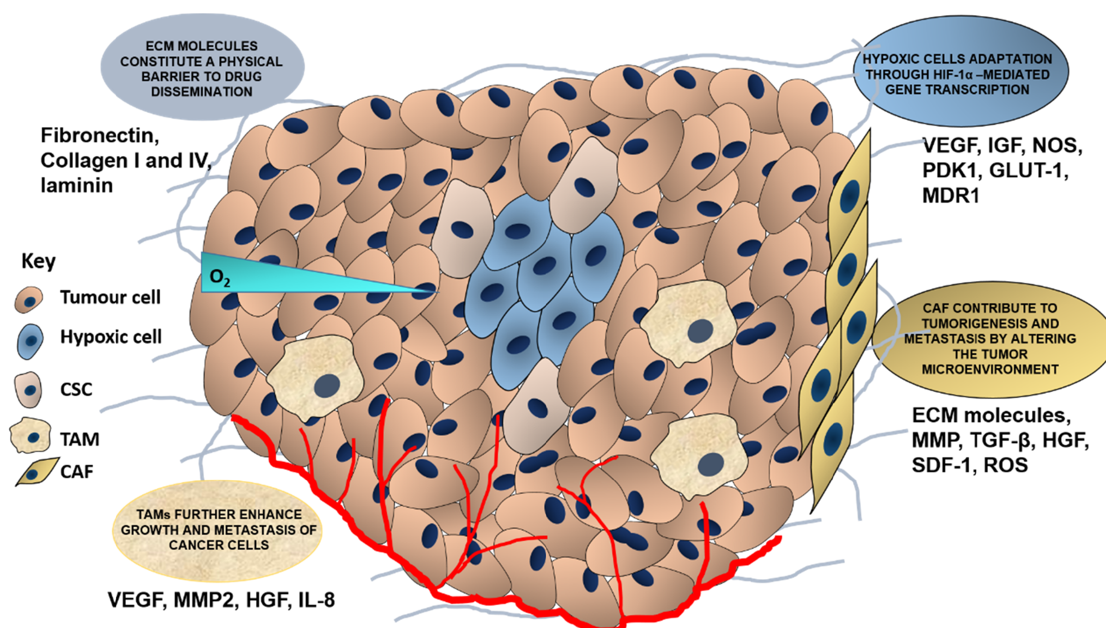

Figure 1. The microenvironment of solid tumors actively modulates cell metabolism, gene expression and chemotherapy response. Solid tumors frequently present inefficient vasculature, resulting in the delivery of suboptimal concentrations of cytotoxic drugs and highly hypoxic tumor cores. Stromal cells and the ECM strongly mediate tumor growth and stress response, and it constitutes a physical barrier for drug delivery.

2.2. Alterations in intracellular drug concentrations

Cisplatin-resistant tumor cells often present reduced intracellular platinum accumulation when compared to non-resistant cells following drug exposure. This may result either from reduced uptake or increased drug export (Stewart DJ, 2007).

2.2.1. Cisplatin uptake

Cisplatin uptake is mainly mediated by high affinity copper transporter protein 1 (hCtr1) and its down-regulation has been associated with resistance to platinum-based drugs *in vitro* (Holzer AK et al., 2004; Liang ZD et al., 2014; Liang ZD et al., 2012) and *in vivo* (Fu S et al., 2012) (Figure 2). An association between hCtr1 rs7851395 and

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rs12686377 polymorphisms and platinum resistance in NSCLC patients has also been described (Xu X et al., 2012). Nevertheless, more clinical studies are required to determine the prognostic value of hCtr1 and its genetic variants in the context of cisplatin treatment. The hCtr1 transporter protein is responsible by regulating intracellular copper homeostasis. In turn, the expression of hCTR1 is regulated at the transcriptional level by copper via the transcription factor Sp1 and at the post-translational level by a mechanism involving copper-stimulated endocytosis and degradation of the transporter (Howell SB et al., 2010; Petris MJ et al., 2003). The exposure to copper-chelating agents has been proven to increase the expression of hCtr1 and sensitize tumor cells to platinum (Galluzzi L et al., 2012; Ishida S et al., 2010; Liang ZD et al., 2012). Furthermore, a pilot clinical trial has shown that administration of a copper-lowering agent can partially resensitize platinum-resistant high-grade epithelial ovarian cancer patients to platinum chemotherapy (Fu S et al., 2012). Altogether, these observations suggest a role for hCtr1 in cisplatin resistance and support larger studies to assess the efficacy of this approach in platinum-resistant cancers treatment. Other transporters such as Solute Carrier Family 22 Members 1 and 2 (Li Q and Shu Y, 2014; Yonezawa A et al., 2006) have also been implicated in cisplatin uptake; nevertheless, more studies are required to delineate their role in cisplatin resistance as well as their potential use as biomarkers for resistance to platinum cytotoxic agents.

2.2.2. Cisplatin efflux

The efflux of platinum-based drugs is believed to be mediated by Transporting P-type Adenosine Triphosphatases ATP7A and ATP7B (Drayton RM et al., 2012; Stewart DJ, 2007; Tadini-Buoninsegni F et al., 2014) (Figure 2). Unsurprisingly, the overexpression of these transmembrane carriers have been implicated in cisplatin resistance and poor patient survival in several cancers (Konkimalla VB et al., 2008; Tadini-Buoninsegni F et al., 2014; Yoshizawa K et al., 2007). Silencing *ATP7A* in cisplatin-resistant tumor cells restored cisplatin sensitivity to a certain extent and enhanced apoptosis, suggesting that ATP7A may also be a target to sensitize cancer cells to cisplatin (Li ZH et al., 2012). Resistance to cisplatin has also been associated with the overexpression of Multidrug resistance-associated proteins (MRP) 1, 2, 3 and

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5, members of the superfamily of ABC transporters (Borst P et al., 2000; Guminski AD et al., 2006; Konkimalla VB et al., 2008; Song Y et al., 2015) (Figure 2). In particular, MRP2 expression seems to determine the efficacy of cisplatin-based chemotherapy in patients with hepatocellular carcinoma (HCC) (Korita PV et al., 2010). Furthermore, a reduction in MRP2 expression using ribozymes has been shown to restore cisplatin sensitivity in cisplatin-resistant adrenocortical carcinoma and melanoma cell lines (Materna V et al., 2005), further suggesting a role for this molecule in cisplatin resistance. Nevertheless, it has been demonstrated that cisplatin is not a substrate for MRP transporters and that its ability to transport the drug across the cell membrane requires the formation of glutathione- or metallothioneins-cisplatin conjugates (discussed in detail in the following section) (Chen ZS et al., 1998; Fujiwara Y et al., 1990; Kelland L, 2007) (Figure 2).

2.2.3. Detoxification by intracellular molecules

Although purine nucleobases are the main therapeutic target for cisplatin, a vast majority of cisplatin molecules will react with other ligands before reaching the nucleus. In particular, cisplatin shows much higher affinity for thiols than for nitrogen groups, hence making intracellular cisplatin detoxification via these functional groups one of the main mechanisms of cisplatin scavenging (Dabrowiak JC et al., 2002). As such, resistance to cisplatin has been associated with the overexpression of metallothioneins, a class of low-molecular weight cysteine-rich proteins that promote intracellular homeostasis of physiological metals, providing protection against metal toxicity and oxidative stress (Knipp M, 2009) (Figure 2). This has been observed in different cancer models (Gansukh T et al., 2013; Peng B et al., 2012) as well as in bladder (Siu LL et al., 1998; Wülfing C et al., 2007), colorectal (Hishikawa Y et al., 2001), esophageal (Yamamoto M et al., 1999), and breast (Bay BH et al., 2006; Lai Y et al., 2011) carcinomas, among other neoplasia.

Glutathione (GSH), a thiol containing tripeptide (Glu-Cys-Gly), highly expressed in mammalian cells, also plays a key role in cisplatin scavenging (Rocha CR et al., 2014) (Figure 2). Glutathione-S-transferase 1 (GSTP1-1) conjugates the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification and catalyzes the formation of cisplatin-GSH conjugates (Peklak-Scott C et al., 2008; Sawers L et al., 2014). As such, increased expression of enzymes involved in GSH

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synthesis and GSH conjugation have been implicated in the generation of cisplatin resistance (Galluzzi L et al., 2012; Rocha CR et al., 2014). Also, elevated levels of glutamate/cysteine transport system (system Xc) were linked with higher intracellular levels of GSH and resistance to cisplatin (Okuno S et al., 2003). The efflux of intracellular glutathione S-cisplatin conjugates is an ATP-dependent process mediated by integral membrane glycoproteins belonging to the MRP family (Ishikawa T and Ali-Osman F, 1993; Keppler D, 1999). Furthermore, MRP transporters are overexpressed in cisplatin-resistant human cancer cells whose GSH levels are substantially enhanced (Chen HHW and Kuo MT, 2010; Ishikawa T et al., 1994), suggesting that GSH and MRP proteins work in synergy for detoxification purposes.

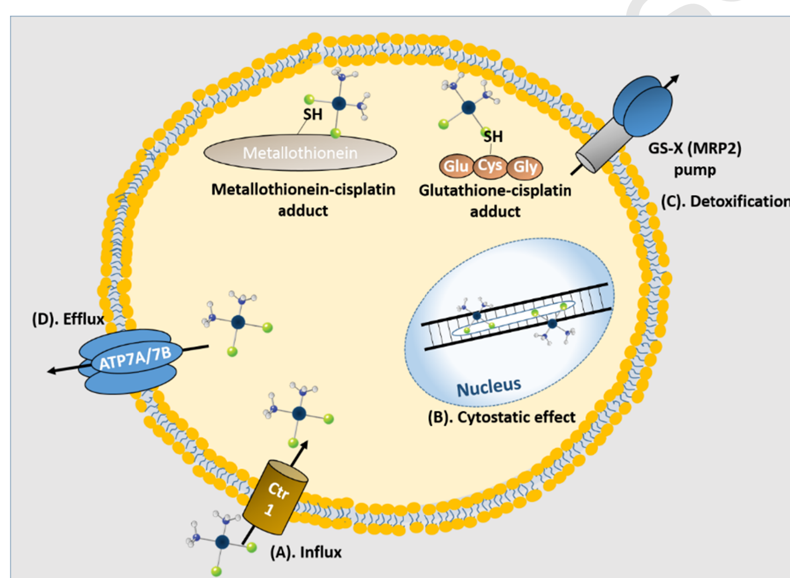

Figure 2. Intracellular cisplatin concentration depends on several detoxification mechanisms. **(A)**. Cisplatin uptake is mainly mediated by high affinity copper transporter protein 1(Ctr1). Once inside the cell, cisplatin has a number of possible targets: DNA; RNA; sulfur-containing enzymes such as metallothionein and glutathione; as well as mitochondria. **(B)**. The alkylating capability of cisplatin enables the formation of adducts with N7 atoms of DNA purine nucleobases, resulting in inter- and intra-strand DNA cross-links that provoke cell cycle arrest. **(C)**. Glutathione-cisplatin and metallothionein-cisplatin conjugates are excreted by MRPs, hence decreasing intracellular cisplatin accumulation. **(D)**. The efflux of platinum-based drugs is mainly mediated by Transporting P-type Adenosine Triphosphatases ATP7A and ATP7B.

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2.3. Alterations in DNA repair systems

Several DNA repair pathways interact to maintain accurate DNA replication, genomic integrity and normal cell functioning. These include: i) nucleotide excision repair (NER); ii) base excision repair (BER); iii) homologous recombination repair (HR); iv) mismatch repair (MMR); v) non-homologous DNA end-joining repair (NHEJ); vi) O^6- alkylguanine DNA alkyl transferase (AGT) mediated repair. Increased efficiency of DNA repair systems is considered essential for the maintenance and longevity of stem cell phenotypes and plays a key role in cytotoxic drugs resistance (Martin LP et al., 2008). This review will discuss the most studied DNA repair pathways involved in platinum response, i.e., nucleotide excision repair (NER) and mismatch repair (MMR), and will briefly refer the role of *BRCA* in DNA repair (Figure 3).

Several reports describe associations between the alterations in the XPF-ERCC1 protein complex of the NER system and platinum-based treatment outcome (Kirschner K and Melton DW, 2010) (Figure 3). This complex is a structure-specific endonuclease involved in recombination, double strand break and inter-strand crosslink repair (Ahmad A et al., 2008). The impossibility to replace damaged nucleotide sequences promotes downstream activation of the mitochondrial BAX protein, inducing apoptosis (Pawlowski J and Kraft AS, 2000) (Figure 4). As such, XPF-ERCC1 overexpression has been found to promote resistance to platinum-based compounds *in vitro* (Arora S et al., 2010; Kirschner K and Melton DW, 2010; McNeil EM and Melton DW, 2012), most likely due to overefficient DNA repair. In agreement with these observations, retrospective studies involving patient's histological samples have highlighted an association between the overexpression of ERCC1, resistance to chemotherapy and poor outcome in squamous cell head and neck (Bauman JE et al., 2013) and bladder carcinomas (Sun JM et al., 2012). A recent clinical trial has also demonstrated that elevated expression of the *ERCC1* gene is an independent prognostic factor for overall survival in first line treatment of advanced gastric cancer (Kwon HC et al., 2007). Furthermore, a phase II trial showed that single nucleotide polymorphisms of *ERCC1* gene (118T/C and 8092C/A) could be used to determine the response of NSCLC patients to cisplatin (Mazzoni F et al., 2013). Another study has demonstrated that 8092C/A could be used as an independent predictive biomarker of better response in esophageal cancer patients treated with cisplatin-based regimens (Bradbury PA et al.,

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2009). An enhanced expression of ERCC1 has also been observed in esophageal (Zhao Y, Bao Q, Schwarz B, Zhao L, Mysliwietz J, Ellwart J, Renner A, Hirner H, Niess H, Camaj P, Angele M, Gros S, Izbicki J, Jauch KW, Nelson PJ, 2014) and ovarian (Abubaker K et al., 2013) platinum-resistant cancer-stem cell sub-populations.

Cisplatin resistance has also been associated with somatic mutations in Mismatch Repair system-associated proteins (MMR), namely in MSH2 and MSH3 proteins (Clodfelter JE et al., 2005; Karran P et al., 2003; Park JM et al., 2013) (Figure 3). MSH2, together with MSH3, form the MutSβ heteroduplex which interacts with interstrand crosslinks induced by drugs such as cisplatin (Muniandy PA et al., 2010). Recognition of mismatched base pairs by MMR proteins may directly activate apoptotic pathways, thereby leading to cell death. As such, cisplatin-resistance is often accompanied by loss of expression of MMR proteins (Topping RP et al., 2009). Takahashi et al., has further demonstrated that MSH3 deficiency contributes to the cytotoxicity of platinum drugs through deficient repair of double strand breaks in the established colorectal cancer cell line HCT116 (Takahashi M et al., 2011). Moreover, Velasco A et al., demonstrated that decreased MMR expression in testicular tumors is associated with a shorter time to recurrence and death despite chemotherapy (Velasco A et al., 2008).

Likewise, disruption of the homologous recombination (HR) system, responsible for DNA double-strand break repair during the S-phase of the cell cycle, has been shown to modulate response to cisplatin (Wang QE et al., 2011; Wiedemeyer WR et al., 2014) (Figure 3). In particular, breast cancer susceptibility proteins 1 and 2 (BRCA1/2) play an important role in DNA repair by interacting with components of DNA repair systems and through gene expression regulation of homologous recombination, non-homologous end joining, and nucleotide excision repair intermediates. As such, BRCA proteins affect transcriptional regulation, cell cycle control, apoptosis, and ubiquitination (Deng CX and Wang RH, 2003; Mullan PB et al., 2006; Wu W et al., 2008). Normally, cancer cells with BRCA1/2 deficiency present defective DNA repair by homologous recombination and are sensitive to interstrand DNA crosslinking agents, such as cisplatin. Therefore, these agents are natural choices for the treatment for BRCA1/2-deficient tumors and were shown to be clinically effective (Shah NP, 2008). In contrast, during tumor expansion or as a result of chemotherapy, genetic reversion of *BRCA1* or *BRCA2* mutations can occur mainly due to secondary mutations and

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restoration of function, leading to cisplatin resistance (Dhillon KK et al., 2011; Peng G and Lin SY, 2011). Loss of function mutations in genes encoding HR system proteins, BRCA1/2 were associated with increased response to cisplatin in breast (Turner NC and Tutt AN, 2012) and ovarian tumors (Kwa M et al., 2014). Also, overexpression of *BRCA1* in cisplatin-resistant human breast and ovarian carcinoma cell lines (MCF-7 CDDP/R and SKOV-3 CDDP/R, respectively), resulted in increased resistance to cisplatin. In turn, antisense inhibition of *BRCA1* expression enhanced cisplatin sensitivity associated with decreased DNA repair by NER and increased apoptosis in the ovarian carcinoma cell line (Husain A et al., 1998). Furthermore, BRCA1 mRNA expression levels were also inversely correlated with sensitivity to cisplatin in malignant pleural effusions of NSCLC patients and in ascites of gastric patients (Wang L et al., 2008). In addition, several retrospective clinical studies have demonstrated that low BRCA1 mRNA expression was associated with longer survival of breast, ovarian, small cell lung cancer and esophageal squamous cell carcinoma (James CR et al., 2007; Margeli M et al., 2010; Quinn JE et al., 2007) subjected to cisplatin-based therapeutic regimens, and could be therefore used as predictive and prognostic marker. Moreover, several studies, recently comprehensively reviewed by Buckley et al., suggest a role for *BRCA1* in stem cell regulation through activation of the p63 and Notch pathways (Buckley NE et al., 2012).Collectively, these studies suggest that functional mutations in *BRCA1* as well as reduced BRCA1 mRNA levels may predict a benefit from DNA-damage-based chemotherapy. Furthermore, they highlight a link between *BRCA1*, stem cell regulation and response to chemotherapy. More detailed information regarding the role of DNA repair systems in resistance to platinum-based treatment can be found in recent reviews by Martin LP et al., (Martin LP et al., 2008).

Some of the above described DNA repair-associated effectors were found overexpressed in CSC, when compared with differentiated tumor cells (Maugeri-Saccà M et al., 2012). For instance, a significant increase in gene copy number of *BRCA1* and *RAD51* has been observed in prostatic CSCs compared with the adherent population isolated from the primary site (Maugeri-Saccà M et al., 2012). Therefore, it has been hypothesized that CSC are endowed with proficient DNA repair mechanisms which allow them to resist conventional chemotherapy and act as tumor initiators (Mathews LA et al., 2011), urging CSC-targeted therapeutics aimed at the elimination of these residual tumorigenic cell sub-populations.

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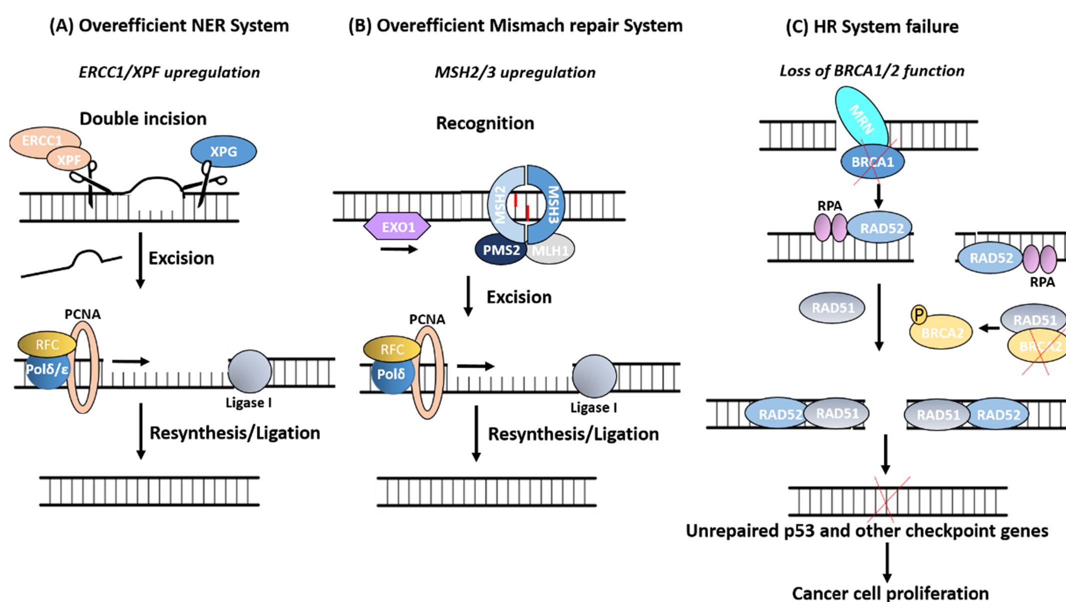

Figure 3. Alterations of DNA repair mechanisms mediate platinum-resistance. (A) Upregulation of XPF-ERCC1 complex results in resistance to platinum-based regimens by promoting NER overefficiency. Both XPG and XPF-ERCC1 are specific for junctions between single- and double-stranded DNA. XPG, which is closely related to the FEN-1 nuclease that participates in base excision repair (BER), cuts on the 3' side of such a junction, while ERCC1/XPF (a heterodimeric protein complex) cuts on the 5' side. The cut made by XPG is 2-8 nucleotides from the lesion, and the cut made by ERCC1/XPF is 15-24 nucleotides away. Next, the replicative gap-repair proteins, RFC, PCNA, and DNA polymerase delta or epsilon, bind to the 3'-OH generated by the ERCC1-XPF cut, and they carry out new DNA synthesis that fills the gap. The final nick is sealed by DNA ligase I. **(B)** Overexpression of MSH DNA-repair proteins leads to cisplatin chemoresistance. The DNA mismatch-repair system includes MSH2, MSH3/6, MLH1 and PMS2. MSH2–MSH3 heterodimers bind to single base-pair mismatches; then heterodimers such as MLH1–MLH3, and PMS2, as well as EXO1, are recruited to this complex. Then, the replicative gap-repair proteins and Ligase I fill and seal the gap, as previously described (Clodfelter JE et al., 2005; Takahashi M et al., 2011). **(C)** BRCA1/2 deficiency is associated with platinum resistance. After DNA damage is introduced, the MRN complex processes the ends of the DSBs. BRCA1 is phosphorylated by ATM and CHK2 (not shown) and regulates the MRN complex. RPA then associates with the 3′ ssDNA overhangs and becomes phosphorylated. Rad52 binds

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RPA and displaces it to allow for Rad51 binding. BRCA2 binds to Rad51 until BRCA2 becomes phosphorylated, releasing Rad51 and allowing it to localize to the DSB with Rad52. Rad51 then forms a nucleoprotein filament that invades a homologous sequence and activates strand exchange to generate a crossover between the juxtaposed DNA (not shown). BRCA. BReast CAncer genes; EXO1. Exonuclease 1; MLH. MutL homologues; MRN complex. This protein complex consists of Mre11, Rad50 andNbs1 proteins; MSH. MutS protein homolog; PCNA. Proliferating-cell nuclear antigen; PMS2. Post-meiotic segregation 2; Pol δ/ε. DNA polymerase δ/ε; RAD. Checkpoint protein; RFC. Replication factor C; RPA. Replication protein A.

2.4. Alterations in apoptosis-regulatory pathways

The cytotoxic effect of cisplatin is primarily mediated by the activation of a multibranched pro-apoptotic signaling cascade in response to the impossibility to repair DNA damage. Details on these mechanisms have been extensively and comprehensively reviewed by several groups (Basu A and Krishnamurthy S, 2010; Siddik ZH, 2003). As such, the response to cisplatin is significantly dependent on functional apoptotic pathways. Furthermore, genetic and epigenetic alterations in genes encoding key mediators of these processes have been long recognized to be associated with drug resistance (Dasari S et al., 2014; Galluzzi L et al., 2012; Shen D et al., 2012). These events may either result in enhanced survival stimuli, diminished death stimuli or diminished sensibility to death signals. Perhaps the most common and well documented apoptosis-related deregulation associated with cisplatin resistance stems from the inactivation of the tumor suppressor protein p53 (Galluzzi L et al., 2012; Siddik ZH, 2003) (Figure 4). Furthermore, several studies demonstrated that cancer patients harboring wild-type TP53 have a higher probability to benefit from cisplatin-based chemotherapy than patients with TP53 mutations (Gadducci A et al., 2002; Liang X et al., 2011). Consistently, mutations occur infrequently (< 3%) in germ cell tumors like testicular cancer, even though p53 protein is overexpressed in the vast majority of tumor samples (Guillou L et al., 1996), reinforcing the key role of p53 as a mediator of chemotherapy response. Other p53-related nuclear transcription factors including p63 and p73, are also part of a network that together with p53, contribute to regulation of apoptosis and tumorigenesis (Flores ER et al., 2005; Flores ER et al., 2002) as well as to

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chemotherapy-induced DNA damage response (Leong CO et al., 2007; Müller M et al., 2006; Rocco JW et al., 2006; Yuan M et al., 2010). Multiple promoters and alternative splicing events result in the expression of several isoforms of these transcription factors, which include full-length isoforms with transactivation domains (TA) homologous to that of full-length p53, and amino-terminally truncated (DeltaN) isoforms, which lack the TA domains. The TA isoforms of p63 and p73 are able to activate downstream target genes and promote apoptosis. Conversely, the DeltaN isoforms may act as dominant inhibitors of the full-length forms of p53, p63 and p73, hence impairing the activation of target genes and inducing apoptosis upon chemotherapy challenge (Courtois S et al., 2002; Marcel V et al., 2012; Takahashi R et al., 2014) (Figure 4). Several studies have further demonstrated that interfering with the expression or function of DeltaNp63 and/or DeltaNp73 and/or mutant p53 in tumor cells contributes to the improvement of tumor response to chemotherapy (Müller M et al., 2005; Müller M et al., 2006).

Alterations in other proteins mediating apoptosis triggered either by DNA damage or oxidative stress, via the mitochondrial pathway or by the extrinsic route, may also influence sensitivity to cisplatin (Henkels KM and Turchi JJ, 1999; Tanida S, Mizoshita T et al., 2012). Several death receptors, cytoplasmic adaptors, pro- and anti-apoptotic members of the Bcl-2 protein family, caspases, calpains, and mitochondrial intermembrane proteins are among the factors shown to modulate the response to cisplatin *in vitro* (Henkels KM and Turchi JJ, 1999; Park MS et al., 2002; van Oosterwijk JG et al., 2012). Moreover, some have been suggested to modulate clinical response (Aggarwal H et al., 2007; Muris JJ et al., 2005). Namely, high endogenous expression of anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-X_L, were associated with increased cisplatin resistance (Michaud WA et al., 2009). Likewise, loss of Bax and Bak causes complete resistance to cisplatin (Qian W et al., 2014) (Figure 4). Moreover, studies involving the combination of cisplatin with the Bcl-2-inhibitor ABT-737 have shown an improvement in the response to cisplatin, through a synergistic effect mediated by Noxa, to promote cell death and loss of clonogenic survival (Li R et al., 2009). Overexpression of survivin, a caspase-inhibitory protein often upregulated in response to cisplatin by phosphoinositide-3-kinase (PI3K)/AKT1-dependent mechanisms (Belyanskaya LL et al., 2005), has also been associated with lack of response to cisplatin and unfavorable outcome in gastric (Sun XP et al., 2014),

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esophageal (Kato J et al., 2001), ovarian (Jiang L et al., 2013) and NSCLC tumors (Wang HQ et al., 2014). Moreover, the administration of survivin inhibitors (for example, YM155, LY2181308) have also been found to improve the outcome of several malignancies (Church DN et al., 2012).

Several of the abovementioned mechanisms have been found to endow CSC with the capability to evade death signals, hence compromising chemosensitivity (Fulda S, 2013; Signore M et al., 2013). More detailed insights on the mechanisms regulating apoptosis in CSC can be found in recent reviews (He YC et al., 2014; Medina V et al., 2009). As recently revised by Signore et al., several strategies exploiting signaling pathways that govern self-renewal and survival of CSC are currently approved or are being tested in clinical trials (Signore M et al., 2013). Strategies to sensitize CSC to chemotherapy include the stimulation of death receptors using Apo2L/TRAIL inducers (Plasilova M et al., 2002; Ravi R et al., 2004). By promoting the activation of the apoptotic extrinsic pathway, this strategy has the potential to overcome drug resistance in many tumors harboring p53-inactivating mutations (Ashkenazi A et al., 2008). Several antibodies and small molecules targeting the mitochondria-associated apoptosis machinery have also been developed including the Bcl-2 inhibitor ABT-727 that was able to sensitize glioma CSC to TRAIL treatment both *in vitro* and in *vivo* (Tagscherer KE et al., 2008). The inhibition of EGF-R/Akt pro-survival signaling is also among the most explored strategies to indirectly induce apoptosis of CSC (Gallia GL et al., 2009; Signore M et al., 2013). Despite the significant success presented by these strategies, it is now consensual that sub-populations of CSC with distinct genetic features may co-exist within the same tumor (He YC et al., 2014; Wang W et al., 2014) and significant molecular variations were also observed between CSC isolated from tumors of the same organ and different organs (Frank NY et al., 2010). This phenotypic heterogeneity accounts for significant variations in response to targeted strategies and highlights the need for a careful evaluation of the signal transduction pathways that drive each sub-population of CSC to evade apoptosis, towards personalized cancer treatments. Furthermore, despite the encouraging reports with patient-derived xenografts capable of recapitulating CSC expression and tumor heterogeneity in immune deficient animals (Dobbin ZC et al., 2014; Whittle JR et al., 2015; Williams SA et al., 2013), more efforts should be devoted to the development of reliable clinical models able to support the development of new anti-cancer strategies.

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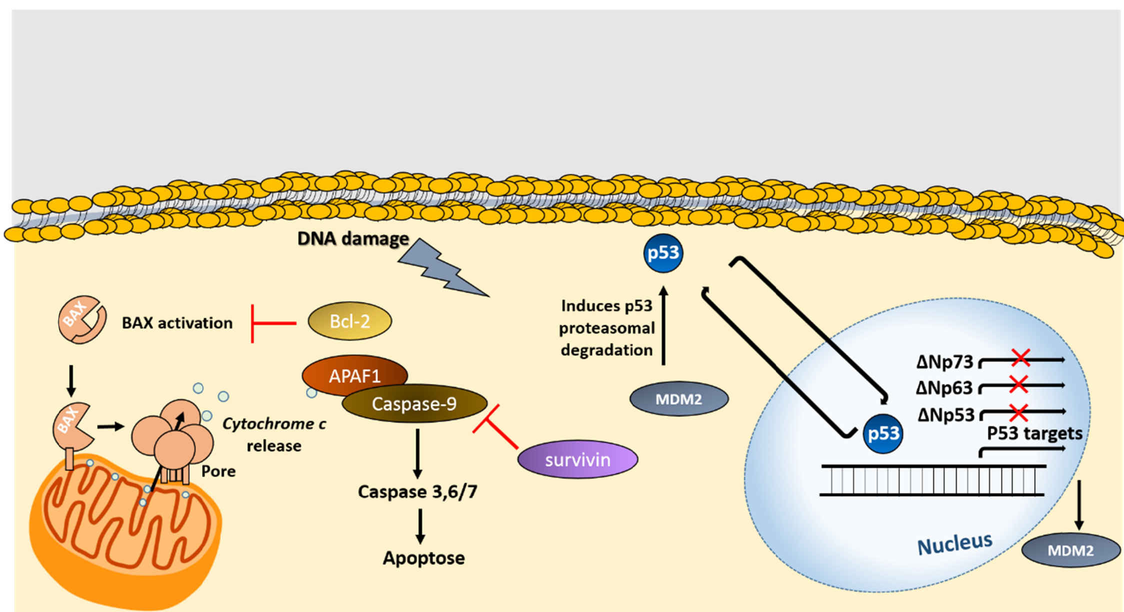

Figure 4 Anti-apoptotic members of the Bcl-2 family such as Bcl-2, Bcl-XL, and Bcl-w regulate the susceptibility to apoptosis through the intrinsic pathway. (A) BAX (B-cell lymphoma 2 (BCL-2)-associated protein X) is sequestered in an inactive state at the outer mitochondrial membrane by binding to members of the anti-apoptotic Bcl-2 family. Upon various stimuli, anti-apoptotic BCL-2 family members are displaced from BAX and lead to the release of cytochrome *c* from mitochondria. After being released, cytochrome *c* binds the apoptotic peptidase activating factor 1 (APAF1), which forms a complex with caspase-9. The complex activates additional caspases leading to cellular death. **(B)** DeltaN isoforms of p53, p63 and p73 may act as dominant inhibitors of the full-length forms, hence impairing the activation of target genes. MDM2 is a major cellular antagonist of p53 and interferes with the tumor suppressor function of p53.

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3. Protein glycosylation in cancer: Implications for chemotherapy response and CSC targeting

3.1. Protein Glycosylation: Structural diversity in cancer

Glycosylation is the most frequent, complex and plastic posttranslational modification of membrane-bound and secreted proteins and results from a coordinated action of nucleotide sugar transporters and biosynthesis pathways, glycosyltransferases

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and glycosidases in the endoplasmic reticulum (ER) and the Golgi apparatus (GA) (Pinho SS and Reis CA, 2015; Spiro RG, 2002). Glycans play a key role in protein folding, trafficking, stability, activity and act as mediators of cell-cell adhesion, cell differentiation, migration, modulation of cell signaling pathways, immune recognition and host-pathogen interactions (Haltiwanger RS et al., 2010; Ohtsubo K and Marth JD, 2006; Pinho SS and Reis CA, 2015; Shental-Bechor D et al., 2009). Two main classes of glycans can be found at the cell-surface: i) *O*-glycans, initiated in the Golgi apparatus by the initial attachment of GalNAc moieties to the hydroxyl groups of Ser or Thr residues of a given polypeptide chain (forming the Tn antigen GalNAcα-Ser/Thr, the simplest form of *O*-glycosylation); ii) *N*-glycans, whose biosynthesis initiates in the ER by the addition of an oligosaccharide chain to an Asn residue within consensus peptide sequences of Asn-X-Ser/Thr (X denotes any amino acid except proline). Less abundant forms of protein glycosylation include *O*-Fucosylation, *O*-Glucosylation and *C*-mannosylation of Thr residues (Pinho SS and Reis CA, 2015; Spiro RG, 2002). Protein glycan chains are often branched or elongated and may present sialic acids in blood group related antigens or ABO(H) blood group determinants as terminal structures (Dall'Olio F et al., 2012). Other modifications may include phosphorylation, *O*-acetylation of sialic acid and *O*-sulfation of galactose and *N*-acetylglucosamine residues, thereby increasing the structural complexity of the glycophenotype (Muthana SM et al., 2012). Furthermore, protein glycosylation patterns do not obey a predefined template (Lazar IM et al., 2011), as it is regulated at the tissue level, and promptly responds to physiological changes and cues (Ohtsubo K and Marth JD, 2006; Palorini R et al., 2013; Shirato K et al., 2011b; Testa R et al., 2015). In fact, variations in glucose and oxygen levels, among other microenviromental stimuli and signaling molecules, have been shown to influence the transcription and activity of glycosyltransferases and glycosidases, the trafficking of these enzymes to the ER and the Golgi apparatus and the availability of sugar donors (Carvalho AS et al., 2010; Dall'Olio F et al., 2012; Gill DJ et al., 2013; Pinho SS and Reis CA, 2015; Shirato K et al., 2011b). Recent studies have challenged the classical view of protein glycosylation as an intracellular event by demonstrating that glycans may experience further structural remodeling by extracellular enzymes (Lee MM et al., 2014; Nasirikenari M et al., 2014). This makes the glycome a highly dynamic molecular entity that mirrors a particular biological milieu and the glycomic/glycoproteomic characterization, a challenging analytical enterprise. Detailed insights on the structure of these glycans and their biosynthesis are

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available in several reviews and textbooks on the subject (Brockhausen I et al., 2009; Stanley P et al., 2009) and will not, therefore, be discussed in detail.

Malignant transformation is often accompanied by the expression of glycosylated structures that promote tumor growth, cell survival, cell-to-cell detachment, migration, immune evasion and metastasis (Figure 5, Christiansen MN et al., 2014; Dall'Olio F et al., 2014, 2012; Gomes C et al., 2013; Pinho S et al., 2007; Radhakrishnan P et al., 2014). In some cases, malignant tissues recapitulate the glycosylated antigens expressed during fetal life (Feizi T, 1985; Hakomori S, 1989; Varki A et al., 2009). Perhaps the most studied cancer-associated glycans include variants resulting from a premature stop in protein *O*-glycosylation, namely the Tn antigen (the simplest *O*-glycan), its sialylated counterpart sialyl-Tn (sTn; Neu5Acα2-6GalNAcα-O-Ser/Thr) and the T or core 1 antigen that results from the addition of a Gal residue to the Tn antigen (Galβ1-3GalNAc-Ser/Thr) (Cazet A et al., 2010; Ju T, Lanneau GS et al., 2008; Julien S et al., 2012; Marcos NT et al., 2004). These antigens have been classically termed simple mucin-type *O*-glycans reflecting the abundance of *O*-glycosylation sites in mucins and stem from the incapability of the cellular glycosylation machinery to produce more elongated glycans. Recently, a precision mapping of the human O-GalNAc glycoproteome has revealed over 6000 glycosites in more than 600 *O*-glycoproteins, the majority of which were found at the cell surface (Campos D et al., 2015; Schjoldager KT and Clausen H, 2012; Steentoft C et al., 2013; Steentoft C et al., 2011), thereby greatly expanding the view of the *O*-glycoproteome and its functional role. Of note, several intracellular proteins, including cytoplasmic, mitochondrial and nuclear proteins, have also been found expressing this type of posttranslational modification (Steentoft C et al., 2013). Despite the need for further analytical validation, these observations may suggest a currently neglected role for *O*-GalNAc glycosylation in intracellular physiological and pathological events. Similar subsequent studies have contributed to the notion that cells of different origins express a minor and unique *O*-glycoproteome that should be carefully explored when envisaging highly specific cancer biomarkers (Campos D et al., 2015; Campos D et al., 2015)

While hindered by extended glycosylation in healthy and benign tissues, simple mucin-type *O*-GalNAc glycans are uncovered in the majority of human carcinomas, particularly in advanced stages of the disease (Figure 5, Dall'Olio F et al., 2012; Freire-de-Lima L, 2014; Julien S et al., 2012; Marcos NT et al., 2004). The Tn, sTn and T

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antigens have been associated with malignant cell phenotypes, disease progression, metastasis and poor prognosis in clinical settings for a variety of different solid tumors (Cazet A et al., 2010; Julien S et al., 2012; Marcos NT et al., 2011; Reis CA et al., 2010a). Recently, we have reported the association between sTn, high-grade bladder cancer and muscle-invasion (Bernardo C et al., 2014; Ferreira JA et al., 2013; Lima L et al., 2013) as well as the capability of sTn$^+$-bladder cancer cells to promote a tolerogenic immune response of dendritic cells (Carrascal MA et al., 2014), demonstrating the involvement of glycans in the mediation of cancer immune responses. The fact that these simple glycans are absent, significantly underexpressed or restricted to some cell types in healthy tissues, renders them potential diagnostic and therapeutic targets. Proteins carrying abnormal glycosylation are often released from the cell-surface, therefore increasing sTn concentrations in the serum (CA72-4 test) of gastric, colorectal and pancreatic carcinoma patients (Carpelan-Holmström M et al., 2004; Reis CA et al., 2010b; Ychou M et al., 2000) as well as in pre-cancerous gastric lesions (Gomes C et al., 2013). The elevation of CA72-4 was shown to be an independent prognostic factor in gastric cancer (Louhimo J et al., 2004) and predictive of tumor recurrence in gastric (Ychou M et al., 2000) as well as pancreatic cancers (Louhimo J et al., 2004). Moreover, monoclonal antibodies have been developed for sTn-MUC1 glycopeptides (Sørensen AL et al., 2006) as well as the therapeutic vaccine Theratope that, despite promising initial results in pre-clinical and in early stage clinical trials for advanced breast tumors (Holmberg LA et al., 2000; Holmberg LA et al., 2004), it remains to be thoroughly assessed in other solid tumors known to overexpress sTn.

Several evidences also point to an increase in the complexity of *N*-linked glycans and glycolipids through β–1,6 branching, mediated by β-1,6-N-acetylglucosaminyltransferase V (Mgat5) in cancer (Figure 5, Fortuna-Costa A et al., 2014; Liu H et al., 2015; Pinho SS et al., 2013; Pinho SS et al., 2011; Taniguchi N et al., 2015). The β-1,6-N-acetylglucosamine-branched glycans are responsible to the high-affinity binding of Galectin-3 to several membrane glycoproteins and glycolipids, with implications to angiogenesis, metastasis, and other tumor progression events (Fortuna-Costa A et al., 2014; Funasaka T et al., 2014). For instance, lysosomal membrane-associated glycoproteins such as (LAMPs)-1and -2, Mac-1 and Mac-3, CD-98, CD-45, and CD-7 as well as EGF, TGF-β, and VEGF are known ligands for Galectin-3. Galectin-3 also binds CEA and MUC-1, among other relevant cancer-associated

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proteins (Fortuna-Costa A et al., 2014). Galectin-3 crosslinks cell surface glycoprotein receptors resulting in functional microdomains that regulate extracellular signal transduction (Boscher C et al., 2011; Lajoie P et al., 2009), glycoprotein receptor turnover and endocytosis (Lakshminarayan Ret al., 2014). As recently highlighted by several reports (Fortuna-Costa A et al., 2014; Liu FT and Rabinovich GA, 2005; Rabinovich GA and Toscano MA, 2009) galectin-3 mediates several hallmarks of cancer, including tumor growth(Peng W et al., 2008), anoikis resistance (Kim HR et al., 1999), inhibition of apoptosis (Takenaka Y et al., 2004), angiogenesis (Nangia-Makker P et al., 2010), cell adhesion (Khaldoyanidi SK et al., 2003), cell motility (Boscher C and Nabi IR, 2013), cell invasion (Tsuboi K et al., 2007) as well as microenvironment modulation (Nangia-Makker P et al., 2008; Reticker-Flynn NE et al., 2015). Increased β-1,6-branching of cadherins, integrins and other cytokine/growth factor receptors has also been found to enhance and promote tumor growth and metastasis (Carvalho S et al., 2015; Granovsky M et al., 2000; Pinho SS et al., 2013; Zhao Y et al., 2008) while the Mgat5 knockout inhibited these phenomena *in vitro* and *in vivo* in different experimental cancer models (Carvalho S et al., 2015; Dall'Olio F et al., 2012; Fortuna-Costa A et al., 2014; Lau KS and Dennis JW, 2008).

Two other common cancer-associated antigens are the α−2,3-sialylated forms of type 1 and type 2 Lewis blood group determinants Le^a and Le^x, sLe^a and sLe^x , that may be found as terminal epitopes of glycan chains in both glycoproteins and glycolipids (Carvalho AS et al., 2010; Dall'Olio F et al., 2012; Julien S et al., 2011; Kannagi R, 2007; Tozawa K et al., 2005). These glycans are specific ligands for E- and P-selectins in endothelial cells, two proteins that mediate leukocyte extravasation at sites of tissue injury in a sLe^a/sLe^x-dependent manner (Dall'Olio F et al., 2012). Similarly, these sialylated glycans are thought to act as regulators of the metastatic cascade by promoting endothelial adhesion (Kannagi R, 1997). In addition to a role in metastasis, selectin ligands are also thought to play a role in tumor growth and angiogenesis (Gomes C et al., 2013; Terraneo L et al., 2013). In particular, several reports associate the overexpression of sLe^a with decreased overall survival in digestive tract tumors (Kannagi R, 2007; Matsui T et al., 2004; Portela SV et al., 2011). Furthermore, serological detection of sLe^a on glycolipids and glycoproteins by the CA19-9 assay is used as a diagnostic tool to monitor clinical response to therapy for these tumors (Humphris JL et al., 2012; Kannagi R, 2007; Yang GY et al., 2013). Furthermore, the

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2,6-oversialylation of lactosamine chains and overfucosylation of terminal motifs are also among some of the commonly observed structural alterations present in cancer cells (Christiansen MN et al., 2014). An association between α-2,6-sialylation and invasive growth has been suggested by several studies (Dall'Olio F et al., 2014, 2012) and the oversialylation of β-integrins has been found to modulate intracellular signaling pathways towards increased cell survival (Dall'Olio F et al., 2014). Different mechanisms may account for the above described structural changes, namely alterations in transcription of biosynthetic and degradation enzymes, derangement of secreting organelles and availability of sugar nucleotides, promoted either by microenvironmental stimuli and/or dysfunctional glycogenes (Dall'Olio F et al., 2012; Itzkowitz SH et al., 1989; Reis CA et al., 2010b). Altogether, abnormal glycosylation is considered a typical hallmark of the transition from healthy to neoplastic tissues with direct influence on cell behavior and clinical outcome. More detailed information about the role of glycosylation in cancer may be found in a comprehensive recent review by Pinho and Reis (Pinho SS and Reis CA, 2015). Since alterations in glycosylation take place at the cell-surface resulting in highly distinct protein glycovariants, these present an opportunity for targeted therapeutics. However, as highlighted by recent publications, both similarities and differences exist in the glycosylation patterns of different tumors, mirroring the dynamic nature of the glycome (Christiansen MN et al., 2014). Furthermore, the same glycans may present opposite biological roles in different tumors, denoting the need for a comprehensive analysis of the glycoproteome in a clinical context, towards personalized cancer treatment.

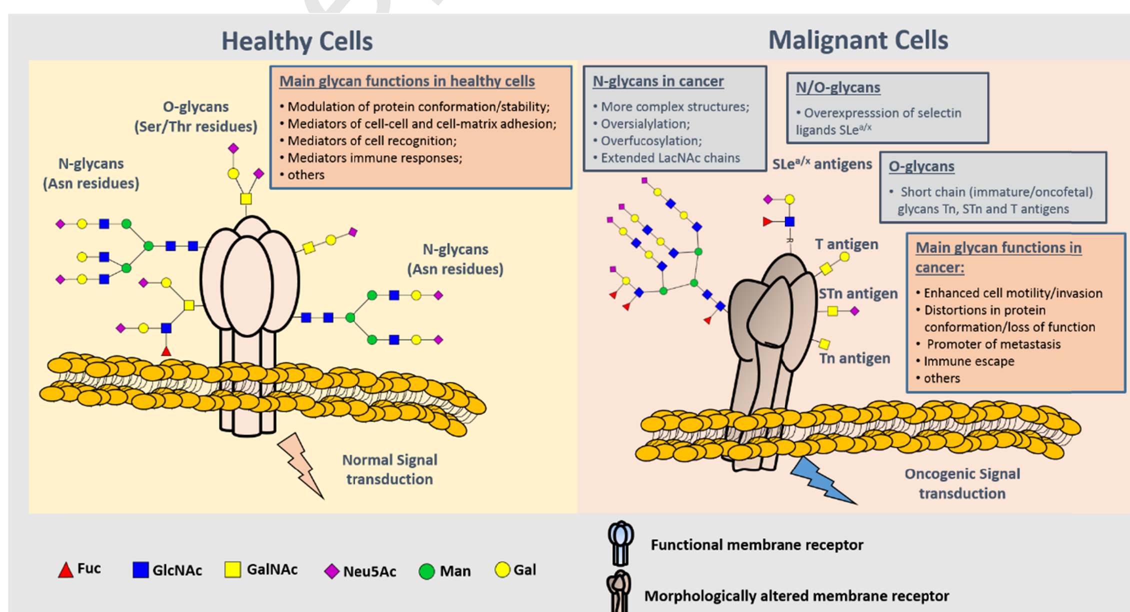

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Figure 5 A) Representation of protein *N*- an *O*-glycosylation in healthy tissues and malignant cells. Protein glycosylation plays a key role in the definition of protein folding and physiological functioning. Glycans contribute to cell-cell and cell-extracellular matrix adhesion, immune cell recognition, among other key biologic processes. Glycosylation is a highly dynamic posttranslational modification resulting from the concerted and highly regulated action of several glycosyltransferases in secretory organelles that rapidly changes in response to physiological stimuli. Several *N*- (Asn residues) and *O*- (Ser/Thr residues) glycans may coexist in the same protein backbone, depending on available glycosites and conformational constrains. In comparison to healthy cells, malignant cells tend to present more complex and branched oversialylated and/or fucosylated *N*-glycans. *N*-glycans may also be more extended by LacNAc chains. Conversely, more malignant clones present less complex and immature *O*-glycans, namely the Tn, sTn and T antigens that may be also found in oncofetal tissues. Some cancer cells may also express selectin ligands sLea/x as terminal structures of both *N*- and *O*-glycans. These structural alterations at the cell-surface favor more motile and plastic cell phenotypes, invasion, lymphatic and hematogenous dissemination and immune evasion. By impairing normal functions of cell-surface receptors, cancer-associated glycans also interfere with normal intracellular signaling transduction pathways towards the activation of oncogenic features.

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3.2. Protein glycosylation: contribution to chemoresistance

Several studies have described that subpopulations of multidrug resistant (MDR) cancer cell lines of distinct tissue origin present altered glycosylation patterns, particularly at the *N*-glycosylation level, when compared with the parental cell lines (Beretta GL et al., 2010; Kudo T et al., 2007; Noda I et al., 1999; Schultz MJ et al., 2013; Zhang Z et al., 2012; Zhao Y et al., 2014). Alterations in glycogene expression were also reported and, in most cases, have been found in agreement with the observed glycosylation patterns (Schultz MJ et al., 2013; Zhao Y et al., 2014). In particular, some studies have further associated the overexpression of cell-surface ATP binding cassette transporters (MRP1 and MRP4) and CD147 showing abnormal *N*-glycosylation patterns with drug resistance (Afonso J et al., 2014; Beretta GL et al., 2010). Furthermore, the exposure of chemoresistant cancer cells to tunicamycin, an *N*-glycosylation biosynthesis

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inhibitor, has been shown to sensitize these cells to anti-cancer agents (Kramer R et al., 1995; Noda I et al., 1999). Although these observations suggest that alterations in *N*-glycosylation may alter the functional properties of cell-surface transporters involved in multidrug resistance, it should be noticed that tunicamycin is responsible by inducing significant ER stress leading to apoptosis (Han C et al., 2008). Several structural alterations, namely α-2,6-oversialylation of cell-surface molecules, have also been shown to significantly modulate downstream oncogenic signaling pathways towards enhanced cell survival, tumor growth and migration (reviewed in detail by Dall'Olio F, et al. and Park J and Lee M) (Dall'Olio F et al., 2014, 2012; Park J and Lee M, 2013). Yet, the contribution of these events to chemotherapy resistance remains to be determined. Adding to these findings, several studies associated elevation of serum sTn antigen (detected by the CA72-4 test) and sLe^a (detected by the CA19-9 test) levels in cancer patients with both recurrence after chemotherapy, metastasis and poor overall survival (Kim DH et al., 2015; Yang GY et al., 2013; Ziske C et al., 2003), suggesting that the presence of these glycans may be associated with drug resistance. However, this hypothesis warrants confirmation in future studies. Altogether, these findings support the notion that glycosylation may constitute a currently neglected posttranslational modification of proteins which may be associated with cancer chemoresistance. Given its cell-surface nature, in-depth studies of these glycans may allow the selective targeting of these aggressive malignant clones and glycosylation remodeling may constitute a novel approach to overcome chemoresistance.

4. Chemotherapy challenge and CSC selection: exploring the role of glycosylation for guided therapeutics

During the last decade, CSC populations were described in various types of solid tumors, albeit their phenotypic and functional characteristics are still under intensive investigation (Tirino V et al., 2013; Visvader JE and Lindeman GJ, 2008). As a result, specific functional features such as self-renewal and long-term repopulation potential were identified in CSC and these characteristics were predominantly related to tumor initiation and maintenance of tumor growth (Al-Hajj M and Clarke MF, 2004). In addition, CSC exhibit a number of genetic and cellular adaptations that confer resistance to conventional chemotherapeutics such as relative dormancy/slow cell cycle kinetics,

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efficient DNA repair, overexpression of multidrug resistance efflux transporters and resistance to apoptosis (Abdullah LN and Chow EK, 2013; Cojoc M et al., 2015), as outlined in detail in the previous sections. Furthermore, several reports suggest that conventional therapy often results in enrichment and maintenance of CSC pools that form the tumor after chemotherapy, triggering relapse as chemoresistant tumors. In particular, Levina et al., (Levina V et al., 2008) suggested that cisplatin-containing chemotherapy leads to propagation of human lung cancer stem cells and inhibition of cell differentiation. Moreover, these cells appear to activate an efficient cytokine network that favors their tumorigenic and metastatic potential (Levina V et al., 2008). Other research groups related lung cancer recurrence with maintenance of treatment-selected CSC (Barr MP et al., 2013; Leung EL et al., 2010). For instance, using whole genome expression analysis, Hamilton et al., (Hamilton G and Olszewski U, 2013) demonstrated that small cell lung cancer (SCLC) cells express CSC markers (CD44, CD133, CD47, ALDH1A1, AKR1C), as well as WNT and Notch pathway intermediates after chemo-radiotherapy, compared to treatment-naïve cells. Bertolini et al., also reported that cisplatin treatment spares highly tumorigenic $CD133^+$ NSCLC cells that display stem-like features (Bertolini G et al., 2009). Zhang Yi et al., found similar correlations in cisplatin-resistant bladder cancer cells, which displayed enhanced self-renewal and tumorigenicity as well as higher levels of sphere formation, a larger proportion of side population cells and stem cell marker expression (Zhang Y et al., 2012). Regarding ovarian cancer, cisplatin-based chemotherapy also appears to preserve CSC-like cells, resulting in increased tumor burden (Abubaker K et al., 2013; Yasuda K et al., 2013).

These findings support the notion that the combination of conventional chemotherapy directed to bulk tumor cells and targeted strategies against CSC may hold promise to improve cancer management compared to monotherapies. Therefore, over the past decade many concentrated efforts have been put on the isolation and molecular characterization of CSC using chemotherapy selection approaches. In fact, the chronic or acute exposure of tumor cell lines, xenografts and patient samples to anticancer drugs has provided a simple strategy for CSC enrichment, as recently reviewed (Freitas DP et al., 2014). This has allowed the establishment of CSC-biomarker panels, which include, for example, the following proteins: ALDH1, CD29, CD24, CD44, CD90, and CD133 (de Beça FF et al., 2013; Langan RC et al., 2013; Yang CH et al., 2014). Nevertheless, these biomarker molecules fall short to discern

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cancer from normal adult stem cells, as previously reviewed in detail (Karsten U and Goletz S, 2013), hence prompting the introduction of more specific molecules for targeted therapeutics. Several authors now suggest that targeting oncofetal stem cell markers, normally not present in adult stem cells, may constitute a novel and more effective treatment strategy.

Exploring cancer-associated glycans, which are often of oncofetal nature, presents a unique and highly specific opportunity to target CSC. In fact, several studies reported alterations in the glycopatterns of CSC of different tissue origins compared to other cancer cells. Many of these findings concern the overexpression of glycans commonly found in glycolipids (CD60a, CD77, GD2, Gb4) and proteins (CD147 or Le^y, CD15 or Le^x) (Karsten U and Goletz S, 2013). More recently cumulative evidences confirm that CSC resistant to gemcitabine (Terao N et al., 2015) and doxorubicin (Azuma K et al., 2014) present distinct glycosylation patterns when compared to drug sensitive parental cell lines. Namely, Terao et al., described that CSC derived from the gemcitabine chemoresistant pancreatic cancer cell line Panc1 display significant overfucosylation and upregulation of fucosyltransferases, GDP-fucose synthetic enzymes, and GDP-fucose transporters (Terao N et al., 2015). However, knockdown of GDP-fucose transporters did not improve gemcitabine response, suggesting that overfucosylation is a result of CSC transformation with little influence on chemoresistance. On the other hand, Azuma K et al., explored the oversialylation of doxorubicin-resistant hepatocellular CSC towards the identification of highly specific glycoprotein species (Azuma K et al., 2014). These approaches have contributed to highlight the importance of an in-depth assessment of the glycome and glycoproteome of different CSC populations towards more specific biomarkers.

In this review we emphasize the growing amount of evidence supporting an association between the protein-specific cancer-associated *O*-glycans sTn and T and CSC proteins (Figure 6). The sTn and sialylated T antigens, which result from a premature arrest in protein glycosylation (as addressed in detail in the previous section), are absent from the majority of healthy tissues, but expressed in several solid tumors (Julien S et al., 2012; Marcos NT et al., 2011; Videira PA et al., 2009). Furthermore, many studies report an increased expression of these proteins in advanced stages of the disease (Julien S et al., 2012). Perhaps the most studied is the sTn antigen (CD72-4), whose presence in adult healthy tissues has been highly restricted to specific cells (Ferreira B et al., 2006; Julien S et al., 2012; Marcos NT et al., 2011). In contrast, the

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sTn antigen has been found overexpressed in many advanced stage solid tumors, where it plays a key role in endowing cancer cells with a metastatic potential; furthermore it is also present in the metastatic lesions (Ferreira B et al., 2006; Julien S et al., 2006; Marcos NT et al., 2011; Pinho S et al., 2007). Although more evidence are required, these findings suggest that sTn-expressing cells may be capable of detaching from the primary tumor and colonize distant tissue locations. Moreover, the sTn antigen was also detected in several fetal organs of both genders (e.g. esophagus, stomach, pancreas, colon, lung, mammary gland, gonadal tissue) (Itzkowitz SH et al., 1989; Julien S et al., 2012; Pistolesi S et al., 2001; Stanick D et al., 1988; Thor A et al., 1986). Although little is known about the biological role of sTn during embryonic development, these evidences suggest that cancer cells undergo, to some extent, similar molecular events. In spite of the possibility of sTn being expressed by all proteins presenting O-glycosylation sites, as demonstrated by several studies using engineered cancer cells to express this antigen (Campos D et al., 2015; Campos et al, 2015b; Julien S et al., 2012), few proteins have emerged from models that naturally express the antigen. Among these proteins are the heavily glycosylated MUC1 (Beatson R et al., 2015; Lakshminarayanan V et al., 2012) and CD44 (Campos D et al., 2015; Carrascal MA et al., 2014; Irimura T et al., 1999; Marcos NT et al., 2011), whose overexpression has been widely observed in CSC of different tumor models (Bourguignon LY et al., 2014; Curry JM et al., 2013; Nath S et al., 2015). Likewise, Karsten and Goletz discussed a similar role for the T antigen (CD176) in their recent review on this topic (Karsten U and Goletz S, 2013). Accordingly, this oncofetal antigen has been found in primary tumors and metastases, and detected in CD44 (Lin W et al., 2011; Singh R et al., 2001), CD45 (Baba M et al., 2007), and CD34 (Cao Y et al., 2008) of CSC from different cancer models.

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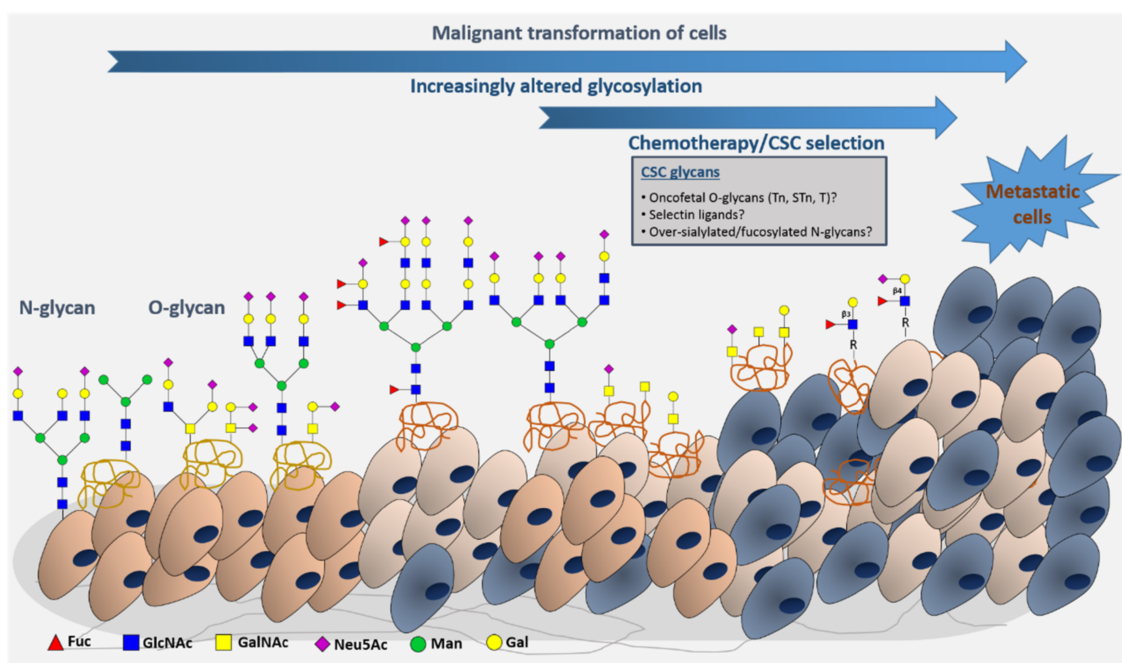

Figure 6. Representation of CSC selection based on chemotherapy with emphasis on cell-surface glycosylation patterns. It has been demonstrated that chemotherapy, while effective against the tumor bulk may promote the selection/development of highly malignant phenotypes, including CSC, responsible for the recapitulation of tumor heterogeneity and ultimately lead to disease dissemination. Increasing evidences support that CSC endowed with chemoresistant properties present distinct proteomes in comparison to more predominant cell populations. Figure 6 illustrates different cancer cells with distinct glycosylation patterns, including oversialylation and fucosylation of both *N*- and *O*-glycans. The selective pressure of chemotherapy towards CSC phenotypes (in blue) and the differentiated nature of CSC glycosylation is also illustrated. The overexpression of oncofetal immature *O*-glycans (Tn, STn, T) and alterations in terminal motifs, namely the overrepresentation of selectin ligands, are amongst the emphasized alterations. A careful glycomic and glycoproteomic evaluation of CSC stemming from chemotherapy challenge may provide unique biomarkers and templates for future drug design.

In summary, while still circumstantial, there is a strong suggestion that the expression of short-chain *O*-glycans is associated with CSC phenotypes. Furthermore, currently there are no direct links between cancer-associated *O*-glycans and chemoresistance. We therefore advocate that a careful investigation of the glycoproteome of CSC focusing on the expression of these fetal glycans may provide

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more specific biomarkers than the currently available panels and provide insights to the possible role of *O*-glycans in chemoresistance.

5. Concluding Remarks

Cancer cells show remarkable capability to resist chemotherapy treatments due to the synergistic effect of intrinsic, acquired as well as microenvironmental factors. Moreover, chemotherapy frequently acts as a positive selective pressure for the emergence of more aggressive cell subpopulations such as CSC, which are responsible for promoting disease relapse and progression. While many molecular mechanisms linking chemoresistance and CSC were already identified, CSC targeting remains a challenge, mostly due to the molecular similarities of CSC to non-malignant stem cells. We now comprehensively propose, for the first time, that alterations in protein glycosylation are amongst the key events accompanying and possibly driving chemoresistance. In fact, chemoresistant cancer cells supported by favorable microenvironments and endowed with intracellular mechanisms to evade cell death, present distinct *N*- and *O*-glycosylation patterns when compared to other cancer and non-malignant cells. Moreover, many studies have brought glycosylation into the spotlight by demonstrating that selective inhibition of *N*-glycosylation pathways may constitute an important therapeutic strategy to overcome chemoresistance. However, we argue that the influence of glycosylation extends beyond these events, given that it plays a pivotal role in the activation of several oncogenic pathways that sustain chemoresistance. Despite these insights, a direct link between protein glycosylation and chemoresistance remains to be established. Furthermore, the specific structural nature of CSC-associated glycans and glycoproteins, envisaging highly specific biomarkers has not yet been fully disclosed. Nevertheless, the context-dependent nature of glycans offers tremendous potential for the identification of unique CSC-glycoproteomes and clinically valuable biomarkers for cancer detection and targeted therapeutics.

As cancer treatment is shifting towards precision medicine and patient-tailored strategies (Kohane IS, 2015), we emphasize the need to integrate the patients' background, clinical history, tumor molecular features and the microenvironment in a single holistic perspective. Furthermore, we conclude that it will be imperative to address chemoresistance as a multifactorial and dynamic feature, considering many of the aspects discussed in the current review. Therefore, we come forward with a novel

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model (Figure 7) that incorporates evaluation of glycomics and glycoproteomics in comprehensive pan-omics approaches envisaging more accurate patient stratification, therapy selection, decision and design. Finally, we highlight that effective cancer treatments will likely benefit from multitargeted approaches selected based on a profound knowledge of the molecular nature of the tumor and patient's physiological and pathological status. The choice of treatment schemes and anti-cancer drugs should be dynamic and should Saccount for individual patient's response to treatment towards true precision medicine.

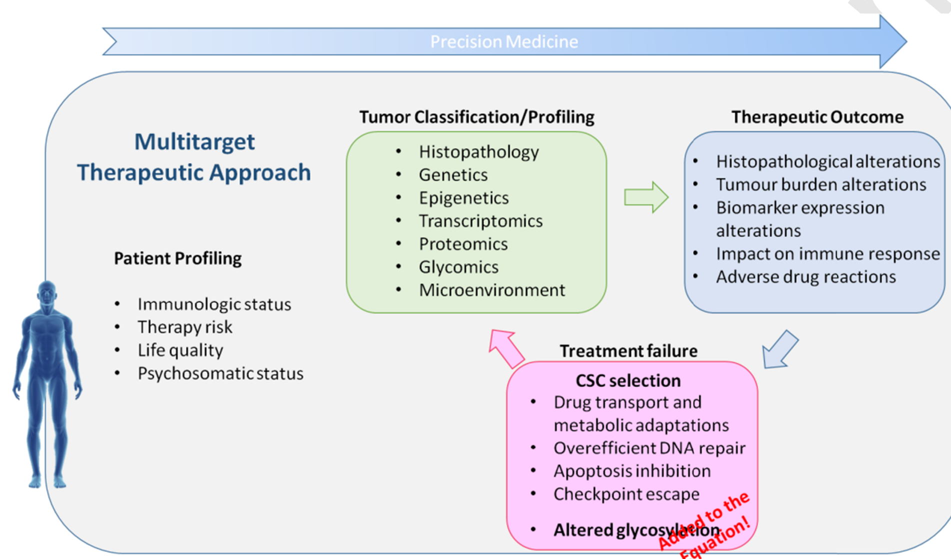

Figure 7. The concept of precision medicine: adding CSC selection to the equation. Effective cancer therapeutics should be based on a dynamic and adaptive decision-making process relying on patient's profile, tumor classification and patient's therapeutic outcome towards individualized treatments and improved responses. This process should include therapeutics specifically designed and targeted to CSC, which should allow the overcoming of chemoresistance mechanisms. Altered protein glycosylation may hold potential for novel targeted therapeutics approaches.

6. Future Perspectives

This review has highlighted the lack of specific biomarkers to target CSC and the necessity of more effective multi-target anticancer therapeutics to address chemoresistance. We believe that glycoproteins carrying oncofetal glycans, such as the sTn and Tn antigens, and other cancer-associated carbohydrates may help paving the

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way towards more specific targeted therapy. Focus should also be put on a careful and comprehensive glycoproteomic evaluation of chemoresistant clones and their microenvironmental context. These studies will greatly benefit from the tremendous advances, over the past two decades, in the development of highly sensitive mass-spectrometry platforms and protocols specifically designed to address the complex structural nature of protein glycosylation (Almeida A et al., 2013; Zhang Y et al., 2013). Moreover, while tumor cell lines are important starting models for proof-of-concept studies, it will be important to expand studies towards models able to more accurately mimic the tumor microenvironment. Future approaches should include direct human cancer xenografts in animal models, the so-called avatar models, which have been shown to preserve the molecular features of human tumors, including tumor glycosylation (Aparicio S et al., 2015; Bernardo C et al., 2014; Hidalgo M et al., 2014). New bioengineering approaches such as 3D Lab-on-a-chip settings may also provide the necessary system complexity to address drug toxicity in cancer cells, as demonstrated in several recent publications (Kim C et al., 2015; Ruppen J et al., 2014; Su M et al., 2014).

As already mentioned, true improvements in patient care and chemoresistance require the introduction of multi-target patient-tailored therapeutics. In this context, altered protein glycosylation may constitute the necessary mean to selectively deliver emerging nanotherapeutic agents to cancer cells, thereby improving the efficacy and reducing the toxicity of the treatments (Fernandes E and Ferreira JA et al., 2015; Livney YD and Assaraf YG, 2013). These nanodelivery sytems may include conventional chemotherapy drugs, genetic-based strategies such as the siRNAs against key oncogenic proteins involved in chemoresistance (Ganesh S et al., 2013; Navarro G et al., 2012; QingShuo M et al., 2013) or the administration of genes capable of rescuing the expression of p53 and other cell cycle checkpoint proteins (Chen GX et al., 2014; Kamal A et al., 2014; Kim SS et al., 2014; Li D et al., 2013). The introduction of antibodies against cancer-associated glycoepitopes may also allow the selective inhibition of oncogenic pathways or the stimulation of immune responses against malignant cells (Azevedo R et al., 2015). Glycan-based therapeutic solutions should also be tested, for the first time, in the context of hematological malignancies that also pose significant chemoresistance issues (Assaraf YG et al., 2014; Assaraf YG, 2007; Gonen N and Assaraf YG, 2012). Nevertheless, the particular differences between blood and epithelial cells should be taken into consideration when designing such strategies.

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In summary, exploiting altered glycosylation for targeted therapeutics is an emerging research topic that holds great potential for overcoming cancer chemoresistance phenomena. However, the roadmap for prototyping glycan-based therapeutics still requires a careful planning addressing the following research topics: i) comprehensive characterization of glycomic and glycoproteomic chemoresistant cells; ii) understanding the biological significance of altered glycosylation in chemoresistance; iii) development of glycan-specific ligands; and iv) establishment of cancer animal models mimicking human glycosylation.

Conflict of interest statement

None declared

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ANEXO B

Over forty years of bladder cancer glycobiology: Where do glycans stand facing precision oncology?

Review

Over forty years of bladder cancer glycobiology: Where do glycans stand facing precision oncology?

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ABSTRACT

The high molecular heterogeneity of bladder tumours is responsible for significant variations in disease course, as well as elevated recurrence and progression rates, thereby hampering the introduction of more effective targeted therapeutics. The implementation of precision oncology settings supported by robust molecular models for individualization of patient management is warranted. This effort requires a comprehensive integration of large sets of panomics data that is yet to be fully achieved. Contributing to this goal, over 40 years of bladder cancer glycobiology have disclosed a plethora of cancer-specific glycans and glycoconjugates (glycoproteins, glycolipids, proteoglycans) accompanying disease progressions and dissemination. This review comprehensively addresses the main structural findings in the field and consequent biological and clinical implications. Given the cell surface and secreted nature of these molecules, we further discuss their potential for non-invasive detection and therapeutic development. Moreover, we highlight novel mass-spectrometry-based high-throughput analytical and bioinformatics tools to interrogate the glycome in the postgenomic era. Ultimately, we outline a roadmap to guide future developments in glycomics envisaging clinical implementation.

INTRODUCTION

Bladder cancer, particularly muscle invasive bladder cancer (MIBC), is amongst the most common and deadliest genitourinary cancers [1]. The mainstay treatment for advanced stage tumours includes surgery and cisplatin-based chemotherapeutic regimens [1], which fail in avoiding tumour relapse and disease progression. Tremendous efforts have been put in the establishment of biomarker panels for early diagnosis, follow-up, patient stratification, prognosis, treatment selection and development of targeted therapeutics [2]. However, the highly heterogeneous molecular nature of bladder tumours has hampered true developments in this field [3]. Moreover, bladder cancer remains mostly an "orphan disease" in terms of targeted therapeutics, leading to few improvements in patient's overall survival over the last decade [2, 4]. More detailed information on the clinicopathological nature of bladder tumours and critical aspects in disease management have been recently

reviewed [5]. A schematic illustration of bladder cancer staging and grading is shown in Figure 1.

Several decades of glycobiology research have disclosed the existence of profound alterations in the glycosylation patterns of bladder tumours, reflecting specific changes in glycan biosynthetic pathways, glycosyltransferases expression, amongst other factors [6]. These events often lead to novel protein and lipid glycoforms, either by incomplete or neo-synthesis of glycan epitopes, that cannot be found in the corresponding healthy tissues and preneoplastic lesions. These events play a key role in tumour progression by affecting ligand-receptor interactions, and interfering with regulation of cell signaling, adhesion, migration, proliferation, angiogenesis, and immune responses [6]. Moreover, cancer-associated glycans may be actively secreted into bodily fluids (e.g. blood and urine) or shed from apoptotic and necrotic cancer cells [7]. As such, glycans and abnormally glycosylated molecules (e.g. proteins and lipids) hold tremendous value for non-invasive cancer detection, while membrane bound glycans may be used to selectively target tumour sites and specific cancer cells. Nevertheless, the structural complexity and heterogeneity of oligosaccharides, and the lack of analytical methods for elucidating structures still pose a major difficulty when addressing the glycome, glycolipidome and glycoproteome [8]. Still, a plethora of mass spectrometry-based analytical approaches have been developed to address these challenges [8, 9] and the standardization of high-throughput glycomics is expected to boost our knowledge on bladder cancer glycobiology in the near future.

Based on these considerations, the present review comprehensively summarizes the clinical significance of the main biomarkers arising from over forty years of bladder cancer glycobiology research and establishes the milestones towards clinical applications. Ultimately, we discuss the need to integrate glycans in holistic panomics models for precision oncology, namely the molecular-based individualization of patient care.

Glycosylation signatures in bladder cancer: biological and clinical implications

Glycosylation is the most frequent, complex and plastic post-translational modification of secreted and membrane-bound proteins, as well as a common substitution in lipids at the cell membrane [10]. Glycans are secondary gene products resulting from the coordinated action of nucleotide sugar transporters, glycosyltransferases and glycosidases in the endoplasmic reticulum (ER) and Golgi apparatus (GA) of mammalian cells [10]. Glycans are involved in several structural, modulatory, molecular mimicry and recognition roles including protein folding, stability, adhesion and trafficking, as recently reviewed [11]. Alterations in glycosylation patterns are common features of solid tumours, being detected even in pre-malignant lesions [12]. Generally, the most frequently described cancer-related glycosylation modifications include the synthesis of highly branched and heavily sialylated glycans, the premature termination of biosynthesis, resulting in the expression of short-chained forms, and the expression *de-novo* of glycosidic antigens of foetal type [13]. These structural motifs are mostly associated with: i) altered glycogenes expression [14, 15]; ii) impaired glycosyltransferases' chaperone function [16]; iii) altered glycosidase/glycosyltransferase activity [15]; iv) reorganization of glycosyltransferases topology [17, 18]; v) bioavailability of sugar nucleotide donors and cofactors [19]; vi) alterations on the conformation of peptide backbone or on the nascent glycan chain structure [19]. The resultant aberrant and cancer-associated glycans seem to be implicated in the activation of oncogenic pathways [20], establishment of tumour-tolerogenic immune responses [21], and in epithelial-to-mesenchymal transition (EMT), a crucial milestone towards invasion and metastasis [22, 23]. Thus, many glycoepitopes, and their related glycosidases/glycosyltransferases, can be considered relevant tumour-associated antigens [24, 25], with possible clinical significance in bladder cancer. Therefore, the following sections will focus on these key findings in bladder cancer glycobiology (summarized in Supplementary Table 1 - Supplementary material). Given their structural complexity and broad distribution, known cancer-associated glycogenes, glycosyltransferases and glycans will be presented in the context of specific classes of biomolecules (glycoproteins, glycolipids, proteoglycans).

Protein glycosylation

Two main classes of glycans can be found altered in cancer cell-surface proteins, namely *N*-glycans, attached to the peptide sequence via an asparagine (Asn) residue, and *O*-glycans, attached by a *N*-acetylgalactosamine (GalNAc) residue to the hydroxyl group of a serine (Ser) or threonine (Thr) residue.

Cancer-associated N-glycans

Protein *N*-glycosylation takes place in the ER, where the oligosaccharide transferase complex (OSTase) scans nascent proteins for Asn-X-Ser/Thr "sequons" ("X" stands for any amino acid residue except proline), and transfers a precursor glycan ($Glc_3Man_9GlcNAc_2$-) from dolichol pyrophosphate to Asn residues [26]. At this point, all *N*-glycans share a common core structure (Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn-X-Ser/Thr), which is further processed in the ER and GA by several glycosyltransferases and glycosidases, yielding mature core structures that may be classified into

three major *N*-glycan types (oligomannose, complex, and hybrid, Figure 2). The *O*-3 linked Man residues in hybrid and complex *N*-glycans may be further *O*-4 substituted with *N*-acetylglucosamine (GlcNAc) residues by GlcNAcT-III (GnT-III) to yield bisecting core structures. The introduction of the bisecting GlcNAc residue by GnT-III alters the composition and conformation of the *N*-glycan, resulting in the suppression of further processing and elongation [27, 28]. More highly branched *N*-glycans may be generated by the action of different GlcNActransferases (GnT-IV, -V, -VI). These structures may be further elongated with galactose, poly-*N*-acetyllactosamine, sialic acid, and fucose residues. Particularly, *N*-glycans frequently exhibit Lewis (Le) blood group related antigens (Le^a, Le^x, Le^b and Le^y) and corresponding sialylated structures or ABO(H) blood group determinants as terminal epitopes. Similar terminal structures may also be found in *O*-glycans (Figure 2). Other sugar modifications may include phosphorylation, *O*-acetylation of sialic acids, and *O*-sulfation of galactose and *N*-acetylglucosamine residues, thereby increasing the structural complexity of the glycome [29].

Several *N*-glycan alterations have been described in bladder tumours, including changes in branching and terminal structures through oversialylation, fucosylation (Supplementary Table 1), which will be discussed in detail in the following sections.

N-glycans branching

Alterations in *N*-glycans branching resulting from impaired GnTs expression have been evaluated in the context of bladder cancer prognosis. Namely, increased GnT-III, *N*-glycans bisection and GnT-IV expression were associated with higher disease stage and grade in bladder cancer patients [30]. Conversely, decreased GnT-V expression, responsible by *O*-6 *N*-glycans branching, was found associated with higher bladder tumour grade and stage, shorter disease-free survival and bladder cancer recurrence [31, 32]. Moreover, low GnT-V expression was found to predict shorter cause-specific survival of bladder cancer patients while overexpression of *O*-6 branched *N*-linked oligosaccharides was associated with lower tumour stage, suggesting that these findings could be applied to risk stratification [32]. The opposing associations of GnT-III and GnT-V in bladder cancer prognosis can be explained by the antagonistic effect of their enzymatic activity [28]. Contrasting with the findings for bladder cancer, reduced GnT-III and increased GnT-V

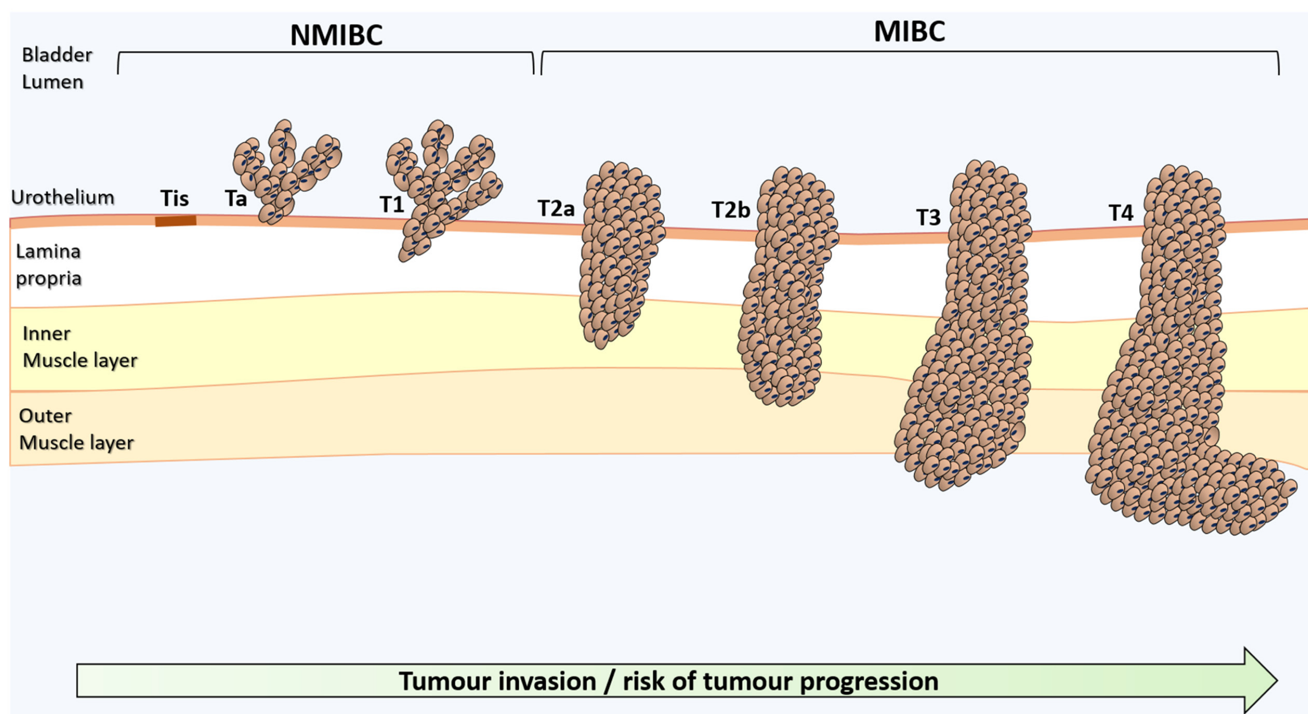

Figure 1: Schematic representation of bladder cancer stage and grade. The stage of the primary tumour (T) is based on the extent of penetration or invasion into the bladder wall. Regarding tumour grading, bladder lesions can be classified as urothelial papilloma (a benign lesion), papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade papillary urothelial carcinoma and high-grade papillary urothelial carcinoma. Of note, PUNLMP lesions do not have cytological features of malignancy and have a very low risk of progression. Nevertheless, they show high tendency to recur. Tis, Tumour in situ: "flat tumour"; Ta, Non-invasive papillary carcinoma; T1, Tumour invades sub-epithelial connective tissue; T2, Tumour invades muscle; T2a, Tumour invades superficial muscle (inner half); T2b, Tumour invades deep muscle (outer half); T3, Tumour invades perivesical tissue; T4, Tumour invades any of the following: prostate, uterus, vagina, pelvic or abdominal wall.

expressions have been found to promote metastasis in different cancer models [33-36] yet no consensus exists between GnT-V expression and prognosis in gastric [35, 36], oral squamous cell [37] and endometrial cancers [38]. These observations suggest GnT-V/III evaluation may hold potential for bladder cancer prognosis and ultimately targeted therapeutics, which warrants confirmation in future studies.

b) *N*-glycans terminal structures (also found in protein *O*-glycans and glycolipids)

Amongst the most common cancer-associated structural features are alterations of terminal glycan epitopes. In fact, the first reported glycosylation alterations in bladder cancer were the loss of ABO(H) blood group determinants in advanced stage carcinomas of secretor individuals [39, 40], as well as changes in Lewis antigens patterns.

The ABO(H) blood group system consists of terminal oligosaccharide antigens carried by glycoproteins or glycolipids in hematopoietic or epithelial cells [41]. Their biosynthesis is presumed to be controlled by the *ABO(H), Se, H, Le*, and *X* blood group genes [41]. These antigens are present on normal bladder epithelium of secretor individuals but not on some low-grade and early-stage papillary urothelial carcinomas [42]. Moreover, initially expressing tumours lose these cell surface antigens upon local recurrence, progression to invasion or metastization [42]. As such, the possibility that loss of genetically predicted blood group antigens precedes the development of recurrent, invasive or metastatic bladder cancer has been extensively explored [43]. Studies have shown that abnormally low or absent expression of these epitopes is frequently found in high grade and invasive bladder disease [44-46] and associated with bladder tumour progression and shorter recurrence-free survival

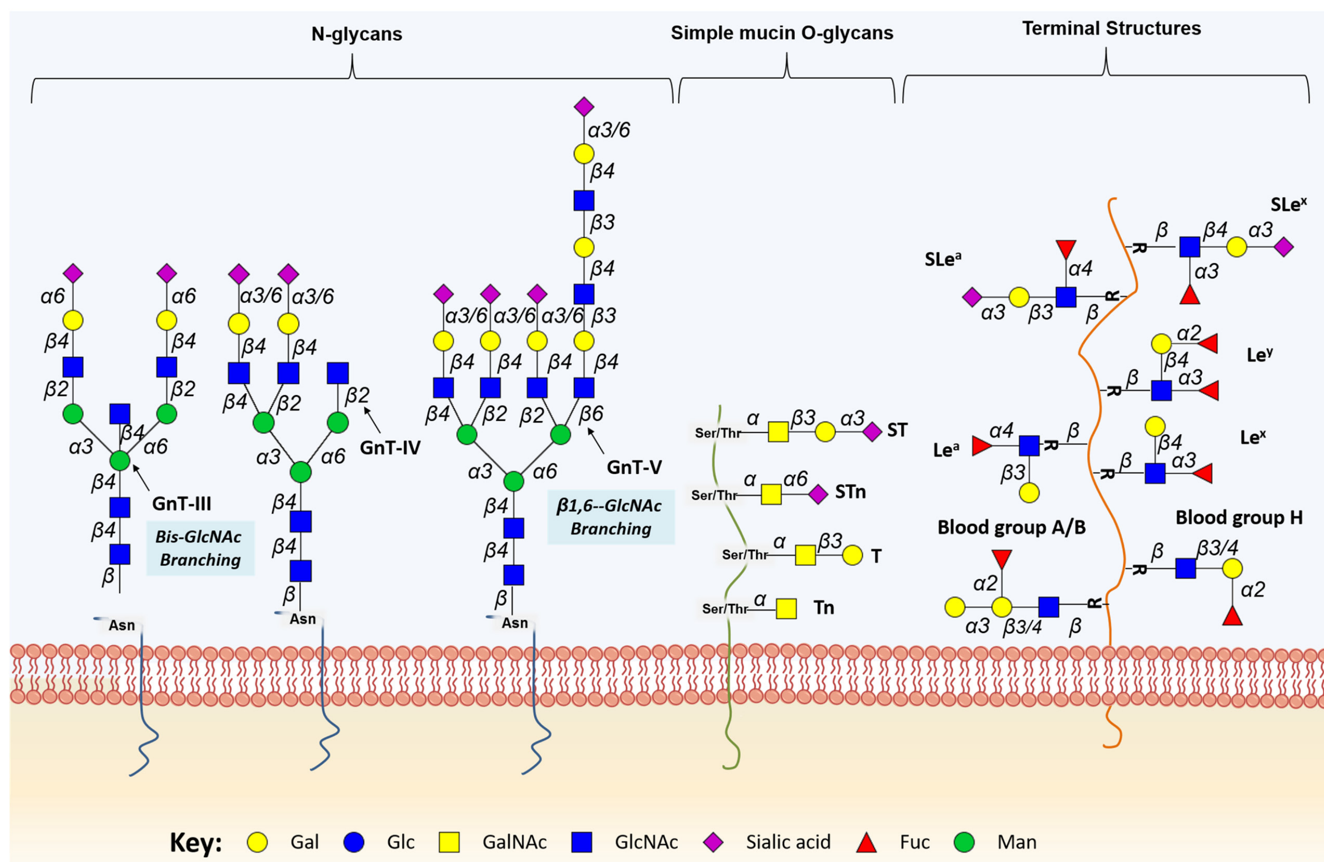

Figure 2: Schematic representation of protein-associated glycan structures relevant in bladder cancer. The figure represents specific *N*-linked and *O*-linked glycan structures, as well as terminal Lewis and sialylated Lewis structures that have biological significance in bladder cancer. Key enzymes mediating the addition of specific sugars are also shown. Protein *N*-glycan alterations include the β1-6 branching of *N*-glycans in result of GlcNAcT-V (GnT-V) overexpression, and the addition of bisecting GlcNAc branches by GlcNAcT-III (GnT-III) glycosyltransferases. Alterations in O-glycosylation pathways are also a common hallmark of malignant transformations of the bladder. Herein, we represent the overexpression of simple mucin-type O-glycans and their sialylated counterparts, T, sialyl T (ST), Tn and sialyl Tn (STn) antigens. Altered expression of terminal structures is also a common feature of bladder tumours. Namely, the abnormally low or absent expression of ABO(H) blood group determinants is frequently found in high grade and invasive disease. Carbohydrate terminal Lewis antigens are significantly under-expressed in healthy urothelium when compared to bladder tumours and are also highlighted here. Lewis type 1 antigens include Lewis^a (Le^a), and sialyl Lewis^a (SLe^a), while the type 2 group includes Lewis^x (Le^x) and sialyl Lewis^x (Sle^x).

[47]. Furthermore, loss of tissue ABO(H) antigens in the initial biopsy of bladder carcinomas predicts a much greater chance of subsequent invasion than in tumours with detectable ABO(H) antigens [44, 45, 47]. However, a significant number of patients whose initial tumours were reported as blood group antigen negative failed to develop an invasive tumour [47]. It is possible that these conflicting results may, at least in part, be explained by differences in methodology, interpretation, or both. Moreover, the loss of activity of the *A* and *B* gene-encoded transferases in bladder tumours from blood group A and B individuals was reported, which explains the deletion of these antigens in bladder tumours [48]. In addition, the loss of the ABO(H) gene and/or its promoter hypermethylation is a specific marker for urothelial carcinoma [39]. In summary, alterations in ABO(H) accompanying bladder malignant transformation and disease dissemination are well established surrogate markers of profound alterations in glycosylation pathways, constituting important starting points for more in depth structural studies.

The ABO(H) determinants have biosynthetic and structural similarities with Lewis antigens, including the fucosylated type 1 Lewis[a] (Galβ(1-3)GlcNAc[Fucα(1-4)]) and type 2 Lewis[x] (Galβ(1-3)GlcNAc[Fucα(1-4)]). Several authors have associated Lewis[a] and Lewis[x] expression patterns with malignant transformations of the bladder, reporting significantly lower expression of this antigen in healthy urothelium when compared to invasive tumours [44, 46]. As such, reduced expression of Lewis[a] and Lewis[x] was associated with higher tumour grade and invasion [44] and shorter recurrence-free survival [49]. As such, the expression of these antigens can be associated with worse bladder cancer phenotypes. Moreover, Lewis[a] antigen expression patterns change at an early neoplastic stage, suggesting that Lewis[a] determination might be useful in the diagnosis of very early premalignant changes in the urothelium [49]. In addition, scoring Lewis[a] expression allows the sub-classification of histologically identical tumours into prognostically different groups, pointing to a relationship between the pathological grade and stage of

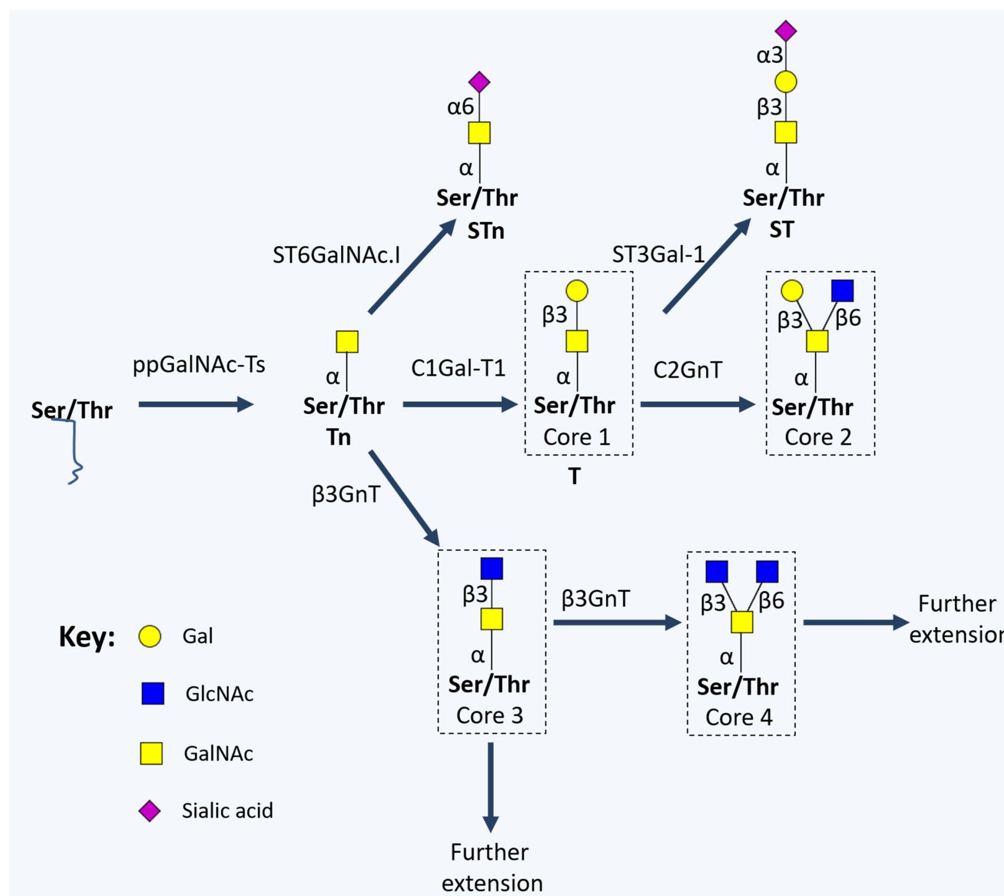

Figure 3: Schematic representation of short-chained O-linked glycan structures. The addition of specific sugar monomers to Ser/Thr residues of a protein backbone begins with the action of polypeptide N-acetylgalactosamine transferases (ppGalNAc-Ts; a family of 20 enzymes, including GalNAc-T1, GalNAc-T2, GalNAc-T3, GalNAc-T4, GalNAc-T5 and GalNAc-T6) giving rise to the Tn antigen, which is generally extended with a Gal residue by C1Gal-T1, originating the Thomsen-Friedenreich or T antigen (core 1). Alternatively, Tn and T antigens can be sialylated by α2,3-sialyltransferases (ST3Gal-Ts) and α-GalNAc ST6Gal-I (ST6GalNAc-I), forming the sialyl-Tn (STn), and sialyl-T antigens. On the other hand, core 1 may be extended originating cores 2-4 by the action of N-acetylglucosamine (GlcNAc) transferases (GnTs; such as GnT-III, GnT-V, core 2 GnTs (C2GnTs) and β3GnT).

the evaluated tumours and a morphological and functional de-differentiation [49]. Given this, Lewis^a antigen is a valuable functional marker of the malignant potential in superficial bladder cancer. In turn, the Lewis^x antigen is not expressed in normal urothelium, except for occasional umbrella cells [46, 50], but has been found in the majority of invasive tumours, regardless of blood type and secretor status of the individuals studied [46]. Lewis^y is expressed in both normal urothelium and bladder tumours, yet its expression was associated with bladder tumour invasion capability [46]. Nevertheless, the number of studies concerning Lewis antigens in bladder cancer is still scarce to withdraw conclusions about their biological and clinical significance.

c) Oversialylation and fucosylation (also occurring in protein *O*-glycans and glycolipids)

Oversialylation of cancer cells often stem from the overexpression of sialylated Lewis antigens sialyl lewis^a (SLe^a; the CA19-9 antigen) and sialyl lewis^x (SLe^x), which can be found as terminal epitopes of *N*-glycans, *O*-glycans and glycolipids [51]. SLe$^{a/x}$ are specific ligands for E- and P-selectins in endothelial cells, thereby promoting the adhesion of malignant cells to the endothelium and the metastatic cascade [50-52]. These antigens also thought to play a role in tumour growth, invasion, and angiogenesis [51, 53]. In line with these observations, the overexpression of SLe^a and SLe^x have also been associated with bladder cancer malignant potential. Particularly, serum overexpression of SLe^a was associated with higher

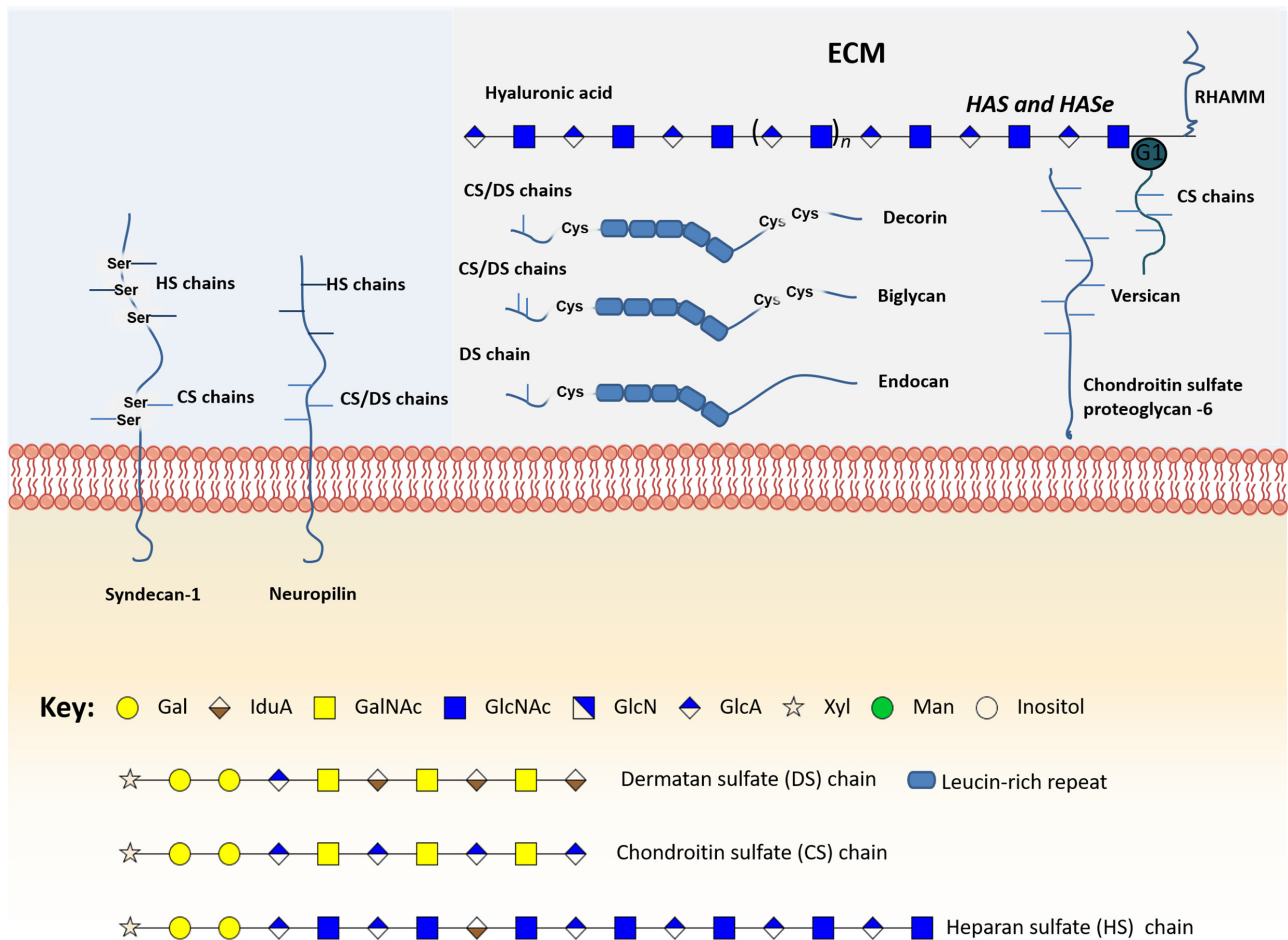

Figure 4: Schematic representation of the main glycomolecules with biological relevance in bladder cancer. The figure represents specific proteoglycans that have one or more glycosaminoglycan (GAG) chains, consisting of linear co-polymers of acidic disaccharide repeating units such as chondroitin sulfate (DS), heparan sulfate (HS) and dermatan sulfate (DS). These glycomolecules can be found attached to the outer leaflet of the plasma membrane (Syndecan-1) or in the extracellular matrix (Versican, Chondroitin sulfate proteoglycan-6, decorin and biglycan). Particularly, some of these structures can bind to each other through their N-terminal globular domain (G1), therefore increasing extracellular matrix (ECM) complexity. Of note, hyaluronic acid is the only GAG primarily found as a free sugar chain in the extracellular matrix. Hyaluronic acid synthases (HAS) and Hyaluronidases (HAse) constantly degrade and remodel hyaluronic acid molecules largely affecting ECM dynamics. Some glycoproteins can also be found linked to the cell membrane through a glycosylphosphatidylinositol (GPI) anchor, an example is glypican-3.

stage, grade and invasion [53] while tissue loss/reduction of SLe[a] expression was associated with higher atypia grade [50]. SLe[x] has been closely link to invasive and metastatic potential of primary bladder tumours and correlated with shorter 5-year and 7-year survival rates [54], but another study demonstrated no associations between SLe[x] with grade or stage in urothelial carcinoma of the renal pelvis, ureter, and urinary bladder [50]. The disialylated form of Le[a] (termed disialyl-Lewis[a], dSLe[a]) was described as preferentially expressed in non-malignant cells, and may be useful for distinguishing benign from malignant diseases mostly expressing SLe[a] [55]. Supporting these observations, the overall increase in cell-surface sialic acid content was shown to reduce the attachment of metastatic tumour cells to the extracellular matrix [56]. These observations support the need for a comprehensive interrogation of bladder cancer cells “sialome” towards understanding tumour progression and dissemination. Moreover, future studies should explore the biological and clinical relevance of structurally identical sialylated forms in the context of bladder cancer.

Fucosylation is another common modification involving oligosaccharides on glycoproteins and glycolipids [57]. Particularly, the quantitative glycome analysis of *N*-glycan patterns in bladder cancer cells often reveals significant differences in *N*-glycan fucosylation compared to normal cells. Namely, bladder cancer cells (KK47, YTS1, J82, T24) showed high expression of complex core-fucosylated *N*-glycans and low expression of terminally fucosylated *N*-glycans [58]. Nevertheless, the implications of these differential fucosylation patterns in bladder cancer malignancy have been so far poorly explored. The transcript levels of fucosyltransferase (FUT) VI (*FUT-VI*) and *FUT-VII* from invasive and non-invasive bladder tumours were also explored using RT-PCR. Particularly, bladder cancer cell lines from invasive tumours that maintained their metastatic properties showed high levels of both enzymes, and cell lines from non-invasive tumours (KK-47) or normal bladder epithelia (HCV-29) were negative for *FUT-VI* and *FUT-VII* [54]. These evidences suggest that *FUT-VI/-VII* expression associates with more malignant cancer cell phenotypes. Another study has described β1-integrin activation by alpha1,2-fucosyltransferase 1 (FUT-I)-mediated fucosylation in J82 human bladder cancer cells, thereby enhancing bladder cancer adhesion and subsequent metastasis [59]. As such, changes in bladder cancer fucosylation patterns seem to be associated with tumour invasion and progression to metastization in cancer cell lines, suggesting that these changes could provide novel strategies for cancer therapy.

Cancer-associated *O*-glycosylation

The most common form of cell-surface protein *O*-glycosylation results from the transfer of a GalNAc residue from a UDP-GalNAc donor to either serine or threonine in a given polypeptide chain (*O*-GalNAc glycosylation), originating the monosaccharide Tn antigen. This reaction is catalysed by several UDP-GalNAc:polypeptide *N*-acetylgalactosaminyl transferases (ppGalNAc-Ts) in the ER, in a substrate dependent manner [60]. As opposed to *N*-glycosylation, no consensus sequence is required for ppGalNAc-Ts recognition. The Tn antigen is generally extended with a Gal residue by Gal-transferase (β(1-3)-galactosyltransferase, C1Gal-T1 or T-synthase) and cosmc chaperone, originating the disaccharide Thomsen-Friedenreich or T antigen (Galβ1-3GalNAcα-O-Ser/Thr, core 1). Alternatively, Tn and T antigens can be sialylated by sialyltransferases, forming the sialyl-Tn (STn), sialyl-T and disialyl-T antigens. Sialylation stops any further processing of the oligosaccharide chain, prompting short-chain GalNAc-type *O*-glycans expression [60]. Alternatively, core 1 may be extended originating cores 2-4 (Figure 3), which are precursors for a vast array of more extended oligosaccharides and terminal structures, similar to the ones found in mature N-glycans.

Recently, a precision mapping of human *O*-GalNAc glycoproteome has revealed over 6000 glycosites in more than 600 *O*-glycoproteins, the majority of which of membrane origin [61], greatly expanding our view on the *O*-glycoproteome and its functional role. Alterations in *O*-glycosylation pathways are a common hallmark of malignant transformations, frequently amplified at the cell-surface as a result of the high number of *O*-glycosylation sites presented by mucins [62]. Such events are particularly pronounced in adenocarcinomas, due to the overexpression of these molecules [63]. While hindered by extended glycosylation in healthy and benign tissues, simple mucin-type *O*-GalNAc glycans are uncovered in most human carcinomas, including bladder cancer [45, 64-67].

a) Premature stop in *O*-glycosylation

Perhaps the most studied cancer-associated *O*-glycans are the Tn antigen, its sialylated counterpart sialyl-Tn (STn) and the T antigen. They result from a premature stop in protein *O*-glycosylation and are classically termed simple mucin-type *O*-glycans, reflecting their overexpression in cancer-associated mucins [68]. Nevertheless, these alterations can also be significantly observed in other densely *O*-glycosylated proteins of relevant importance in bladder cancer, namely CD44 and different types of integrins [69, 70]. Several reports attribute the expression of simple mucin-type *O*-glycans to a disorganisation of secretory pathway organelles in cancer cells, mutations on *Cosmc*, a gene encoding a molecular chaperone of T-synthase [16, 71], and absence or altered expression and/or activity of glycosyltransferases [72]. In particular, the overexpression of *ST6GalNAc-I* has been found to promote the premature sialylation of the Tn antigen and consequent formation of

the STn antigen in bladder cancer [64, 69]. Specifically, the STn antigen is absent in the healthy urothelium, while being present in more than 70% of high-grade NMIBC and MIBC, denoting a cancer specific nature [64]. This post-translational modification of cell surface proteins is mostly expressed in non-proliferative tumour areas, known for their high resistance to cytostatic agents currently used to improve the overall survival of advanced stage bladder cancer patients [64]. Recently, a novel STn-dependent mechanism for chemotherapeutic resistance of gastric cancer cells to cisplatin has been described, in which STn protects cancer cells against chemotherapeutic-induced cell death by decreasing the interaction of cell surface glycan receptors with galectin-3 and increasing its intracellular accumulation [73]. Nevertheless, the relationship between chemoresistance and STn overexpression remains to be fully explored in bladder cancer. Furthermore, STn expression is significantly higher in MIBC when compared to NMIBC, denoting its association with muscle invasion and poor prognosis [20]. Studies *in vitro* have further demonstrated that this antigen plays an important role in bladder cancer cell migration and invasion through mechanisms so far unexplored [64, 69]. Recent glycoproteomics studies of bladder cancer cell models highlighted that STn was mainly present in integrins and cadherins, further reinforcing a possible role for this glycan in adhesion, cell motility and invasion [69]. Also, recent work from our group has demonstrated the presence of STn in lymph node and distant metastasis, strengthening the notion that STn expression may influence cancer cell motility and metastization (unpublished data). Furthermore, STn-expressing bladder cancer cells have shown the ability to induce a tolerogenic microenvironment by impairing dendritic cells maturation, allowing cancer cells to evade innate and adaptive immune system responses [21]. Interestingly, the tolerogenic effect of short-chained *O*-glycans has also been correlated with bladder tumour metastasis through a mechanism

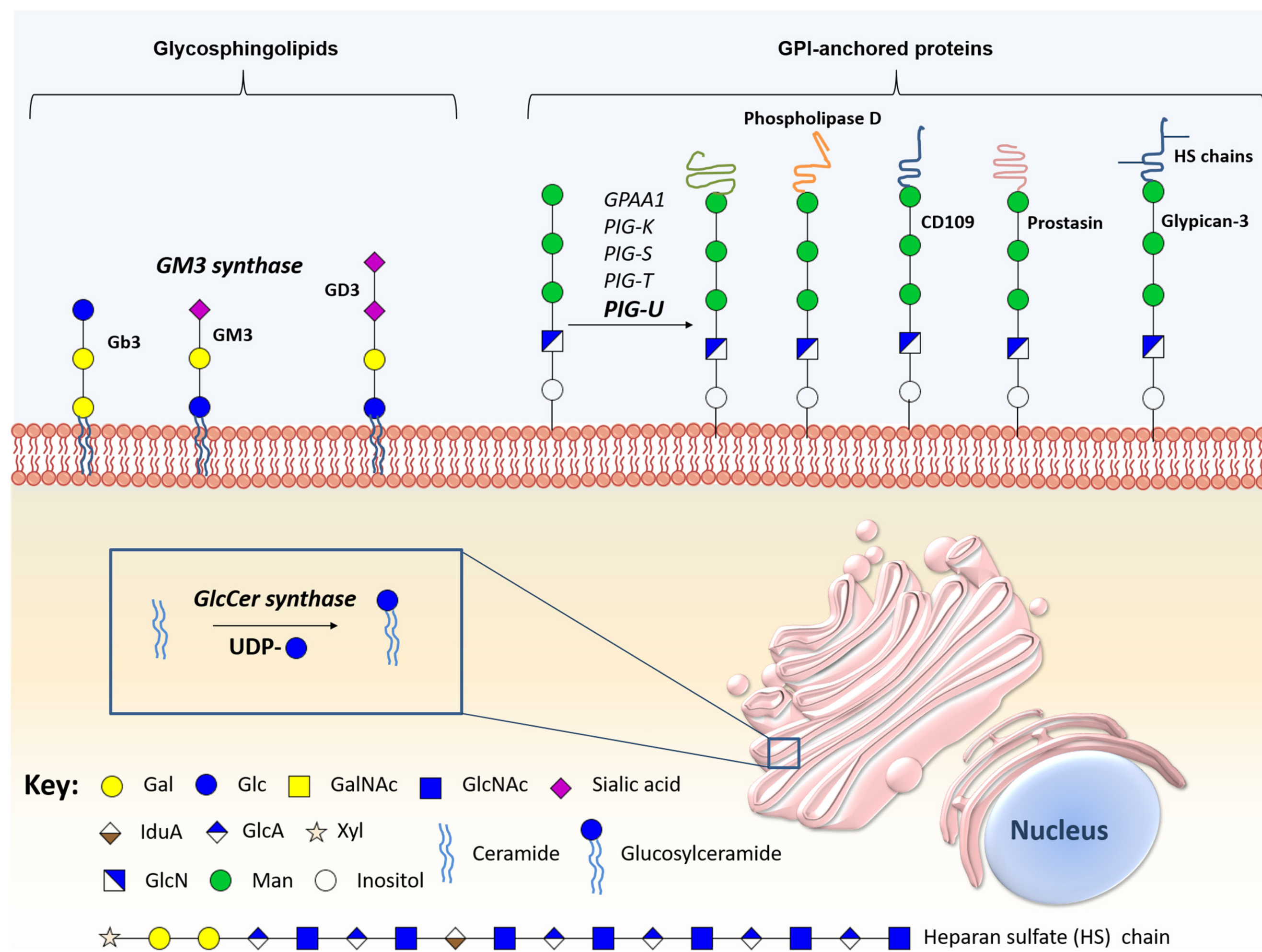

Figure 5: Schematic representation of the main biologically relevant glycosphingolipids and glycosylphosphatidylinositol-anchored proteins in bladder cancer. The figure represents certain glycosphingolipids, especially the sialic acid-containing glycomolecules, glycosylphosphatidylinositol-anchored proteins, and the enzymes implicated in the synthesis and hydrolysis of these conjugates have been implicate in bladder cancer malignancy.

in which MUC1 carrying core 2 *O*-glycans functions as a molecular shield against NK cells attack, thereby promoting metastization [74]. In addition, STn expression in bladder cancer tissues has been used in combination with other surrogate markers of tumour aggressiveness envisaging patient stratification regarding disease stage and therapeutic benefit. Specifically, expression of STn and sialyl-6-T (s6T), a sialylated form of T antigen, are independent predictive markers of BCG treatment response and were found useful in the identification of patients who could benefit more from this immunotherapy [75]. Moreover, STn was found to be a marker of poor prognosis in bladder cancer and, in combination with PI3K/Akt/mTOR pathway evaluation, holds potential to improve disease stage stratification [20]. In turn, it was observed that the reduction of Tn antigen expression was associated with higher bladder cancer stage [67].

Several reports associated the presence of T antigens with higher grade, stage and poor prognosis in bladder cancer [66, 76], suggesting that these antigens may be surrogate markers of profound cellular alterations. Also, there is growing evidences linking the overexpression of ST3Gal.I, the enzyme responsible for T antigen sialylation, with higher stage and poor prognosis [65]. Moreover,

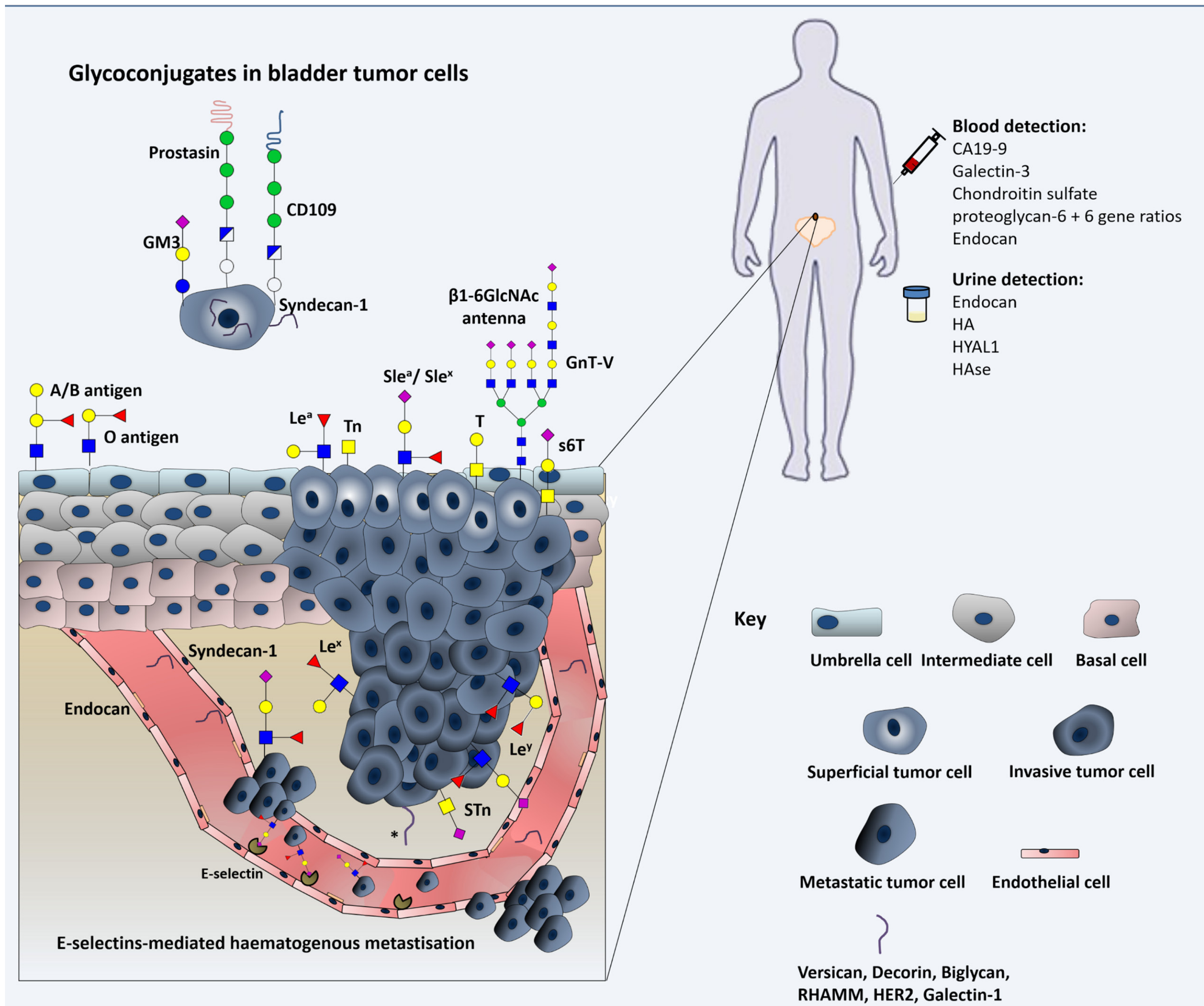

Figure 6: Schematic representation of the glycomolecule-mediated metastization model and diagnostic value of glycans. Herein we represent the process of tumour cell invasion, dissociation and metastization in which glycans interfere with cell-cell adhesion and haematogenous tumour cell spread. We emphasize the modification of epithelial cadherin with β1,6-*N*-acetylglucosamine (β1,6GlcNAc)-branched *N*-glycan structures, the loss of ABO(H) blood group determinants, changes in Lewis antigens patterns, and the oversialylation of glycans resulting in the over-expression of simple mucin type O-GalNAc glycans. Furthermore, expression of glycolipids, proteoglycans and gangliosides in cancer cell membranes can modulate signal transduction, activating various cellular pathways that induce tumour growth and progression. As such, some of these relevant glycomolecules are represented as well. The diagnostic value of some of these macromolecules is also highlighted.

the expression of T antigen is significantly associated with higher risk for subsequent recurrences with deep muscle invasion and metastatic involvement of regional lymph nodes [67]. In agreement with these observations, we have recently reported that short-chain *O*-glycans are preferentially accumulated in hypoxic tumour areas [69], known to harbor more malignant sub-populations. It has been suggested that HIF-1α directly or indirectly modulates the expression of glycosyltransferases involved in the initial steps of *O*-glycosylation while repressing core elongation, thereby promoting an accumulation of precursor structures [69]. The fact that these simple glycans are absent, significantly under-expressed or restricted to some cell types in healthy tissues, makes them ideal diagnostic and therapeutic targets for bladder cancer therapy [77].

Overexpression of cancer-associated membrane glycoproteins

Alteration in *N*- and *O*-glycosylation and other types of protein glycans are often amplified in cancer cells by the overexpression of key cancer-associated glycoproteins. Namely, HER2 (also known as ErbB2 or HER2/neu) is an heavily glycoprotein [78], member of the EGF receptor (EGFR) family, that is overexpressed in several malignancies, including advanced stage bladder cancer [79-81]. Curiously, the incidence of HER2 overexpression in bladder cancer (12.4%) is even higher than that found in breast carcinomas (10.5%), where it is associated with tumour aggressiveness, prognosis and responsiveness to therapy [81]. In fact, HER2 expression is also associated with poor prognosis in bladder cancer [82]. Thus, HER2 could serve as a useful biomarker for clinical prediction and trials of anti-HER2 agents are warranted in patients with advanced bladder cancer. Nevertheless, the glycosylation of HER2 in bladder cancer remains to be addressed, which would be critical for the establishment of a more sensitive and specific biomarker.

EpCAM, also known as CD326, is a glycoprotein predominantly located in intercellular spaces of epithelial, progenitor and normal stem cells [83, 84]. This transmembrane macromolecule regulates both normal and cancer-associated cellular adhesion, proliferation, differentiation, migration and invasion [84, 85]. Its expression is associated with increased tumour stage and grade, as well as with poor prognosis and decreased overall survival in bladder cancer patients [86, 87]. Despite these evidences, the glycosylation pattern of EpCAM in bladder cancer has also not yet been evaluated.

Frequently, cancer cells also overexpress galectins, *N*-acetyllactosamine-binding glycoproteins yielding either one or two carbohydrate-recognition domains. Galectins cross-link glycoproteins depending on their glycan structures and concentrations, forming galectin-glycan molecular lattices [88]. Particularly, the correlation between increased galectin expression and tumour progression is proposed to be linked to their interaction with poly-*N*-acetyllactosamines on matrix proteins such as laminin, aiding cellular invasion [89]. Moreover, these glycoproteins are known to modulate cell growth, differentiation, adhesion, and apoptosis [90-92]. The altered expression of galectins has been implicated in bladder cancer malignancy [93], and both galectin-1, -2, -3, and -8 were suggested as potential disease markers and possible targets for bladder cancer therapy [94]. Specifically, galectin-1 is a possible independent prognostic marker of urothelial carcinoma [95], with its positive immuno-expression being significantly correlated with tumour stage, grade, vascular invasion and nodal status [96]. Moreover, galectin-1 mRNA and protein levels are markedly increased in most high-grade bladder tumours compared with low-grade and normal bladder tissue [97, 98]. Furthermore, this glycoprotein is associated with bladder cancer cell invasion by mediating the activity of MMP9 through the Ras-Rac1-MEKK4-JNK-AP1 signalling pathway [95]. Recently, a photodynamic therapeutic approach targeting galectin-1 in bladder cancer cells and xenografts has inhibited tumour growth and enabled selective cytotoxicity in cancer cells, preventing undesired phototoxicity in the surrounding healthy tissues [99]. This study ultimately suggests that galectin-1 constitutes a valid bladder cancer cell biomarker capable of being used in effective targeted therapies. In turn, galectin-3 mRNA and protein levels were also found increased in bladder tumours when compared with normal urothelium [94, 97, 98, 100]. Moreover, galectin-3 levels are increased in invasive tumours compared with non-muscle invasive lesions [101-103]. Furthermore, its expression patterns are also correlated with tumour stage, grade, proliferation (Ki67), apoptosis (apopdetek and bcl-2), and overall survival in patients with T1G3 tumours [101]. These observations suggest a role for galectin-3 as a biomarker for bladder cancer staging and prognosis. In succession, galectin-7 was pointed as a predictive marker of chemosensitivity to cisplatin in urothelial cancer [104]. Finally, the loss of galectin-8 in bladder tumours increases tumour recurrence, while decreased immunohistochemical staining is associated with higher tumour stage and grade [105]. As such, the loss of galectin-8 might be an early step in the development of malignant lesions of the bladder and is a significant independent predictor of recurrence [105].

Several studies have recently pointed out the unique biological properties of basal-like bladder tumour cell subpopulations in their anchorage-independent growth ability and their association to poorly differentiated bladder cancer [106]. In this context, CD44, a member of the transmembrane glycoprotein family commonly implicated in cell-cell and cell-matrix interactions, cell proliferation, differentiation, migration, angiogenesis, presentation of cytokines, chemokines, and growth factors to the corresponding receptors, docking of proteases at the cell membrane, and cell survival [107-

109], has been implicated as a cancer stem cell (CSC) marker in several malignancies [110-114]. Particularly, both CD44 and its splicing variants have been involved in bladder cancer carcinogenesis and progression. CD44+ cells exhibit an enhanced capacity to form xenografts in immunocompromised mice as well as chemoresistance compared to CD44− cells [115, 116]. CD44v6, a CD44 isoform containing the CD44v6 exon, has also been shown increased in bladder CSCs [117, 118]. CD44v6 expression on CSCs is supported by a study that correlates CD44v6 expression on bladder cancer cell lines with stem cell properties [119]. Both expression levels of CD44 and CD44v6 were higher in invasive bladder tumours than in pre-invasive tumours and normal urothelium [120]. Also, CD44 and CD44v6 upregulation is associated with higher tumour grade and stage [120, 121]. However, other studies have demonstrated an inverse association between CD44v6 expression and bladder cancer grade and stage [121, 122]. Moreover, the loss of CD44v6 expression was demonstrated as an independent factor for increased recurrence and shorter overall survival [123]. Also, the loss of CD44 expression was associated with shorter progression-free survival [124]. These discrepancies can be explained by the lack of standard immunohistochemical assays, the use of antibodies with different specificities, and differences in the clinicopathological status of bladder tumours used in the different studies. Therefore, integrative and standardized studies are necessary to elucidate the role of CD44 and CD44v6 in bladder cancer, as they hold an important biological and clinical value and may serve as therapeutic targets. In turn, CD44 variant 9 (CD44v9) overexpression has been associated with shorter progression-free and cancer-specific survival in bladder cancer [125], likely impacting invasion and migration via the epithelial-mesenchymal transition (EMT). Therefore, its expression might be a useful predictive biomarker in basal-type muscle invasive and high-risk NMIBC [125]. Nevertheless, the specific glycosylation patterns of CD44 in the context of bladder cancer also remains an open research topic.

Mucins are large membrane-bound glycophosphoproteins, commonly overexpressed in

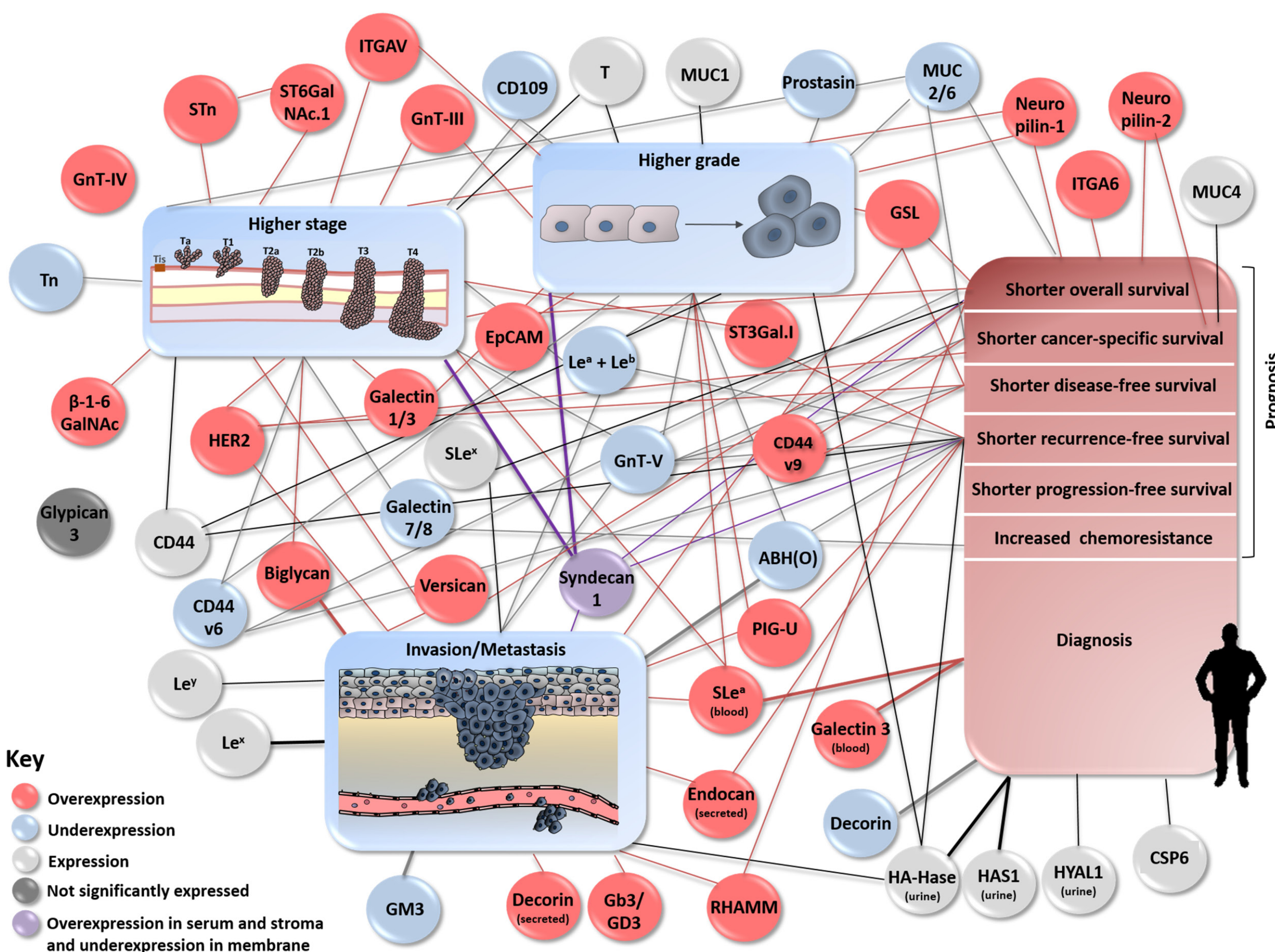

Figure 7. Schematic representation describing associations between (altered) expression of glycans and glycoconjugates and bladder tumour stage, grade, invasion/metastasis, patients' diagnosis and prognosis. The figure clusters using a gradient of colored circles and lines the biological and clinical role of the altered expression of glycans and glycoconjugates in bladder cancer.

several malignancies [126], including bladder cancer [127-129]. Mucin 1 (MUC1) is restricted to the apical membranes of umbrella cells in normal urothelium, while there is an aberrant MUC1 expression in basal and intermediate layers of neoplastic epithelium [128, 130]. Additionally, the pattern, intensity and depth of MUC1 immunostaining are correlated with bladder cancer grade [129]. Notwithstanding, other study reported no correlation of MUC1 expression with survival, tumour stage or grade [131]. Yet, patients overexpressing MUC1 only had a favourable survival when HER3 was also overexpressed [131]. This may be at least partially explained by the existence of several MUC1 glycoforms, including underglycosylated, sialylated, and fully glycosylated forms. As previously mentioned, several studies have been focusing on the identification of extracellular cell surface markers for urothelial CSCs, envisaging diagnosis and drug targeting. Of note, it has been shown that urothelial CSCs are enriched in an MUC1−CD44v6+ subpopulation of cells. This conclusion was based on the observation that MUC1− and CD44v6+ cells were only present in the basal layer of normal urothelium, which is thought to comprise urothelial stem cells. Subsequently, MUC1− and CD44v6+ cells were isolated, and a slightly increased clonogenicity was observed for these cells compared with unsorted bladder tumour cells [117]. Expression of other mucins such MUC2 and MUC6 were associated with a less aggressive behavior of bladder tumours and demonstrated to be useful predictors of better bladder cancer survival while MUC4 demonstrated an opposite role [129]. In addition, MUC16 STn+ glycoforms, characteristic of ovarian cancers, were recently described for the first time in bladder cancer and demonstrated to be expressed in a subset of advanced-stage bladder tumours facing worst prognosis [132]. Nevertheless, with the exception of MUC16, the specific glycosylation patterns of this class of glycoproteins also remains unknown in bladder cancer.

Integrins are a family of transmembrane adhesion receptors for extracellular matrix components participating in the metastatic cascade. Particularly, normal urothelium presents a polarized expression of alpha6beta4 integrin (ITGA6) on basal cells, while neoplastic urothelium frequently overexpresses this receptor [133]. Moreover, the evaluation of alpha6beta4 integrin tumour expression may provide valuable prognostic information on bladder cancer patients clinical outcome, since patients with alpha6beta4 integrin overexpression hold a significantly worst survival [133]. Throughout EMT-driven carcinogenesis, disseminated cancer cells often acquire a stem cell-like self-renewal capability [134, 135]. Moreover, during EMT, epithelial markers such as ITGAV (αv integrin receptors) are upregulated in several solid tumours [136-138], including bladder cancer with a trend increase in ITGAV expression with disease stage and grade [139]. Furthermore, the functional inactivation of ITGAV (targeting with the integrin receptor antagonist GLPG0187 or knockdown of ITGAV) leads to a less malignant bladder cancer phenotype with significantly impaired migration, EMT response, clonogenicity and a reduction in the size of the stem/progenitor pool. In line with these *in vitro* observations, knockdown of ITGAV or treatment with GLPG0187 significantly inhibited metastasis and secondary tumour growth [140]. In turn, a central role was also suggested for the beta1-integrin subunit in forming the cell-cell and cell-matrix bonds necessary for adhesion, extravasation and migration of bladder cancer cells [141] through enhanced transmission and generation of contractile forces [142] and possible microenvironmental involvement [69]. Despite its role in bladder carcinogenesis there are also no reports about the specific glycosylation of this class of glycoproteins.

In summary, increased levels of several glycoproteins have been associated with the severity of disease and as part of the molecular signature of more malignant bladder cancer sub-populations. These events not only amplify structural alterations that stem from deregulations in glycosylation pathways but also synergically contribute together with altered glycosylation, to a net effect favouring disease progression. Nevertheless, a comprehensive and context-oriented glycomapping of relevant glycoproteins has not been provided yet, which would be crucial for achieving highly specific cancer biomarkers holding true therapeutic potential. Moreover, the glycomic mapping of relevant glycoproteins may provide highly cancer-specific epitopes in comparison to glycans or glycoproteins alone. This would pave the way for designing more effective targeted therapeutics for more malignant bladder cancer cells.

Proteoglycan glycosylation

Proteoglycans are structurally and functionally complex glycoconjugates, exhibiting one or more high molecular weight glycosaminoglycan (GAG) chains covalently attached to a protein core [143]. These structures can be found as: i) transmembrane syndecans or glypicans, at the cell surface; ii) hyalectans (aggrecan, versican, brevican and neurocan) or small leucine-rich proteoglycans (decorin, biglycan and lumican) at the extracellular matrix (ECM); iii) basement membrane proteoglycans (perlecan, agrin and collagen XVIII) [144]. Serglycin is the only characterized proteoglycan found at intracellular level, normally in secretory compartments [145].

The biosynthesis and modification of proteoglycans occurs in the Golgi apparatus (GA) through the action of glycosyltransferases, sulfotransferases, epimerases, sulfatases, glycosidases, and heparanases, revealing multiple layers of regulation of these macromolecules [143]. The length and structure of each GAG chain may differ greatly within a certain proteoglycan molecule, while the number of chains linked to the protein core

is determined by the number of sugar attachment sites, marked by Ser-Gly dipeptide motifs [143, 146]. The biosynthesis of GAGs, such as chondroitin sulfate, heparan sulfate, dermatan sulfate, hyaluronic acid, and heparin is initiated by the sequential addition of four monosaccharides (Xyl, Gal and GlcA) to a Ser-Gly motif on the core protein. Then, the sugar chains are extended by the addition of two alternating monosaccharides containing an acetylated or sulfated hexosamine (GalNAc, GlcNAc) and uronic acid (GlcA acid or idoA) [143]. In the case of keratan sulfate, the GAG is initiated as *N*-linked or *O*-linked repeating disaccharides, and extended by the addition of *N*-acetyl-glucosamine and galactose residues [143]. Once synthesized, the GAGs are linked to a core protein and proteoglycans are transported from the GA to the cell surface or ECM [144, 147]. Notably, unlike all other GAGs, hyaluronic acid is primarily found as a free sugar chain at the ECM, and its synthesis is epigenetically regulated [148]. Interestingly, hyalectans have the ability to bind hyaluronic acid through their N-terminal globular domain (G1), therefore increasing ECM complexity [149]. Of note, proteins such as MHC class II invariant chain, transferrin receptor, thrombomodulin and CD44 can be considered proteoglycans, since some of their alternative splicing variants present GAG-initiation sites [150]. Other proteoglycans like endocan and versican also present alternatively spliced forms with variable sugar modifications [150]. In particular, a versican variant without chondroitin sulphate attachment sites has been described, [149].

Proteoglycans present high affinities for various ECM constituents and cell adhesion molecules, playing a crucial role in intercellular interactions [144]. These glycoconjugates can also bind growth factors, cytokines and chemokines, allowing them to escape proteolysis. Some can also act as co-receptors for growth factors and tyrosine kinase receptors, changing the duration of their signaling reactions or lowering their activation thresholds [143, 144]. Therefore, the altered expression of proteoglycans, including syndecan-1, neuropilins, versican, chondroitin sulfate proteoglycan 6, decorin, biglycan, endocan, hyaluronic acid and its metabolic enzymes, has been linked to several cancers and, specifically, with bladder cancer carcinogenesis, metastasis and prognosis (Figure 4, Supplementary Table 1).

Cancer-associated transmembrane proteoglycans

Syndecans are a family of heparin sulfate proteoglycans, commonly presenting three to five heparin sulfate chains, and are known to modulate cellular adhesion, migration, proliferation, differentiation, and growth factor signaling [151]. These macromolecules are commonly found at bladder cancer cell surfaces, along with other transmembrane proteoglycans and glypicans [152, 153]. Syndecans can also be found in their soluble form, due to a post-translational modification causing the release of their ectodomains through juxtamembrane region proteolysis [154]. Particularly, syndecan-1 (CD138), frequently expressed in epithelial cells and some leukocytes [155], was found to be increased in bladder cancer patients serum and stroma, especially in muscle-invasive cases [156-158]. Serum overexpression of syndecan-1 was associated with lymph node metastasis, while stromal overexpression was related with poorer overall survival [158]. The loss of transmembrane syndecan-1 expression in tumour cells was related to higher tumour stage and grade [159, 160], as well as reduced recurrence-free survival in bladder cancer [160, 161]. Still, high-grade superficial, and deep invasive bladder carcinomas were also characterized by elevated expression of syndecan-1, while low-grade and non-invasive phenotypes do not [161]. Cytoplasmic overexpression of syndecan-1 in cancer cells, often accentuated close to the nucleus, was demonstrated in Ta tumours compared to normal urothelium, suggesting a failure in intracellular trafficking caused by the loss of functional syndecan-1 [162]. This directly affects carcinogenesis through the loss of cellular adhesion properties, thereby promoting more invasive phenotypes [160]. Also, syndecan-1 altered expression can affect tumour cells via junB-FLIP long signals, involving apoptosis resistance and increased proliferation [161]. Simultaneous loss of syndecan-1 expression in tumour cells and its overexpression in high-stage and high-grade bladder cancer patients serum suggest the importance of syndecan-1 in tumour progression; therefore, this molecule could be a new therapeutic target in human urinary bladder cancer [158].

Neuropilins are co-receptors of two structurally and functionally unrelated ligands classes, the class 3 semaphorins and selected VEGF family members [163]. Neuropilin-1 has multiple heparan and/or chondroitin/dermatan sulfate GAG chains [163]. Recent reports demonstrate neuropilin-1 expression on non-endothelial cells in bladder urothelium [164], as well as its overexpression in high grade/stage bladder tumours [165]. Moreover, neuropilin-1 upregulation was associated with shorter overall survival in bladder cancer patients [165]. In addition, neuropilin-2 is expressed in neural and endothelial cells and, upon ligand stimulation, induces neural development and the growth of newly formed blood and lymphatic vessels [163]. Overexpression of neuropilin-2 demonstrated to have prognostic value in bladder cancer, as it was associated with shorter overall and cancer-specific survival and earlier cancer-specific death after transurethral resection and radiochemotherapy [166]. Additionally, the co-expression of neuropilin-2 and the family member VEGF-C is also a prognostic marker for overall survival of bladder cancer patients [166]. Therefore, syndecan-1 and neuropilins may play an important role in the progression of bladder cancer and their altered expression may serve as a biomarker for

prognosis.

Cancer-associated extracellular matrix proteoglycans

Versican, also known as chondroitin sulfate proteoglycan 2, a central component of cancer-related inflammation, is highly expressed in metastatic bladder carcinomas and its overexpression is correlated with poor survival [167]. In tumour cell lines, versican overexpression was associated with increased cell migration and tumour stage [168]. A correlation between versican overexpression, RhoGTP dissociation inhibitor 2 (RhoGDI2) underexpression, metastasis and poor clinical outcome was also demonstrated [167, 169]. Particularly, RhoGDI2 underexpression and versican overexpression are associated with metastasis through the involvement of macrophages and the CCL2/CCR2 signaling axis [167, 169]. In fact, RhoGDI2 is a regulator of several Rho GTPases that play important roles in cell cycle progression, neovascularization, invasiveness, and metastasis [170]. Therefore, targeting this mechanism may provide novel therapeutic strategies for delaying the appearance of clinical metastasis [170].

The role of decorin, a key component of the tumour stroma, in cancer progression and its therapeutic potential has been the focus of several studies. Increased secretion of decorin in the MB49/MB49-I murine bladder cancer model and in muscle-invasive tumours was associated with the promotion of angiogenesis and tumour cell invasiveness [171]. Nevertheless, other studies demonstrate a possible tumour suppressor role for decorin, where bladder tumour tissues are entirely devoid of decorin expression while non-malignant stromal areas express this proteoglycan [172, 173]. A mechanism through which decorin exerts its tumour suppressor role has been proposed, where decorin may act as a natural antagonist of the oncogene insulin-like growth factor receptor I (IGF-IR) [173, 174]. Therefore, in bladder tumours, the loss of decorin expression eliminates IGF-IR activity and signaling repression, promoting cellular motility, invasion, and cancer progression [173, 174].

Biglycan is a small leucine-rich proteoglycan with immune and growth factor activity modulating properties, as well as matrix assembly involvement [175]. This proteoglycan has been demonstrated to be overexpressed on invasive bladder cancer tissue [172, 176]. Interestingly, while biglycan overexpression is associated with higher tumour stages and muscle invasiveness, it's up-regulation was related with tumour cell proliferation inhibition and increased patients' 10-year survival [176].

Endocan, also known as endothelial cell-specific molecule 1, is a secreted proteoglycan that has a single dermatan sulfate side chain attached to serine 137 and demonstrated to be highly elevated on tumour vessels from invasive bladder cancer tissues [177]. Moreover, its expression correlated with stage, and invasiveness as well as predicted a shorter recurrence-free survival time in non-invasive bladder cancers [177]. Therefore, endocan expression impacts the prognosis of bladder cancer patients and, as described ahead, also is a possible diagnosis marker.

Hyaluronic acid (HA), an unsulfated anionic linear GAG, its implicated in cell adhesion, migration and angiogenesis [178]. Particularly, hyaluronidases (HAse) are enzymes that hydrolyze HA molecules into small angiogenic fragments, participating in the degradation of tumour surrounding ECM, and enabling cancer cells invasion and dissemination [178]. As such, HA, HAses (e.g. HYAL1), hyaluronic acid synthases (HAS) 1, 2 and 3, as well as hyaluronic acid receptors (e.g. CD44 and receptor for hyaluronan-mediated motility, RHAMM) have been suggested as possible diagnosis and prognosis biomarkers. Also, both HAS, HYAL1, CD44 and RHAMM were found to be overexpressed in bladder cancer tissues [179, 180]. In addition, HYAL1 expression was also correlated with disease-specific mortality and recurrence [179, 181]. Finally, elevated expression of RHAMM was found in invasive bladder tumours and associated with poor prognosis, due to increased tumour cell proliferation and shorter overall and disease-specific survival [180].

Alterations in cancer-associated proteoglycans were demonstrated as associated with bladder cancer progression and may present prognosis value, yet more studies are necessary in order to confirm these associations and transpose these markers to clinical practice.

Lipid glycosylation

Glycolipids are a major class of glycoconjugates that include glycosphingolipids (GSLs) and GPI anchors.

Alterations in sphingolipids' glycosylation

Glycosphingolipids (GSLs) are neutral or anionic molecules composed by a hydrophilic glycan covalently β-linked via glucose (glucosylceramide) or galactose (galactosylceramide) to the terminal hydroxyl group of a hydrophobic ceramide backbone [182, 183]. Specifically, GSL biosynthesis is initiated in the ER with the condensation of sphingosine and acyl-CoA by a group of six ceramide synthases, giving rise to a long-chain amino alcohol base (sphingosine) in amide linkage to a fatty acid, namely a ceramide lipid [183, 184]. Ceramide can then be galactosylated by galactosylceramide synthase, to produce galactosylceramide, which in turn can be transported to the GA where it is sialylated to produce GM4 ganglioside, or sulfated to produce sulfogalactolipids [185-187]. Also, ceramide is frequently glucosylated in the GA by glucosylceramide synthase to form glucosylceramide, the core structure of 90% of GSLs [185-187]. Subsequently, the C-4 hydroxyl of glucosylceramide can be galactosylated by β4-galactosyltransferases V and VI, forming lactosylceramide [188]. Once produced,

lactosylceramide will serve as the metabolic precursor of more than 300 structurally different classes of complex GSLs through the action of specific glycosyltransferases and sulfotransferases, depending on nucleotide sugar donors availability [189]. Particularly, lactosylceramide is a template for: 1) GA2, through β,4-N-acetylgalactosylaminyltransferase (B4GALNT1) activity; 2) GM3 ganglioside, through α-2,3-sialyltransferase (ST3GAL5Gb3); 3) Gb3, by α-1-4-galactosyltransferase (A4GALT) activity; and, 4) Lc3, by the β-1,3-N-acetylglucosaminyltransferase (B3GNT5) [189]. GSLs glycan chains can be further extended and terminated with structural moieties similar to those found in glycoproteins, namely and Lewis blood type antigens. After synthesis, these structures leave the GA and are redirected to the plasma membrane [190], constituting approximately 5% of all membrane lipids

GSLs are also implicated in key cellular functions, such as cell adhesion, proliferation, differentiation, apoptosis, motility and immune recognition [191-193]. Particularly, ceramide is implicated in apoptosis and regulates several cell cycle and senescence pathways [194]. Consequently, GSLs have received considerable attention as promising biomarkers for disease progression, as well as pharmacological targets for bladder cancer therapy. Pioneering studies demonstrated that glucosylceramide and glucosylceramide synthase are overexpressed in several multidrug resistant cancer cell lines, being related with drug resistance [195-197]. Particularly, in bladder cancer, glucosylceramide synthase overexpression was demonstrated to be associated with higher histologic grade [198]. In accordance, the overexpression of this enzyme is an indicator of poor prognosis, showing associations with lymph node metastasis, reduction in the 5-year overall and disease-free survival [198]. The glycosphingolipid composition of human bladder cancer tissue has been assessed, showing large amounts of ganglioside GM3 in superficial bladder tumours, but not in invasive tumours [199]. This overexpression can be caused by the simultaneous overexpression of GM3 synthase and downregulation of both Gb3 and GD3 synthases [199]. Moreover, high levels of GM3 are associated with reduced invasive potential [199], proliferation, motility, tumour growth and increased apoptosis [200]. Since exposure to exogenous GM3 have been proved to inhibit tumour cell lines proliferation and adhesion, this approach was proposed as bladder cancer therapy. Also, the direct instillation of GM3 in orthotopic models inhibited tumour growth [201]. It has also been reported that the expression of GM2, GM3, or GM2/GM3 complexes inhibited bladder cancer cell motility and growth [202] (Figure 5, Supplementary Table 1).

Alterations in glycosylphosphatidylinositol-anchored molecules

The anchoring of proteins and proteoglycans to cell membranes through the lipid portion of a GPI anchor is a conserved post-translational modification [203]. These anchors present conserved core structures consisting of ethanolamine phosphate, three mannose monomers, and a non-*N*-acetylated glucosamine attached to a inositol phospholipid (EtNP-6Manα-2Manα-6Manα-4GlNα-6myoInositol-P-lipid) [204]. This backbone can be modified with phosphoethanolamine and/or various glycan side-branches. More detailed information about the biosynthesis of this class of glycans may be found in [204-206] and has been summarized in Figure 5.

Alterations in several enzymes involved in glycosylation of GPI-anchored molecules such the multi-protein transaminase complex were first mentioned as relevant in cancer after the discovery of the oncogenic activity of PIG-U in bladder cancer [207, 208]. This was demonstrated through the induction of tumourigenesis mediated by PIG-U overexpression in mice [208]. In addition, PIG-U overexpression *in vitro* was correlated with increased cell proliferation and upregulation of GPI-anchored proteins, such as urokinase receptor, increasing STAT-3 phosphorylation and subsequent cellular migration, and apoptosis [208]. PIG-U overexpression is also associated with higher tumour grade and muscle invasion, suggesting its role in tumour development and progression [209]. Moreover, overexpression of PIG-U is an independent predictor of recurrence for superficial bladder cancer [209]. Consequently, the expression of PIG-U and other multi-protein transaminase complex subunits was explored and confirmed in different cancer types using microarrays [210].

GPI-anchored proteins are almost exclusively located on cell surfaces, and are functionally diverse, presenting key roles in cell-cell interaction, adhesion, host defense, and signaling transduction [204, 211]. Thus, these proteins have been explored regarding their potential as biomarkers for carcinogenesis and metastatic potential in bladder cancer (Figure 5, Supplementary Table 1). The expression of GPI-specific phospholipase D, a highly specific GPI-anchored enzyme, is significantly increased in highly malignant murine bladder carcinoma cells when compared to less malignant controls [212]. In addition, CD109, a GPI-anchored glycoprotein that negatively regulates the transforming growth factor (TGF)-β/Smad signaling *in vitro*, is overexpressed in the basal layer of NMIBC and low-grade tumours. Interestingly, CD109 shows a similar expression pattern to cancer stem cell marker CD44, and its overexpression was associated with better cancer-specific survival [213]. Prostasin, a GPI-anchored serine protease crucial for epithelial differentiation [214] and epidermal growth factor receptor (EGFR) proteolysis [215], was shown to be downregulated

in high-grade urothelial bladder cancer cell lines [216]. This loss of expression was associated with EMT, marked by a reduced E-cadherin expression and loss of epithelial morphology, which may have implications in the invasive potential and resistance to anti-EGFR therapy [216]. However, the clinical relevance of prostasin was not yet evaluated in bladder cancer. Notwithstanding, multi-protein transaminase complex subunits and GPI-anchored proteins have potential to serve as markers of tumourigenesis and metastatic capability, being relevant targets for bladder cancer therapy.

Beyond GPI-anchored proteins, alterations in glypicans, a class of GPI-anchored proteoglycans, also have been studied in cancer. Glypicans are a family of GPI-anchored heparan sulfate proteoglycans known to interact with growth factors through heparan sulfate chains [217]. This class of proteoglycans is predominantly expressed during fetal development, being critical to organogenesis [218]. Moreover, glypicans were described in several cancers [219, 220], including bladder cancer. Particularly, glypican-3 is expressed in squamous cell and invasive urothelial carcinomas; however, it is not a good biomarker for diagnosis using tumour tissues [217]. Furthermore, glypican-3 expression was not associated with tumour stage, grade, lymph node metastasis, concomitant CIS, soft tissue surgical margins, disease recurrence or cancer specific mortality after radical cystectomy [221].

In conclusion, alterations in PIG-U and GPI-anchored proteins seem to be promising regarding their prognosis value while GPI-anchored proteoglycans appear to be bad prognosis biomarkers. Notwithstanding, further studies are necessary to evaluate these GPI-anchored molecules and enzymes from multi-protein transaminase complex in bladder cancer biological and clinical context.

PROGNOSIS GLYCOMARKERS FOR BLADDER CANCER

The most challenging aspect in bladder cancer management stems from its highly heterogeneous nature, irrespectively of disease stage. This constitutes a true obstacle for individualized therapeutic decision, disease monitoring and prognosis with tremendously negative impact on patient care and life expectancy. In this context, several clinical studies have unveiled glycan-associated signatures (glycans, glycan binding proteins, glycosyltranserases, heavily glycosylated proteins/lipids/GPI-anchored molecules) associated with more aggressive cancer cell phenotypes, tumour recurrence, progression, metastization, or decreased overall survival. Particularly, in bladder cancer, the presence/overexpression of GnT-III/IV, Le^x, Le^y, SLe^x, STn, ST6GalNAc.I, T-antigen, ST3Gal.I, HER2, EpCAM, galectin-1, galectin-3, CD44, CD44v9, MUC1, MUC4, ITGA6, ITGAV, neuropilin-1, neuropilin-2, versican, decorin, biglycan, endocan, HYAL1, hyaluronic acid synthase 1, RHAAM, glucosylceramide synthase, and PIG-U was associated with more aggressive phenotypes and/or poor prognosis [20, 30, 46, 50, 54, 64-67, 76, 82, 86, 87, 96, 97, 101-103, 120, 121, 124, 125, 129, 133, 139, 165-168, 171, 172, 176, 177, 179-181, 198, 208, 209]. By another hand, (over) expression of β-1-6GalNAc antennae, galectin-7, CD44v6, MUC2, MUC6, glycolipid enzyme GM3, and CD109 were linked to a less aggressive phenotype and/or better prognosis [32, 104, 121, 122, 129, 199, 213]. In turn, the loss of GnT-V, ABO(H) terminal structures, sLe^a, Tn, galectin-8 and prostasin is associated with more aggressive cancer phenotypes, making them potential markers of poor prognosis [31, 32, 44-47, 50, 67, 105, 158-160, 200, 216]. Yet, glypican-3 tissue expression was not associated with aggressive phenotype nor prognosis [217, 221] and studies for syndecan-1 demonstrated contradictory results [158-162, 222], warranting elucidation of its prognostic and diagnostic role. For other molecules, the co-expression with another allowed to have or enhanced a significant prognostic and therapeutic outcome impact, such as Le^a/Le^b antigens [44, 49], s6T/STn [75] neuropilin-2/VEGF-C [166], Gb3/GD3 synthases [199] and GPI-specific phospholipase D/H-ras oncogene [212] (Figure 6 and 7, Supplementary Table 1).

While these studies support the potential of glycans for patient stratification significant bias hampers conclusive remarks to support introduction in clinical practice. Namely, most studies differ in cohort size and distribution of the cohort by bladder cancer type, stage and grade, which often translate into conflicting results. Moreover, most studies disregard patient ethnicity, lack endpoints standardization and rely on different biological samples, as well as in different sample processing and analysis techniques. Nevertheless, several molecular markers hold promise for becoming widely available and cost-effective tools for a more reliable risk assessment. Thus, efforts need to be conducted to validate glycobiomarkers in larger series for prospective use, ideally in the context of prospective clinical multicenter randomized trials, using current clinicopathological parameters for risk assessment. The inclusion of relevant glycobiomakers in current patient stratification parameters may help accurately assess patient's prognosis and response to different treatment options. Therefore, future studies should also evaluate the impact of glycomarkers in the therapeutic outcome and as novel targets for bladder cancer therapy. Finally, recent evidences support the need to integrate this knowledge in multiplex risk assessment tool combining standard clinicopathological factors with molecular markers [223] envisaging individualization of responses. Although much effort has been spent on glycobiomarkers research, clinicians and medicinal chemists rarely consider glycans as biological targets or drugs [224], hindering advances in bladder cancer management and in the development of targeted therapeutics. Notwithstanding, this unfamiliarity is beginning to change as improved methods for

carbohydrate synthesis [225-227], sequencing [228, 229], and biological analysis [230-232] become more sensitive and widely available.

EXPLORING GLYCANS FOR NON-INVASIVE BLADDER CANCER DETECTION

Non-invasive disease detection remains a major challenge despite the pressing need for tools capable of reducing the burden of disease-follow up, treatment monitoring and early detection [233]. The active secretion of cancer-associated glycoconjugates into bodily fluids, such as urine and blood, or shed from apoptotic and necrotic cancer cells holds tremendous potential to address this challenge [7, 51]. Accordingly, several FDA-approved cancer biomarkers for non-invasive cancer detection, follow-up and prognosis are either glycans, such as CA19-9 (SLe[a]), or heavily glycosylated glycoproteins, such as CA125 (MUC16), CA15-3 (MUC1), CA-72-4 (tumour-associated glycoprotein 72, TAG-72), PSA (prostate-specific antigen), and CEA (carcinoembryonic antigen) [233]. In bladder cancer, serum CA19-9 is a marker of aggressiveness and advanced stage disease, being almost invariably raised in patients with metastatic cancer. As such, it constitutes a valuable marker of poor prognosis [234]. Notwithstanding, its value as a screening tool has been opposed by its low sensitivity (29%) [234]. On the other hand, urinary CA19-9 is a better screening parameter, with optimum sensitivity and specificity, than its serum counterpart for diagnosis of low grade and early stage bladder cancer. Furthermore, it can be suggested that urinary CA19-9 can be used as better prognostic marker for low grade bladder cancer than its serum equivalent [235]. Moreover, urinary CA19-9 levels could be a new effective diagnostic tool for bladder cancer patients with both Le and Se alleles. Particularly, 70% of bladder cancer patients with both Le and Se alleles presented CA19-9 levels over the cut-off value, and only 16% of patients with other urological conditions were over the cut-off [236]. Furthermore, simultaneous elevation of CA19-9 and CEA serum levels correlated with tumour invasion and grade in patients with CA19-9-expressing urothelial carcinomas [53]. In addition, SLea antigen has been observed in bladder dysplasia, Tis, non-invasive, and invasive carcinomas of the bladder [53, 236, 237], suggesting that these patients may present elevated CA19-9, which warrants confirmation in broad clinical studies.

Considering the cancer-associated glycoproteins, galectins also hold potential for bladder cancer detection. In fact, bladder cancer patient's serum levels of galectin-3 are considerably higher than control groups, and are correlated with tumour type, stage and grade [98, 100, 103]. Particularly, patients with high-grade urothelial carcinoma have higher serum levels of galectin-3 than those with low-grade tumours [103]. Moreover, patients with muscle-invasive tumours also have higher serum levels of galectin-3 than those with Ta tumours [103], conferring to this glycoprotein a diagnostic and stratification value for bladder cancer patients. In addition, serum levels of galectin-3 also have diagnostic value for bladder squamous cell carcinoma (SCC) patients [103]. More recently, a multiplexed immunosensor has been developed for the detection of specific biomarkers galectin-1 and lactate dehydrogenase B present in different grades of bladder cancer cell lysates. This approach has allowed not only the identification of different grades of bladder cancer cells but also the real time detection of multiple analytes on a single chip, providing more practical benefits for clinical diagnosis [238].

Regarding proteoglycans, syndecan-1 was also explored as a diagnosis biomarker in a non-invasive urine-based assay, however with low predictive value (sensitivity of 70%, specificity of 48% and accuracy of 59%) [222]. Therefore, further validation is warranted in larger and prospective studies. The chondroitin sulfate proteoglycan 6, also known as structural maintenance of chromosomes 3 (SMC3), has also been found overexpressed in bladder cancer. Moreover, when used in combination with six gene transcripts (insulin-like growth factor-binding protein 7, sorting nexin 16, cathepsin D, chromodomain helicase DNA-binding protein 2, nell-like 2, and tumour necrosis factor receptor superfamily member 7), it could discriminate bladder cancer from control blood samples with a sensitivity of 83% (95% confidence interval, 67-93%) and a specificity of 93% (95% confidence interval, 76-99%), constituting a possible blood diagnosis biomarker [239]. Beyond the prognosis role of endocan, recently this proteoglycan also demonstrated non-invasive diagnosis potential for bladder cancer. In fact, serum expression of endocan could discriminated bladder cancer patients with a sensitivity of 50% and specificity of 77% while the urinary endocan expression resulted in a sensitivity of 62% and specificity of 71% [240]. Moreover, HA and its related enzymes may have potential for detecting bladder cancer patients. In fact, increased HA levels are detected in all bladder cancer grades, while HAse levels are preferentially elevated in urothelial bladder cancer grades 2 and 3 [241, 242]. Lokeshwar *et al*. and Passerotti *et al*. have reported an optimal sensitivity (91.9% and 81.9%, respectively) and specificity (92.8% and 80.5%, respectively) of the HA test for bladder cancer detection [243, 244]. Urinary HAse measurement demonstrated a 100% sensitivity and 88.8% specificity to detect grade 2 and 3 bladder tumours [242]. Measurements of both urinary HA and HAse (the HA-HAse test) demonstrated an 89% sensitivity and 83% specificity for detecting urothelial bladder cancer [241]. Similarly, other study evaluated this non-invasive method in urine samples, showing that the HA test has 83.1% sensitivity, 90.1% specificity and 86.5% accuracy to detect bladder cancer [245]. Also, the urinary HAse test

demonstrated 81.5% sensitivity, 83.8% specificity and 82.9% accuracy in detecting grade 2 and 3 bladder cancer [245]. Other studies also reported high sensitivity and specificity for detecting bladder cancer using both urinary HA and HAse by a non-invasive approach [246-249]. In fact, the sensitivity of HA-HAse test demonstrated to be superior to ImmunoCyt® and cytology (83.3% *versus* 63.3% and 73.0%, respectively), as well as to BTA STAT® test (94.0% *versus* 61.0%). In turn, specificity was comparable between HA-HAse and ImmunoCyt® or cytology (78.1% *versus* 75.0% and 79.7%, respectively) and between HA-HAse and BTA STAT® test (63.0% *versus* 74.0%) for detecting bladder cancer or bladder cancer recurrence [248, 249]. The evaluation of HAS1 and HA expression resulted in a 79% and 88% sensitivity, as well an 83.3% and 100% specificity, respectively, for detecting bladder cancer. Moreover, both expressions correlated with a positive HA urine test [247]. In addition, the combined expression of HAS2-HYAL1 detected bladder cancer with overall sensitivity of 85.4% and 79.5% specificity, predicting recurrence within 6 months [179] (Figure 6).

Despite significant research efforts and promising upfront results, neither glycans nor glycoconjugates have yet been approved for non-invasive bladder cancer detection. Again, reduced study dimensions, biased patient series and variations in detection methods are amongst the factors hampering the generalization of these approaches. As such, comprehensive clinical studies on the glycome, glycoproteome and glycolipidome of bodily fluids are warranted to broaden our understanding about alterations accompanying malignant transformations, disease progression and dissemination. Moreover, efforts should be undertaken to incorporate glycans in broad biomarker panels envisaging highly sensitive and specific detection methods.

GLYCOMICS AND GLYCOPROTEOMICS: INSIGHTS TOWARDS PRECISION MEDICINE

The establishment of clinically useful glycan-based/assisted molecular models have been significantly delayed by serious analytical limitations. In fact, the majority of the studies presented to this date are target-directed and based on immune assays with antibodies and/or lectins whose specificity for the target ligands has not been fully disclosed yet. These aspects delay the generalization of glycans biomarker potential. Moreover, most studies are one-dimensional, failing to provide a comprehensive overview of the glycome in clinical settings. The low abundance of relevant glycoforms in biological milieus also poses a major analytical difficulty, which adds to the significant structural diversity and complexity presented by this class of biomolecules.

In the past decade, mass spectrometry has emerged as a core analytical technology to interrogate the glycome due to the rapid advance in resolution, mass accuracy, sensitivity, and reproducibility provided by modern hybrid mass analyzers. Currently, glycans and glycoconjugates analysis relies on hyphenated techniques comprehending separations by liquid chromatography or capillary electrophoresis, as well as detection by electrospray tandem mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS). Complementary tandem experiments are required for unambiguous assignments. Even though, the identification of isomeric and/or isobaric species remains a challenging task. Nevertheless, it has been demonstrated that certain isomeric glycans produce characteristic product ion spectra that can be used for identification, irrespectively of the type of mass spectrometer [250, 251]. Moreover, the introduction of graphitized LC columns has significantly improved the analysis of native glycans by mass spectrometry [252]. More in depth insights on analytical advances in glycomics may be found in recent reviews on the subject [253-255]. Recent developments in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) on formalin-fixed paraffin-embedded (FFPE) tissue sections may also provid a key tool for evaluating spatio-temporal investigation of glycosylation changes in cancer tissues. [269]. However, while the field still struggles with technical difficulties associated with variations in accuracy depending on analytical method and variety of mass spectrometry architectures, significant efforts are ongoing to standardize protocols and implement robust glycoanalytical platforms [256, 257]. In addition, rapid expansion of high-throughput mass spectrometry studies has generated significant amounts of experimental "omics" data that require more sophisticated bioinformatics tools and databases. In this context, several groups have started the development of algorithms for computerized annotation of mass spectra and fragmentation data, as revised by Hu *et al*. [258]. However, while significant advances have been observed for glycan analysis, glycopeptide data interpretation remains immature compared to proteomics data analysis. This is partly due to lack of consensus regarding the best way of estimate the false discovery rate, and the existence of multiple formats of data storage. Nevertheless, guidelines for reporting mass spectrometry-based glycoanalytic data are being developed [257, 259].

Despite the enormous potential of glycomics, few quantitative and comprehensive studies were conducted on bladder cancer. Yang *et al*. quantitatively analyzed and compared glycan expression patterns in normal and bladder cancer cells through an integrated methodology using lectin microarray and mass spectrometry [58]. It has been demonstrated that SLe^x and high mannose-type *N*-glycans were highly expressed in bladder cancer cells [58]. In addition, a high expression of core-fucosylated

N-glycans but a low expression of terminally fucosylated *N*-glycans was observed in bladder cancer cells [58]. In turn, Pocheć *et al*. determined the *N*-glycan patterns of integrin α3β1 in bladder cancer cells compared to normal bladder cells using an integrated methodology of lectin-binding assays and mass spectrometry [260]. Accordingly, bladder cancer cell-associated integrins have been found to express high-mannose, hybrid and predominantly complex type *N*-oligosaccharides, as well as the sialylated tetra-antennary complex type glycan $Hex_7HexNAc_6FucSia_4$ [260]. Weather by focusing on the whole proteome or in a single glycoprotein, both studies have given an important *in vitro* view of the glycome pattern of bladder tumour cells compared to a normal state, paving the way for new and more comprehensive studies.

In summary, early glycan-based clinical studies have created the molecular basis to drive the emerging area of advanced glycomics in bladder cancer. We expect that the generalization of these approaches leads to the discovery of key glycan biomarkers with clinical and therapeutic potential in the near future. The incorporation of glycogenomics and glycoinformatics datasets are expected to accelerate a comprehensive understanding of the glycome. Furthermore, the integration with other "omics" will be crucial to deepen the understanding of glycosylation's role in human systems and provide models capable of disclosing the polymorphic nature of disease and ultimately help tailoring medical decisions and achieve precision medicine settings.

Concluding remarks and future perspectives

Bladder cancer is a heterogeneous disease encompassing distinct biological features and clinical outcomes. This is responsible for elevated recurrence rates, often accompanied by disease progression facing existing treatments. Moreover, it has hampered the establishment of precision medicine settings capable of molecular-based individualization of disease management. These aspects make bladder cancer one of the costliest malignancies to manage, constituting a burden to both patients and healthcare systems. We believe that true advances in this field will require an integrative panomics approach capable of providing robust models for molecular-based patient tailored clinical decisions. So far, most efforts have been put in genomics, transcriptomics and proteomics fields, with upfront enthusiastic results; however, over 40 years of glycobiology research has yet to retrieve solid evidences capable of boosting clinical implementation. Even though many studies have highlighted glycans and glyconjugates (glycoproteins, glycolipids and proteoglycans) holding true clinical potential, few have engaged in a comprehensive interrogation of the glycome. Notwithstanding, the relevance of these molecular entities for disease progression and dissemination suggests potential for more in depth targeted omics studies. Moreover, the standardization of glycomics protocols backed by high-throughput analytical and novel bioinformatics tools opens now a unique opportunity for real advances in this area. Therefore, the field must now focus on large scale multicentric translational studies integrating glycomics data with novel molecular findings, including recently proposed models for bladder cancer risk-stratification [261-263]. Moreover, our understanding on the glycobiology of chemoresistance, formation of the pre-metastatic and metastatic niches is still scarce and warrant careful evaluation on the near future. Complementary understanding of glycan biosynthesis pathways and the biological significance of these alterations will be warranted, envisaging theragnostic applications. Taking into consideration the glycome's strong dependence on the microenvironment and physiological status, significant efforts should put on developing models capable of recreating tumour glycoheterogeneity. Patient derived xenografts have been proven useful in this context [264] and may constitute valuable tools for understanding the dynamics of glycosylation in malignancy as well as for the identification of prognostic glycobiomarkers. Ultimately, this will be of key importance for developing targeted therapeutics while exploring the cell-surface nature of glycans. Such approaches would strongly benefit from the identification of glycoconjugates (proteins, peptides or lipids) yielding cancer-associated carbohydrate antigens, which would significantly narrow biomarker specificity for malignant cells. While the development of novel high-affinity glycan ligands, namely humanized monoclonal antibodies and antibody fragments, for theragnostics applications still poses a major task due to the complex structural nature of these molecules, several advances in cell glycoengeneiring and glycosynthesis [265-267] hold potential to overcome these limitations. Finally, it has been highlighted that glycans play a key role in immune modulation, especially by favoring tumour tolerogenic mechanisms [21, 268]. A deeper understanding of influence of glycosylation in immunological mechanisms is a hot research topic that will pave the way for circumventing these events and for more effective and less toxic immunotherapies. In summary, an intervention roadmap has been established to boost glycobiology towards omics settings capable of generating key data to improve the management of bladder cancer patients.

Abbreviations

BCG: bacillus Calmette-Guérin; CSC: cancer stem cell; ECM: extracellular matrix; EGFR: epidermal growth factor receptor; EMT: epithelial-to-mesenchymal transition; ER: endoplasmic reticulum; FDA: Food and Drug Administration; FUT-I: alpha1,2-fucosyltransferase 1; FUT-VI: alpha1,3-fucosyltransferase VI; GA: Golgi apparatus; GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglucosamine; GPAA1: GPI anchor attachment 1; HAS: hyaluronic acid synthases; IGF-IR: insulin-like growth factor receptor I; ITGA6: alpha6beta4 integrin;

ITGAV: αv integrin receptors; MIBC: muscle invasive bladder cancer; MUC1: mucin 1; MVAC: methotrexate, vinblastine, cisplatin and doxorubicin; NMIBC: non-muscle invasive bladder cancer; PD-L1: programmed death-ligand 1; PGAP1: post-GPI attachment to proteins 1; ppGalNAc-Ts: UDP-GalNAc:polypeptide N-acetylgalactosaminyl transferases; NMP22: nuclear matrix protein; OSTase: oligosaccharide transferase complex; RHAMM: hyaluronan-mediated motility; RhoGDI2: RhoGTP dissociation inhibitor 2; SLea: sialyl lewisa; SLex: sialyl lewisx; STn: sialyl-Tn; TGF: transforming growth factor; TURBT: transurethral resection of the bladder tumour.

Author contributions

LL, LLS and JAF presented the idea of making this review. RA, AP, CG, EF, MN, LL and JAF structured the manuscript. RA, AP, CG, EF and MN searched the state of art. RA and AP produced the first draft of the review. All authors contributed with alterations on the manuscript. All alterations were approved by LL, LLS and JAF.

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CONFLICTS OF INTEREST

The authors declare no potential conflict of interests.

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ANEXO C

Targeted O-glycoproteomics explored increased sialylation and identified MUC16 as a poor prognosis biomarker in advanced-stage bladder tumours

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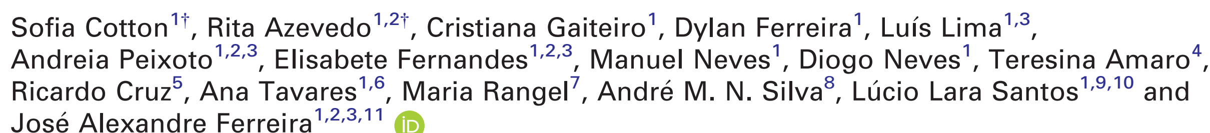
Targeted *O*-glycoproteomics explored increased sialylation and identified MUC16 as a poor prognosis biomarker in advanced-stage bladder tumours



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Bladder carcinogenesis and tumour progression is accompanied by profound alterations in protein glycosylation on the cell surface, which may be explored for improving disease management. In a search for prognosis biomarkers and novel therapeutic targets we have screened, using immunohistochemistry, a series of bladder tumours with differing clinicopathology for short-chain *O*-glycans commonly found in glycoproteins of human solid tumours. These included the Tn and T antigens and their sialylated counterparts sialyl-Tn(STn) and sialyl-T(ST), which are generally associated with poor prognosis. We have also explored the nature of T antigen sialylation, namely the sialyl-3-T(S3T) and sialyl-6-T(S6T) sialoforms, based on combinations of enzymatic treatments. We observed a predominance of sialoglycans over neutral glycoforms (Tn and T antigens) in bladder tumours. In particular, the STn antigen was associated with high-grade disease and muscle invasion, in accordance with our previous observations. The S3T and S6T antigens were detected for the first time in bladder tumours, but not in healthy urothelia, highlighting their cancer-specific nature. These glycans were also overexpressed in advanced lesions, especially in cases showing muscle invasion. Glycoproteomic analyses of advanced bladder tumours based on enzymatic treatments, *Vicia villosa* lectin-affinity chromatography enrichment and nanoLC-ESI-MS/MS analysis resulted in the identification of several key cancer-associated glycoproteins (MUC16, CD44, integrins) carrying altered glycosylation. Of particular interest were MUC16 STn^{+}-glycoforms, characteristic of ovarian cancers, which were found in a subset of advanced-stage bladder tumours facing the worst prognosis. In summary, significant alterations in the *O*-glycome and *O*-glycoproteome of bladder tumours hold promise for the development of novel noninvasive diagnostic tools and targeted therapeutics. Furthermore, abnormal MUC16 glycoforms hold potential as surrogate biomarkers of

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poor prognosis and unique molecular signatures for designing highly specific targeted therapeutics.

1. Introduction

Bladder cancer is the fifth most common cancer in Western society and a growing concern in developing countries, as a result of demographic expansion, increased life expectancy and, in some areas, *Schistosoma haematobium* infection (Antoni *et al*., 2016; Burger *et al*., 2013; Ploeg *et al*., 2009). At the time of clinical diagnosis, most cases are non-muscle-invasive bladder cancers (NMIBC), conservatively treated by complete transurethral resection (TUR) (Babjuk *et al*., 2016; Bryan, 2011). In turn, high-grade tumours are generally characterized by high recurrence rates and elevated risk of progression to muscle invasion (Babjuk *et al*., 2016; Bryan, 2011). Muscle-invasive bladder cancer (MIBC) is amongst the most common and deadliest genitourinary cancer (Witjes *et al*., 2013). The mainstay treatment includes cisplatin-based regimens (Witjes *et al*., 2013), which fail to avoid tumour relapse and disease dissemination (Chen *et al*., 2015; Weight *et al*., 2009), urging the introduction of predictive biomarkers and novel therapeutics (Azevedo *et al*., 2015; Ecke, 2015).

Glycosylation is the most common post-translational modification of proteins, and more than 50% of human proteins are thought to be glycosylated (Ferreira *et al*., 2016a; Spiro, 2002). The patterns of protein glycosylation are cell and tissue specific, closely reflecting the physiological status of cells (Moremen *et al*., 2012; Pinho and Reis, 2015; Spiro, 2002). Therefore, glycosylation changes have been described for several pathological conditions, including cancer (Abou-Abbass *et al*., 2016; Maverakis *et al*., 2015; Nardy *et al*., 2016). Taking advantage of their cell surface nature, many cancer-associated glycobiomarkers (CA72-4; CA19-9; CA125 which detects MUC16, CEA) have been exploited for noninvasive cancer detection, follow-up and therapy development (Bottoni and Scatena, 2015; Santos *et al*., 2014; Silva, 2015). Moreover, alterations in glycosylation often render protein glycoforms holding tremendous potential for targeted therapy (Azevedo *et al*., 2015; Fernandes *et al*., 2015; Ferreira *et al*., 2016b). In this context, it has also been long demonstrated that advanced-stage tumours present significant deregulations in glycosylation pathways, translated by the loss of ABO blood group determinants (Sheinfeld *et al*., 1992). Nevertheless, there are little insights on bladder cancer glycome remodelling accompanying malignant transformation, disease progression and dissemination. Still, few reports have suggested that bladder cancer cells mimic other advanced-stage solid tumours by promoting a premature stop in protein *O*-glycosylation (Ferreira *et al*., 2013; Langkilde, 1995; Yamada *et al*., 1988) (biosynthesis pathway depicted in detail in Fig. S1). This causes the accumulation of short-chain *O*-glycans as a consequence of (a) altered glycosyltransferase expressions (Vazquez-Martin *et al*., 2004); (b) mutations in key enzymes involved in *O*-glycans biosynthesis (Guda *et al*., 2009); (c) mislocalization of glycosyltransferases in secretory organelles (Rivinoja *et al*., 2009); (d) metabolic deregulations (Pinho and Reis, 2015), amongst other factors. The accumulation of short-chain *O*-N-acetylgalactosamine (*O*-GalNAc) glycans at the cell surface of tumour cells affects their adhesive properties while promoting their invasive, metastatic and angiogenic potential, as well as immune scape (Bapu *et al*., 2016; Carrascal *et al*., 2014; Ferreira *et al*., 2013). Moreover, it may modulate intracellular signalling and activate key oncogenic pathways (Bapu *et al*., 2016). Reinforcing these notions, we have previously demonstrated that 70% of advanced-stage bladder tumours express the cancer-associated carbohydrate antigen sialyl-Tn (Costa *et al*., 2015; Ferreira *et al*., 2013); conversely, the healthy urothelium and most superficial tumours do not (Ferreira *et al*., 2013). STn expression favours cell invasion, motility (Ferreira *et al*., 2013; Peixoto *et al*., 2016) and immune tolerance (Carrascal *et al*., 2014) and has been associated

Abbreviations

CD44, cluster of differentiation 44; CEA, carcinoembryonic antigen; CID, collision-induced dissociation; CSS, cancer-specific survival; DAGR, Database of Anti-Glycan Reagents; dST, disialylated sialyl-T; DTT, 1,4-dithiothreitol; ESI, electrospray ionization; FFPE, formalin-fixed, paraffin-embedded tissue section; HRP, horseradish peroxidase; ITGB1, integrin beta 1; LTQ-Orbitrap XL, hybrid linear ion trap-Orbitrap mass spectrometer; MIBC, muscle-invasive bladder cancer; MS/MS, tandem mass spectrometry; MS, mass spectrometry; MUC16, mucin-16; nanoLC, nanoliquid chromatography; NMIBC, non-muscle-invasive bladder cancer; *O*-GalNAc, *O*-N-acetylgalactosamine; PLA, *in situ* proximity ligation assay; S3T, sialyl-3-T; S6T, sialyl-6-T; STn, sialyl-tn; ST, sialyl-T; TUR, transurethral resection; WHO, World Health Organization.

with poor overall survival (Costa *et al*., 2015). In addition, solid tumours often accumulate the more complex T antigen and its sialylated form ST, whose overexpression has also been associated with poor prognosis (Dow *et al*., 1989; Videira *et al*., 2009). However, the ST antigen comprises a heterogeneous group of mono- (sialyl-6-T: S6T; sialyl-3-T: S3T) and disialylated glycoforms that remain to be individually evaluated in cancer. Building on these insights, we aimed to screen bladder tumours and corresponding urine samples for the above-mentioned cancer-associated short-chain *O*-glycoproteins, envisaging a molecular rationale for the development of novel non-invasive diagnostic tools and highly specific targeted therapeutics towards precision medicine.

2. Materials and methods

2.1. Patient and sampling

The screening of cancer-associated short-chain *O*-glycans (Tn and STn; T and ST, S6T and S3T) was performed on 47 formalin-fixed, paraffin-embedded tissue sections prospectively collected from 37 male and 10 female patients, mean age of 70 years (ranging 45–89 years old), who underwent bladder surgery in the Portuguese Institute for Oncology of Porto (IPO-Porto, Portugal), between July 2011 and May 2012. Based on urothelial carcinoma grading and staging criteria of the World Health Organization (Eble *et al*., 2004), three different groups were considered: low-grade (LG; n = 17), high-grade (HG; n = 12) non-muscle-invasive papillary bladder cancers (NMIBC) and muscle-invasive (n = 18) bladder cancers (MIBC). For molecular target validation, a larger subset of samples was used, composed by a retrospective series of 176 bladder cancer cases (74 NMIBC and 102 MIBC). In NMIBC, the male/female gender ratio was of 61 : 13 and the median age was 64 years. The male/female gender ratio in MIBC was of 9 : 1 and the median age was 71 years. Forty cases were considered stage Ta, 34 stage T1, 25 stage T2, 48 stage T3 and 29 stage T4 (for further analysis, T1- to T4-staged tumours were compared against Ta-staged tumours). All MIBC patients were treated with cystectomy, 27 of which were also treated with adjuvant chemotherapy (cisplatin+gemcitabine). All tumour samples were revised by a pathologist (TA) according to 2004 WHO grading criteria. As such, 38 cases were considered low-grade and 138 high-grade tumours. All procedures were performed under the approval of Institutional Ethics Committee of IPO-Porto after obtaining informed patient's consent. All clinicopathological information was obtained from patient's clinical records.

2.2. Immunohistochemistry

FFPE urothelium sections were screened for the glycans of interest by immunohistochemistry using the avidin/streptavidin peroxidase method, as described by Ferreira *et al*. (2013). The expression of the Tn, sialyl-Tn and T antigens was directly evaluated using in-house mouse monoclonal antibodies 1E3, TKH2 and 3C9, respectively (Clausen *et al*., 1988; Kjeldsen *et al*., 1988; Marcos *et al*., 2004). All available information on these antibodies including immunogens, specificity and associated bibliography is deposited in the Database of Anti-Glycan Reagents (https://ccr2.cancer.gov/resources/Cbl/Tools/Antibody/About.aspx) (Sterner *et al*., 2016). The expression of sialylated T antigens (mono- and disialylated glycoforms) was determined by comparing histological sections probed for the T antigen before and after digestion with an α-neuraminidase from *Clostridium perfringens* (Sigma Aldrich, St. Louis, MO, USA). The S3T antigen expression was determined by comparing histological sections probed for the T antigen before and after digestion with an α-(2,3)-neuraminidase from *Streptococcus pneumonia* (Sigma Aldrich) according to Fig. S1A. The S6T antigen expression was accessed by comparing histological sections probed for STn before and after digestion with a recombinant β-(1,3)-galactosidase from *Xanthomonas campestris* (R&D systems, Minneapolis, MN, USA) according to Fig. S1B. The chromogen 3,3-diaminobenzidine tetrahydrochloride (ImmPACT DAB; Vector Laboratories, Burlingame, CA, USA) was used to visualize antibody-binding sites, and sections were counterstained with Harris's haematoxylin. Negative controls were performed by replacing the primary antibody with 5% bovine serum albumin (BSA). Positive controls were known positive tissues for the antigens under study. Bladder tumours and metastasis were also screened for MUC16 using rabbit anti-human CA-125 monoclonal antibody EPR1020 (1 : 200 in PBS; Abcam, Cambridge, UK) at room temperature for 1 h. Furthermore, tumour tissues were screened for CD44 using anti-CD44 (1 : 150 in PBS; EPR1013Y; Abcam) and anti-ITGB1 (1 : 100 in PBS, A-4 clone; Santa Cruz Biotechnology, Dallas, TX, USA). In addition, prior to glycoproteomics studies, FFPE tissues were screened for blood group A determinants using mouse monoclonal anti-human blood group A antibody HE-195 (1 : 100 in PBS; Thermo Fisher Scientific, Waltham, MA, USA) after 1-h incubation at 37 °C. This approach aimed to elect negative

cases for downstream glycoproteomics studies. The immunoreactive tissue sections were assessed double-blindly through light microscopy by two independent observers (LL and DF) and validated by an experienced pathologist (TA). Although the interobserver agreement was high ($k = 0.961$, $P < 0.001$), discordant readings were re-analysed using a double-headed microscope (Olympus BX46; Olympus Corporation, Tokyo, Japan), and consensus was reached. A semi-quantitative approach was established to score the immunohistochemical labelling based on the percentage of positively stained cells. For the evaluation of glycans, the tissues were categorized as follows: negative (−), when no staining was observed; positive (+), 1–19% of positive cells; positive (++), 20–49% of positive cells; positive (+++), 50–79% of positive cells; positive (++++), 80–100% of positive cells. Regarding MUC16 evaluation, samples were classified as positive whenever the antigen was present or negative in the absence of the antigen.

2.3. Glycoprotein extraction and enrichment

Proteins were extracted from FFPE STn-positive bladder tumours of male MIBC patients ($n = 5$) using Qproteome FFPE tissue kit (Qiagen, Hilden, Germany) according to the supplier's instructions. To avoid false positives in downstream glycoprotein enrichment steps based on GalNAc affinity chromatography, only Tn and blood group A antigen-negative tumours were included in this study. Five 10-μm-thick tumour sections from each patient were used for this propose. The amount of protein in each extract was estimated with RC DC protein assay kit (Bio-Rad, Hercules, CA, USA). The extracted glycoproteins were then blotted for STn as previously described (Peixoto *et al.*, 2016) to confirm the presence of glycoproteins yielding the STn antigen. For proteomics analysis, 20 μg of the protein pool was separated by 4–16% gradient SDS/PAGE under reducing conditions; the bands were excised from the gels; and proteins were reduced with 5 mM 1,4-dithiothreitol (Sigma Aldrich) for 40 min at 60 °C, alkylated with 10 mM iodoacetamide (Sigma Aldrich) for 45 min in the dark and digested with trypsin (Promega, Madison, WI, USA) *in situ* for MS analysis (Ferreira *et al.*, 2011) (according to Fig. S3A). For glycoproteomics analysis, approximately 1 mg of total protein was precipitated by the addition of four volumes of −20 °C acetone to a sample extract and dried under vacuum on a speedvac. The extract was resuspended in 0.05% RapiGest (Waters, Milford, MA, USA), digested with PNGase F (10 U PNGase F from *Elizabethkingia meningoseptica*; Sigma Aldrich) to remove *N*-glycans, facilitating downstream trypsin digestion and peptide identification. Subsequently, the extract was subjected to neuraminidase treatment [10 U *C. perfringens* neuraminidase type VI (Sigma Aldrich)] to remove neuraminic acids from STn, thereby exposing the GalNAc residue (Tn antigen). The sample was then loaded on 300 μL of agarose-bound *Vicia villosa* agglutinin (VVA; Vector Laboratories) column to enrich the extract in Tn-expressing glycoproteins. The column was then washed with 10 column volumes of 0.4 M glucose in LAC A buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 M urea, 1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$) followed by 1 mL 50 mM NH_4HCO_3 (all reagents were purchased from Sigma Aldrich). The glycoproteins were then eluted by 4 × 500 μL 0.05% RapiGest (Waters) with heating to 90 °C for 10 min. The glycoprotein fraction was then directly reduced, alkylated and digested with trypsin as previously described (Ferreira *et al.*, 2011) (according to Fig. S3B).

2.4. NanoLC-ESI-LTQ-Orbitrap-CID-MS/MS

A nanoLC system (3000 Ultimate nano-LC; Dionex, Sunnyvale, CA, USA) was coupled online to a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a nano-electrospray ion source (EASY-Spray source; Thermo Scientific). Eluent A was aqueous formic acid (0.2%), and eluent B was formic acid (0.2%) in acetonitrile. Samples (20 μL) were injected directly into a trapping column (C18 PepMap 100, 5 μm particle size) and washed over with an isocratic flux of 95% eluent A and 5% eluent B at a flow rate of 30 $\mu L \cdot min^{-1}$. After 3 min, the flux was redirected to the analytical column (EASY-Spray C18 PepMap, 100 Å, 150 mm × 75 μm ID and 3 μm particle size) at a flow rate of 0.3 $\mu L \cdot min^{-1}$. Column temperature was set at 35 °C. Peptide separation occurred using a linear gradient of 5–40% eluent B over 117 min, 50–90% eluent B over 5 and 5 min with 90% eluent B. In order to favour the separation and identification of peptides presenting high hydrophobicity, samples were also analysed with a two-step gradient protocol: 5–35% eluent B over 37 min, 35–65% eluent B over 80 min, followed by 65–90% eluent B over 5 min and 5 min with 90% buffer B. The mass spectrometer was operated in the positive ion mode, with a spray voltage of 1.9 kV and a transfer capillary temperature of 250 °C. Tube lens voltage was set to 120 V. MS survey scans were acquired at an Orbitrap resolution of 60 000 for an m/z range from 300 to 2000. Tandem MS (MS/MS) data were acquired in the linear ion trap using a data-dependent method with dynamic exclusion: the

top six most intense ions were selected for collision-induced dissociation (CID). CID settings were 35% normalized collision energy, 2-Da isolation window, 30-ms activation time, and an activation Q of 0.250. A window of 90 s was used for dynamic exclusion. Automatic gain control was enabled and target values were 1.00e+6 for the Orbitrap and 1.00e+4 for LTQ MSn analysis. Data were recorded with XCALIBUR software version 2.1 (Thermo Fisher Scientific).

2.5. MS/MS data curation

Data were analysed automatically using the SequestHT search engine with the Percolator algorithm for validation of protein identifications (Proteome Discoverer 1.4; Thermo Scientific). Data were searched against the human proteome obtained from the SwissProt database on 22/11/2015, selecting trypsin as the enzyme and allowing for up to two missed cleavage sites, a precursor ion mass tolerance of 10 p.p.m. and 0.6 Da for product ions. Carbamidomethylcysteine was selected as a fixed modification, while oxidation of methionine (+15.994u), modification of serine and threonine with HexNac (+203.08u), and/or HexNacNeuNac (STn) (+494.17u), considering the possibility of partially inefficient α-neuraminidase treatment, and/or T (+365.13u) were defined as variable modifications. For whole tumour proteome analysis, only high confidence peptides were considered. In glycoproteomics studies, due to the high lability of the sugar moieties under CID conditions, and the consequent difficulty in identifying modified peptides, Sequest results of low confidence peptides were also considered. Protein grouping filters were thus set to consider glycosylations with low confidence and ΔCn better than 0.05. The strict maximum parsimony principle was applied. A protein filter counting peptides only on top-scored proteins was also set. Peptides were filtered for $\text{Xcorr} \geq 1.0$ and $\Delta\text{Cn} \leq 0.05$. Cytoplasm membrane proteins with at least one annotated glycosylation site were selected and the modifications were validated manually. Membrane proteins were sorted using NetOGlyc version 4.0 (http://www.cbs.dtu.dk/services/NetOGlyc/) (Steentoft *et al.*, 2013) to generate the final protein list. Protein molecular and biological functions were interpreted using Panther (Mi *et al.*, 2016).

2.6. *In situ* proximity ligation assays on tissue sections

The simultaneous detection of mucin-16 (MUC16), ITGB1 and CD44 STn^{+}-glycoforms was made by *in situ* proximity ligation assays (PLA) using the Duolink *in situ* detection reagents Brightfield and Red, respectively (Olink Bioscience, Uppsala, Sweden) according to the manufacturer's instructions and based on previous reports (Campos *et al.*, 2015; Ricardo *et al.*, 2015). Briefly, FFPE tissues were deparaffinized, rehydrated and subjected to acid- and heat-induced antigen retrieval, followed by incubation with 3% hydrogen peroxide and blocking solution in a humidity chamber, as previously described (Ferreira *et al.*, 2013). MUC16 was detected by direct PLA using monoclonal antibody CA125 (clone M11; DAKO, Santa Clara, CA, USA) conjugated with PLA probe PLUS (concentration of 0.005 mg·mL^{-1}) and B72.3 monoclonal antibody against STn, which showed similar recognition but lower background when compared with TKH2 monoclonal antibody used for immunohistochemistry, with PLA probe MINUS (concentration of 5 ng·mL^{-1}). Antibodies were conjugated according to the instructions of Duolink *in situ* Probemaker and were hybridized for 1 h at 37 °C. Next, ligation was performed for 30 min at 37 °C and amplification was carried out for 120 min at 37 °C to produce rolling circle products, followed by incubation with horseradish peroxidase (HRP)-labelled probes and addition of the chromogen. Finally, sections were counterstained with haematoxylin, dehydrated, cleared and mounted for optical microscope analysis. Regarding the indirect PLA for ITGB1 and CD44, FFPE tissues were incubated with anti-CD44 (EPR1013Y; Abcam) and anti-ITGB1 (A-4 clone; Santa Cruz Biotechnology) overnight at 4 °C in a humidity chamber. Then, the PLA probes anti-rabbit MINUS and anti-mouse PLUS were both added and sections were incubated at 37 °C for 1 h. The following steps of ligation and amplification were performed in the same conditions of the direct PLA. Sections were incubated with 4′,6-diamidino-2-phenylindole for 10 min at room temperature and mounted for fluorescence microscopy. PLA results were evaluated by two observers and validated by an experienced pathologist, who independently registered cytolocalization of staining. PLA validation was conducted using MUC16-expressing cell lines OVCAR3 wild-type which do not express STn (Kui *et al.*, 2003) and sequential ovarian cancer tissue sections showing MUC16 and STn colocalization by immunohistochemistry (Ricardo *et al.*, 2015).

2.7. *MUC16* transcription in bladder tumours

RNA was isolated from FFPE tissue samples using the Absolutely RNA FFPE Kit (Stratagene, San Diego, CA, USA), as previously described (Lima *et al.*, 2014). Up to 2 mg of total RNA was reverse-transcribed with

random primers, using the 'High Capacity cDNA Reverse Transcription Kit' (Applied Biosystems, Foster City, CA, USA). Real-time PCR amplification of cDNA samples was performed in a StepOne Real-Time PCR System (Applied Biosystems) using TaqMan Gene Expression Master Mix, primers and probes provided by Applied Biosystems. *MUC16* expression was measured with TaqMan expression assay (ID: Hs01065189_m1) from Applied Biosystems. The raw $-\Delta C_t$ was used to analyse *MUC16* expression and therefore used as an estimate of the mRNA relative levels. ΔC_t stands for the difference between the cycle threshold (C_t) of the amplification curve of the target gene and that of the *GAPDH* (ID: Hs03929097_g1). The efficiency of the amplification reaction for each primer/probe is more than 95%, as determined by the manufacturer.

2.8. Immunoprecipitation for CD44 and ITGB1

CD44 and ITGB1 were immunoprecipitated from total protein extracts (IP) with anti-CD44 (EPR1013Y; Abcam) and anti-ITGB1 (A-4 clone; Santa Cruz Biotechnology) monoclonal antibodies using Pierce Direct IP Kit (Thermo Scientific) according to the supplier's instructions. Protein samples were separated in reducing SDS/PAGE gels, transferred to 0.45-mm nitrocellulose membrane (GE Healthcare Life Sciences, Uppsala, Sweden) and blotted for the CD44 and ITGB1, respectively, as well as for STn with TKH2 monoclonal antibody. Protein extracts treated with α-neuraminidase (Sigma Aldrich) were used as controls.

2.9. Statistical methods

Statistical data analysis was performed with IBM Statistical Package for Social Sciences – SPSS for Windows (version 20.0; IBM, New York, NY, USA). Chi-square analysis was used to compare categorical variables. Kaplan–Meier survival curves were used to evaluate correlation between MUC16-positive tumours and cancer-specific survival (CSS) and were compared using log-rank statistical test. CSS was defined as the period between the tumour removal surgery and patient death from cancer and the last follow-up information.

3. Results and discussion

3.1. Expression of short-chain *O*-glycans in bladder cancer

Despite the biological and clinical relevance of altered *O*-glycosylation in cancer, few studies have comprehensively addressed this matter in the context of bladder malignancies. Herein, 47 bladder cancer sections were screened by immunohistochemistry for short-chain *O*-glycans, using specific monoclonal antibodies. These included the Tn, STn and T antigens, as well as sialylated T glycoforms (mono- and disialylated forms) exposed after digestion of the histological sections with a neuraminidase. Particular emphasis was given to the expression of T antigen monosialylated forms S3T and also S6T, which is regarded as rare *O*-glycan, until now mostly observed *in vitro* (Pinho *et al.*, 2007) and more recently in superficial bladder tumours (Lima *et al.*, 2013). Table 1 summarizes the expression of these glycans in the studied samples according to their disease subtype.

3.1.1. Expression of nonsialylated short-chain *O*-glycans (Tn and T antigens)

Table 1 highlights that Tn and T antigens are poorly expressed in bladder tumours (20–50% of total cases) in comparison with their sialylated counterparts (62% and 100%, respectively). More importantly, these antigens are mostly found in high-grade tumours, irrespective of the degree of invasion. Nevertheless, the number of T antigen-positive cases largely exceeds the Tn-positive cases (53% *vs* 23%), which was particularly notorious in advanced tumours when compared to low-grade superficial lesions. These observations suggest a possible overexpression of C1GalT1 (core 1 synthase, T-synthase) or downregulation of other glycosyltransferases involved in *O*-glycan extension in bladder tumours, which warrants careful evaluation in future studies. Possible modulation by secreted galactosidases, sialidases are also a possibility that should be investigated. Noteworthily, we have previously observed that bladder cancer cells exposed to hypoxia, a common microenvironmental feature in advanced tumours, promoted a striking downregulation in *C2GnT* accompanied by an increase in *C1GalT1* (Peixoto *et al.*, 2016). It is possible that similar events may account for T antigen accumulation in bladder tumours. More importantly, neither Tn nor T antigens were found in the six studied healthy urothelia cases, demonstrating the malignant nature of these molecular alterations. Finally, our observations reinforce early studies in bladder cancer glycosylation describing an association between T antigen expression and tumour invasion (Langkilde *et al.*, 1992). Focus should now be set on understanding the biological and clinical implications of this profound alteration in *O*-glycosylation.

Table 1. Expression of short-chain *O*-GalNAC glycans in bladder tumours of different clinicopathological natures determined by immunohistochemistry

		Tn	STn	T	ST	S6T	S3T
Non-muscle invasive bladder cancer (NMIBC) (%)							
Low Grade	17						
−		15 (88)	13 (76)	13 (76)	0 (0)	2 (12)	10 (59)
+		2 (12)	4 (24)	4 (24)	5 (29)	11 (65)	6 (35)
++					10 (59)	4 (24)	1 (6)
+++					2 (12)		
++++							
Positive cases, *n* (%)		2 (12)	4 (24)	4 (24)	17 (100)	15 (89)	7 (41)
High Grade	12						
−		5 (42)	3 (25)	9 (75)	0 (0)	0 (0)	3 (29)
+		7 (58)	7 (58)	3 (25)	3 (25)	5 (42)	7 (57)
++			2 (17)		2 (17)	7 (58)	2 (14)
+++					7 (58)		
++++							
Positive cases, *n* (%)		7 (58)	9 (75)	3 (25)	12 (100)	12 (100)	9 (71)
Muscle invasive bladder cancer (MIBC)	18						
−		16 (89)	2 (11)	0 (0)	0 (0)	5 (28)	7 (39)
+		2 (11)	16 (89)	4 (22)	3 (17)	8 (44)	7 (39)
++				6 (33)	5 (28)	6 (33)	4 (22)
+++				5 (28)	6 (33)		
++++				3 (16)	4 (22)		
Positive cases, *n* (%)		2 (11)	16 (89)	18 (100)	18 (100)	14 (78)	11 (61)
Total positive cases	47	11 (23)	13 (62)	25 (53)	47 (100)	38 (81)	27 (57)

Scoring: −, negative; +: > 0–19%; ++: 20–49%; +++: 50–79%; ++++: ≥ 80%.

3.1.2. Expression of sialylated short-chain *O*-glycans in bladder (STn and mono- plus disialyl-T)

Contrasting with neutral short-chain *O*-glycans, sialylated Tn and T antigens, including mono- and/or disialyl-T, are widely detected in bladder tumours irrespective of their grade and degree of invasion (62–100%; Table 1). In agreement with previous studies (Carrascal *et al.*, 2014; Costa *et al.*, 2015), the STn antigen was found in high-grade and invasive tumours (75 and 89%, respectively), whereas only 24% of low-grade cases were positive. The majority of the positive cases presented a low extension of expression (< 20%), of focal and polydisperse nature, throughout the tumour. STn was mostly found in cells of the basal layer (Fig. 1A); yet in tumour areas presenting extensive staining (> 50%) (Fig. 1B), it could also be detected in papillary urothelium and invasive fronts (Fig. 1B). Moreover, whenever present in the tumour, STn was also detected in the adjacent but not in the distal mucosa, also in agreement with previous reports (Carrascal *et al.*, 2014; Costa *et al.*, 2015). Hence, cells neighbouring the tumour are thought to carry significant alterations that result in the expression of this antigen. We also note that increase in STn is generally accompanied by a loss of Tn, reinforcing the association between increase in sialylation of *O*-glycan precursors and the severity of the lesions (Table 1). On the other hand, the sialylated forms of the T antigen, including mono- and/or disialylated glycans, are diffusely expressed by all studied bladder tumours (Table 1 and Fig. 2). However, a significant increase in the extension of sialylated T antigen could be observed in more advanced cases, suggesting an overexpression and/or increased activity of sialyltransferases (Fig. 2). In agreement with these observations, it has been demonstrated that advanced-stage bladder tumours overexpress ST3Gal-I (Videira *et al.*, 2009), the glycosyltransferase responsible for T antigen sialylation. In summary, while superficial tumours mostly present sialylated T antigens, more advanced-stage tumours also co-express more immature *O*-glycans, including the STn antigen (Table 1) that has been frequently associated with more malignant phenotypes and poor outcome (Bernardo *et al.*, 2014; Cabral *et al.*, 2010; Costa *et al.*, 2015; Ferreira *et al.*, 2013).

3.1.3. Exploring the nature of T antigen sialylation

Despite the widespread nature of sialylated T antigens in healthy and, particularly, malignant tissues, few studies have focused on disclosing the nature of

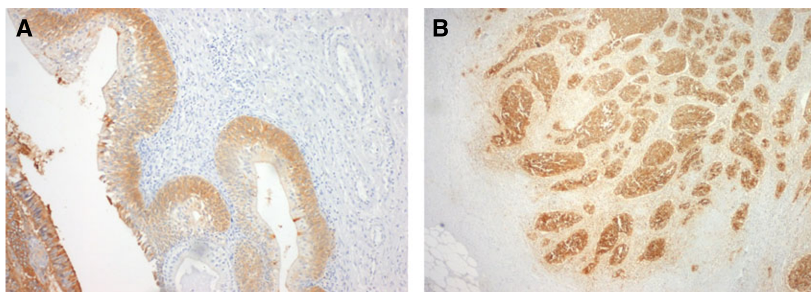


Fig. 1. Immunohistochemistry for sialyl-Tn (STn) antigen evidencing (A) expression in cells longing and invading the basal layer in high-grade NMIBC and (B) extensive staining including in cells invading the muscle layer in MIBC.

T antigen sialylation, most likely due to the lack of specific monoclonal antibodies and limitations in glycomics approaches. Facing these problems, we digested bladder tumour sections with a β-(1-3)-galactosidase prior to incubation with the anti-STn monoclonal antibody, to address the possibility of *O*-6 GalNAc sialylation (S6T). This procedure was responsible for the removal of *O*-3-linked Gal residues from S6T antigens exposing STn antigens for recognition (Fig. S2A). Accordingly, we observed positive staining after enzymatic treatment in STn-negative tumours (Fig. S2A), as well as an increased STn expression in several cases (Table 1), suggesting the presence of the S6T antigen. This glycan was found in approximately 80% of the studied tumours, with similar percentage of positive cases between NMIBC and MIBC. However, increased extension of expression could be observed in advanced tumour (Table 1 and Fig. 2). The S6T was further evaluated in FFPE healthy urothelium from six necropsied male individuals, which confirmed its cancer-associated nature. Recently, we have described that the presence of S6T and STn in bladder tumours was associated with a better response to BCG immunotherapy for more aggressive NMIBC, suggesting that *O*-6 sialylation plays a key role in *bacillus* binding to the epithelium (Lima *et al.*, 2013). Such observations reinforce the importance of including alterations in glycosylation in panomics predictive molecular models. Moreover, we have described an overexpression of ST6GalNAc-I, a key glycosyltransferase involved in *O*-6 sialylation of Tn antigens (Sewell *et al.*, 2006) in advanced-stage bladder tumours (Ferreira *et al.*, 2013). Future studies should be conducted to disclose the transcription of ST6GalNAc-I/II and possibly ST6GalNAc-IV, known to be involved in the *O*-6 sialylation of Tn antigens (Spiro, 2002), gaining more insights on the biological mechanisms underlying these molecular alterations and its clinical relevance.

On the other hand, incubation with a α-neuraminidase specific for cleaving *O*-3-linked sialic acids allowed T antigen detection in some negative tissues (Fig. S2B) and increased the extension and intensity of expression in T antigen-positive cases (Table 1), strongly suggesting the presence of the S3T antigen. Contrasting with the ubiquitous nature of S6T, the S3T antigen was mostly found in high-grade NMIBC (41% low-grade NMIBC; 71% high-grade NMIBC; 61% MIBC). Nevertheless, we should note that many high-grade tumours co-express both T sialylated forms. These observations support previous associations between the overexpression of both sialyl-T and ST3Gal-I, the sialyltransferase responsible for T antigen *O*-3 sialylation, in high-grade tumours (Videira *et al.*, 2009). Moreover, similar to S6T, the S3T antigen was also not detected in the healthy urothelium, reinforcing the cancer-associated nature of these antigens.

In summary, we have demonstrated that there are minor subsets of advanced-stage tumours that co-overexpress nonsialylated short-chain *O*-glycans (Tn and T antigens) in association with their sialylated glycoforms. Moreover, we have highlighted the structural diversity of T antigen sialylation in bladder tumours, its cancer-associated nature and the prevalence of up until now neglected *O*-6 sialoforms. Interestingly, this mimics the sialylation of the Tn antigen, whose biological and clinical significance has been extensively studied by our group. Furthermore, we have again reinforced the association between STn antigen expression and aggressive disease, raising to over 300 the number of evaluated tumour sections of different clinicopathological classifications and aetiologies (Bernardo *et al.*, 2014; Cabral *et al.*, 2010; Costa *et al.*, 2015; Ferreira *et al.*, 2013; Lima *et al.*, 2013; Peixoto *et al.*, 2016; Santos *et al.*, 2014). Significant efforts should be put on providing accurate quantification of these antigens using high-throughput glycomics approaches and on developing highly specific ligands. This would set the necessary means for large-scale clinical studies and targeted therapeutics. Moreover, it will be crucial for

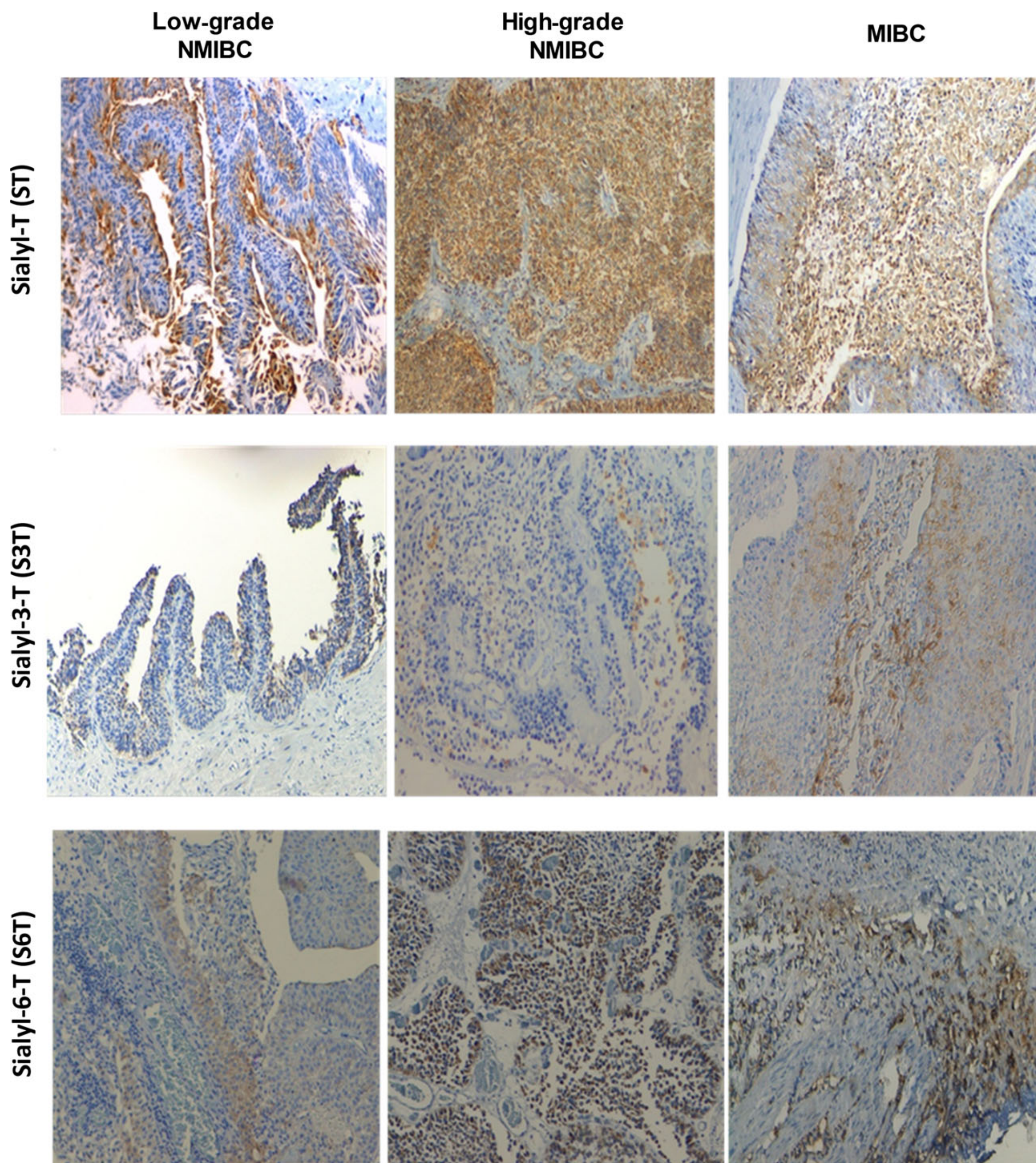


Fig. 2. Immunohistochemistry for sialylated T antigens (ST: corresponding to mono- and disialylated T glycoforms; S3T and S6T) for low- and high-grade superficial papillary muscle-invasive bladder tumours. The figure highlights the increase in T sialylation with the severity of the lesions. As the S6T antigen was determined based on comparisons with STn expression after β-(1,3)-galactosidase digestion, only STn-negative tumour lesions are being presented in this figure. Moreover, because the S3T antigen expression was determined based on comparisons with T antigen expression after α-(2,3)-neuraminidase treatment, only T-negative tissues are being presented.

understanding the molecular mechanisms underlying glycomic alterations, including (a) to determine the events modulating the expression and activity of glycosyltransferases and glycosidases in bladder tumours; (b) to access the distribution of glycosyltransferases throughout the secretory organelles and

pathways; (c) to explore mutations in key enzymes involved in *O*-glycans biosynthesis and its functional impact. Such information will be crucial for accessing the biological and clinical significance of altered *O*-glycosylation in bladder cancer, providing relevant insights for glycoproteomics studies and ultimately the design of novel and more effective therapeutics (Ferreira *et al.*, 2016a).

3.2. Bladder cancer-targeted glycoproteomics

Based on our previous and current observations, the STn constitutes a key cancer-associated antigen highly associated with advanced disease and poor prognosis (Bernardo *et al.*, 2014; Costa *et al.*, 2015; Ferreira *et al.*, 2013; Lima *et al.*, 2013; Peixoto *et al.*, 2016). Moreover, we have observed that STn expression significantly favours cell motility and capacity to invade (Ferreira *et al.*, 2013; Peixoto *et al.*, 2016) as well as immune escape (Carrascal *et al.*, 2014). Therefore, mapping the STn-glycoproteome is crucial for developing highly specific targeted therapeutics against advanced-stage bladder tumours. However, while the majority of glycoproteomics studies presented so far have focused mostly on body fluids and, to lower extent, human tissues, none has attempted to address protein glycosylation in FFPE tissues. Herein, we extracted proteins from five MIBC tumours and screened the samples for STn expression by western blot, which retrieved similar expressions patterns (Fig. S5A). These samples were then pooled and analysed by a conventional gel-based and nanoLC-MS/MS proteomics approach (Fig. S3A), which allowed the identification of 2578 peptides, corresponding to 294 proteins (Table S1). This illustrated that the feasibility of using FFPE has starting material for retrospective proteomic studies on clinical samples, despite the significant modifications and degradation induced by paraffin embedding. Gene ontology interpretation of the results using Panther highlighted the presence of proteins from all cell compartments, including plasma membrane proteins known to yield the STn antigen (4%; Fig. S4A); nevertheless, an overrepresentation of cytoplasmic and cytoskeleton proteins could be observed (Fig. S4A), in accordance with its higher abundance in the cellular milieu. The main represented molecular functions included binding, structural and catalytic activities, whereas the main biological functions were set on metabolic and cellular processes (Fig. S4B,C), in accordance with the wide range of identified proteins. Nevertheless, due to the low abundance of STn-expressing membrane glycoproteins potentially yielding the STn antigen, an enrichment step was introduced based on affinity to VVA lectin that selectively binds terminal GalNAc residues. To render the glycoproteins with affinity for the chosen lectin, the extracts were digested with a α-neuraminidase prior to the enrichment step, which removed sialic acids from STn exposing the GalNAc residue (Tn antigen). The absence of Tn and blood group A determinants in the chosen cases ensured the specificity of the enrichment for STn-expressing proteins (Fig. S3B). Subsequent nanoLC-MS/MS analysis led to the identification of over 400 *O*-glycosites and 143 membrane glycoproteins putatively expressing the STn antigen (Table S2), which may be potential targets for targeted therapies. These glycoproteins were found associated with a wide array of molecular and biological functions, as depicted in detail in Fig. 3. In particular, STn-expressing proteins mostly mediate binding to other proteins and have hydrolase catalytic activities. They also mediate cell–cell communication and signalling and regulate primary metabolic processes. These observations strongly suggest that altered glycosylation may influence a wide array of cell functions, thereby providing key preliminary insights to understand the role of STn expression in bladder cancer. Approximately half of the identified glycoproteins had been previously studied in the context of bladder cancer and could be comprehensively distributed according to its association with disease on an analysis *in silico* with Oncomine (Rhodes *et al.*, 2007) (Fig. 4). This list included CD44, a typical bladder cancer stem-cell associated glycoprotein also associated with drug-resistant phenotypes and poor prognosis (Kobayashi *et al.*, 2016), and several integrins, in accordance with previous observations (Peixoto *et al.*, 2016). For validation purposes, we have immunoprecipitated CD44 and ITGB1 in these samples and confirmed the expression of STn by western blot (Fig. S5B). Furthermore, immunohistochemistry showed the co-expression of these antigens in the same tumour area, which was confirmed by PL (Fig. S5C), which allows the simultaneous detection of the protein and the glycan whenever there is close proximity. In addition to these glycoproteins, we have also identified, for the first time, MUC16 and abnormal MUC16 glycoforms in bladder tumours (Table S1). Interestingly, these high molecular weight glycoproteins are generally found in ovarian tumours facing poor prognosis, being frequently used for serological monitoring and as diagnostic marker of ovarian cancer (CA125 test) (Duffy *et al.*, 2005; Felder *et al.*, 2014; Vasudev *et al.*, 2011). Again, we have confirmed the presence of STn in MUC16-derived glycopeptides based on characteristics of CID-MS/MS fragmentation spectra (Fig. 5A).

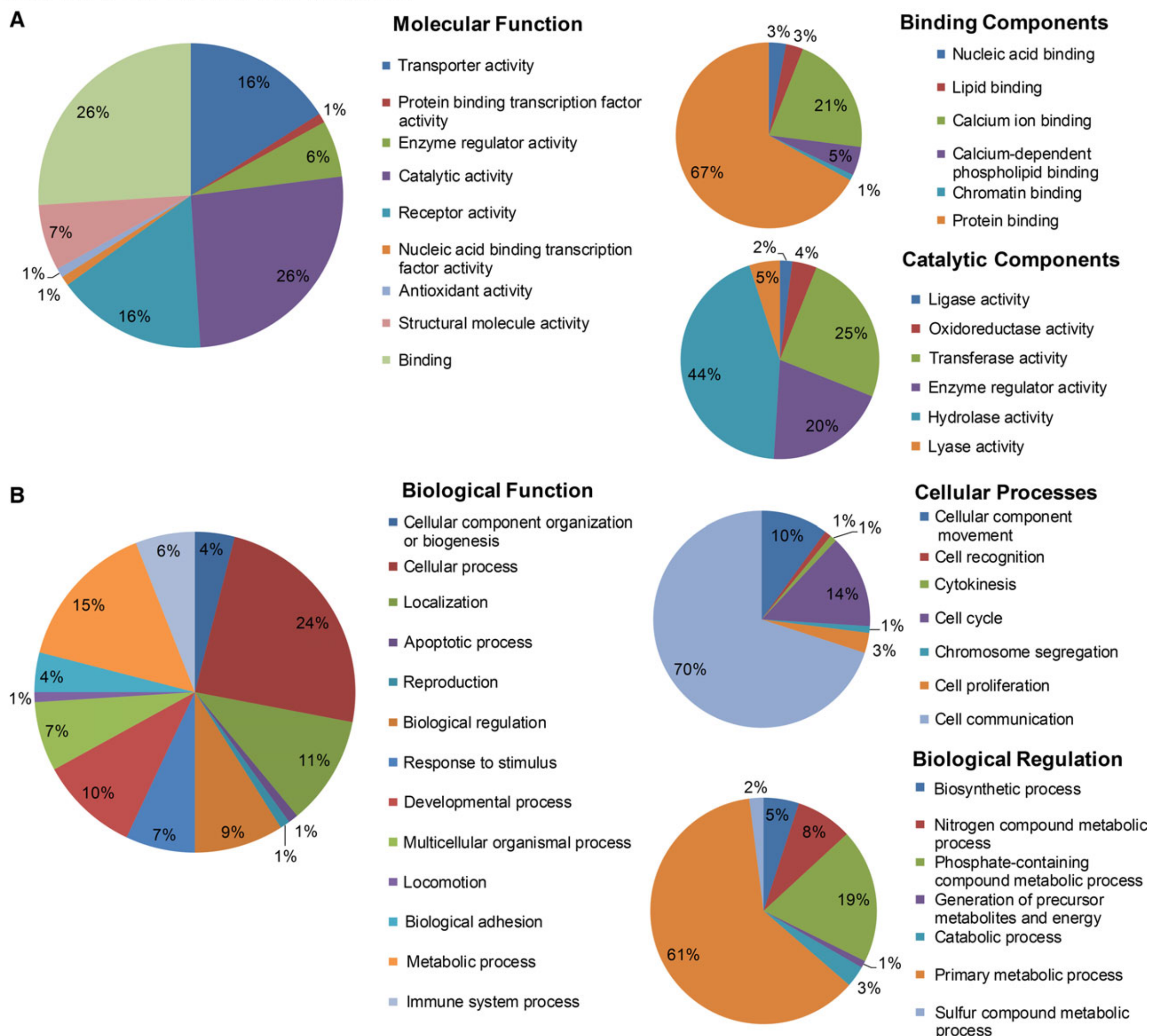


Fig. 3. Distribution of candidate STn-expressing glycoproteins in muscle-invasive bladder tumours (detailed in Table S2) comprehensively integrated according to cellular localization (A), molecular (B) and cell functions (C) based on gene ontology analysis by Panther bioinformatics tool. STn-expressing proteins were found to be associated with a wide array of molecular and biological functions as depicted in detail in the figure. Accordingly, the identified glycoproteins were involved in nine main classes of molecular functions, with an overrepresentation of catalytic activities (hydrolase, lyase and transferase activities) and protein binding mediation. Moreover, 13 main biological functions were highlighted, being the most representative cellular processes such as cell communication and, to some extent, cell cycle control. These observations suggest that altered glycosylation may influence a wide range of key cell events, which warrants evaluation in future studies.

Moreover, we found glycopeptides carrying both GalNAc and Gal-GalNAc substituents, highlighting the complex antigenic glycoarray presented by bladder cancer-associated glycoproteins (Fig. S6). In addition, the analysis of consecutive bladder tumour sections revealed that MUC16 expression is associated and colocalized with STn expression in 95% of the cases, irrespective of their histological classification (Fig. 5B). Moreover, PLA confirmed the presence of MUC16 STn^+ glycoforms in clinical samples (Fig. 5C). Despite unequivocal data supporting the existence of MUC16 STn- glycoforms, we have further attempted to IP and blot this glycoprotein as it has been done for CD44 and ITGB1; however, its high molecular weight

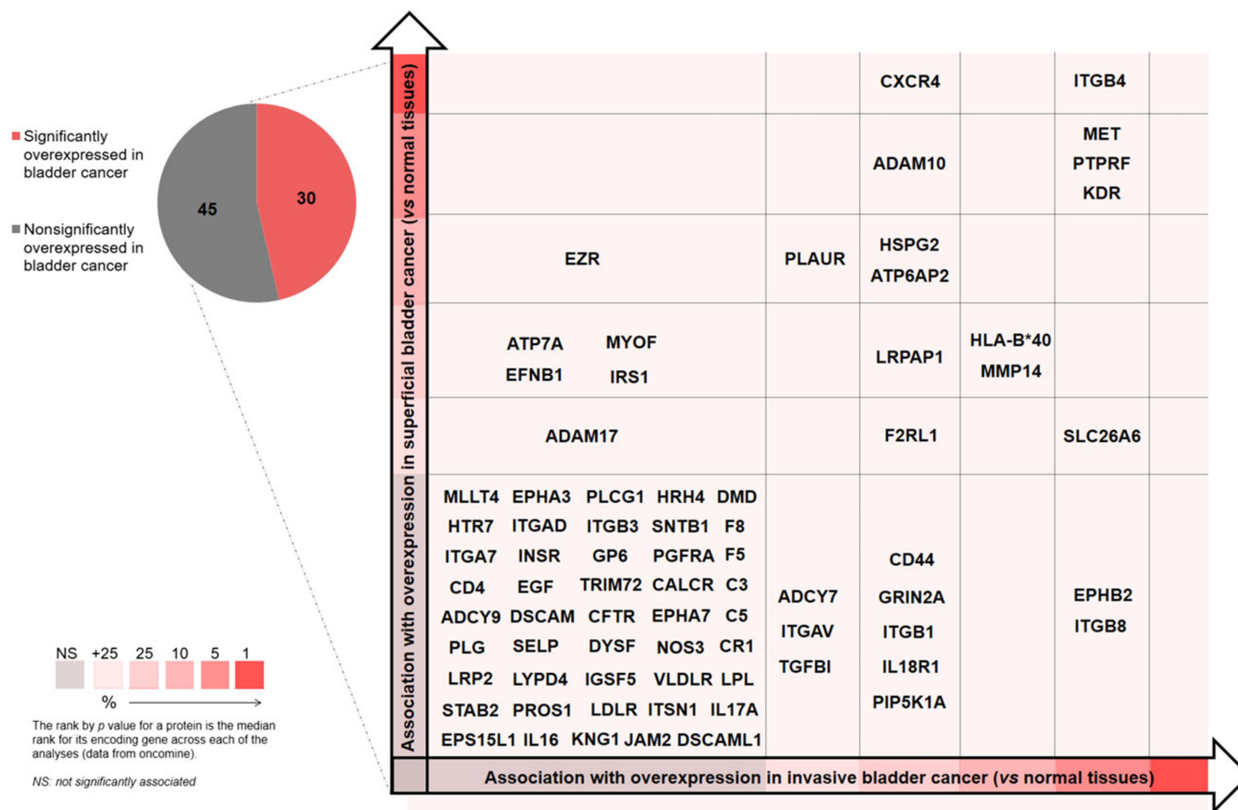
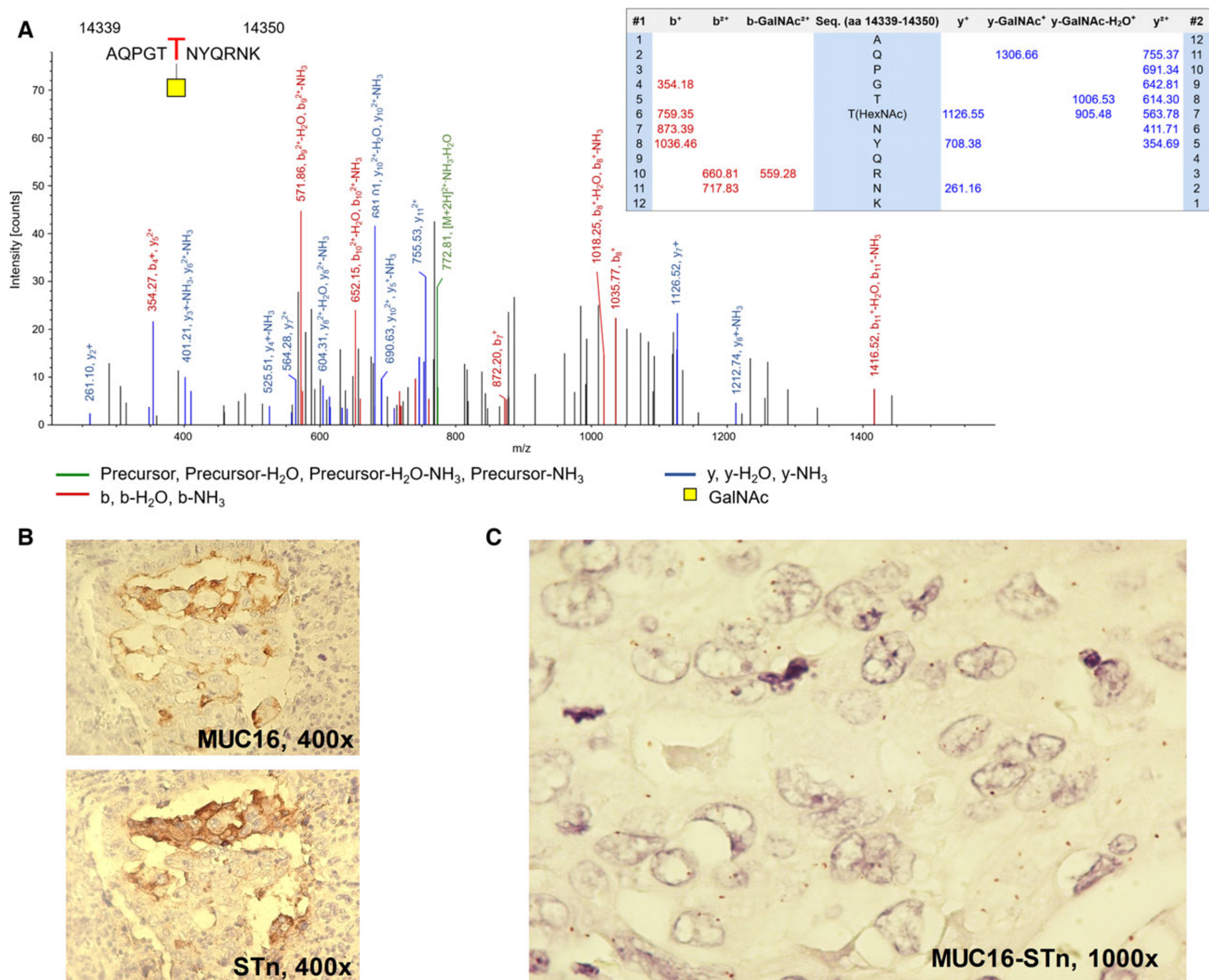


Fig. 4. Candidate STn-expressing glycoproteins in muscle-invasive bladder tumours comprehensively distributed according to its association with the severity of the lesions. Briefly, the identified glycoproteins were distributed according to associations with the type of lesion based on an *in silico* analysis with Oncomine. Proteins identified for the first time in bladder tumours have not been included in the graph due to the lack of associations with the type of disease.

(Mw < 2000 kDa) and the lack of suitable antibodies for this procedure have posed as a significant analytical limitation that will be addressed in future studies. Even though this is the first report regarding MUC16 expression in bladder cancer, CA125 elevation has been previously observed in the serum of patients with advanced pathological stage in comparison with lower-stage disease, suggesting that this antigen may predict advanced bladder cancer (Margel *et al.*, 2007; Vasudev *et al.*, 2011). Furthermore, abnormal CA125 levels have been associated with unresectable tumours, again reinforcing its association with worse prognosis (Vasudev *et al.*, 2011).

3.3. Clinical significance of MUC16 expression in bladder cancer

Given the key role of MUC16 in ovarian cancer (Felder *et al.*, 2014; Ricardo *et al.*, 2015), and building on the lack of clinical data for bladder cancer, we have screened a retrospective series of 176 tumours spanning different classifications (74 NMIBC and 102 MIBC). MUC16 was mainly expressed in the cell membrane and cytoplasm, with moderated and focal expression that did not exceed 20% of tumour cells for the majority of the positive cases (Fig. 5B), irrespective of their histological/TNM classification. The MUC16 antigen was observed in approximately 27% of cases (48 of 176), mainly in tumours showing *lamina propria* (T1; 30%) and *muscularis propria* (≥ T2; 20–40%) invasion; conversely, the number of MUC16-positive Ta tumours was lower than 15% (Fig. 6A; $P < 0.005$). Concerning WHO criteria, MUC16-positive cells were mostly observed in the high-grade cases ($P = 0.008$; Fig. 6B), reinforcing the association between MUC16 expression and poor prognosis. In agreement with these observations, we have also observed an increased transcription of *MUC16* gene in MUC16-positive tumours in comparison with MUC16-negative tumours (Fig. S7, $P = 0.005$). Moreover, we found that MUC16 expression associates with lower CSS in MIBC patients treated with cisplatin and



#1	b^+	b^{2+}	b-GalNAc^{2+}	Seq. (aa 14339-14350)	y^+	y-GalNAc$^+$	y-GalNAc-H_2O^+	y^{2+}	#2
1				A					12
2				Q		1306.66		755.37	11
3				P				691.34	10
4	354.18			G				642.81	9
5				T			1006.53	614.30	8
6	759.35			T(HexNAc)	1126.55		905.48	563.78	7
7	873.39			N				411.71	6
8	1036.46			Y	708.38			354.69	5
9				Q					4
10		660.81	559.28	R					3
11		717.83		N	261.16				2
12				K					1

Fig. 5. (A) Exemplificative annotated nanoLC-ESI-LTQ-Orbitrap-CID-MS/MS spectra for a MUC16 glycopeptide substituted with a HexNAc residue evidencing the specific glycosite; (B) colocalization of MUC16 and STn in bladder tumours by immunohistochemistry; (C) expression of MUC16 STn glycoforms in bladder tumours based on PLA analysis. This work identified for the first time MUC16 in bladder tumours and its association with abnormal glycoforms such as the STn antigen. The mass spectrum shows a MUC16 glycopeptide substituted with a HexNAc residue, strongly suggesting the presence of STn. The colocalization of MUC16 and STn (B) in bladder tumours also reinforces this hypothesis. Finally, the red dots on the PLA image (C) in areas of colocalization result from the simultaneous detection of both antigens, reinforcing this evidence.

gemcitabine, suggesting a possible role in drug resistance that is being currently evaluated. These observations are in agreement with the findings from serological CA125 evaluation (Felder *et al.*, 2014; Rao *et al.*, 2015) and strongly support the need for a deeper investigation on the biological and clinical significance of MUC16 in bladder cancer.

4. Conclusions

It has been long known that advanced bladder tumours present significant alterations in glycosylation that relate to worst prognosis; however, there is a lack of information on the structural nature of cancer-specific glycans. This work highlights that advanced bladder tumours overexpress and frequently co-express an array of short-chain *O*-glycans resulting from a premature stop in the glycosylation of membrane and secreted proteins. Moreover, it clearly demonstrates a predominance of sialylated over neutral glycoforms, with emphasis on sialylated Tn and T antigens. In addition, for the first time, we provide key insights on the nature of the T antigen sialylation, which will be crucial for guiding

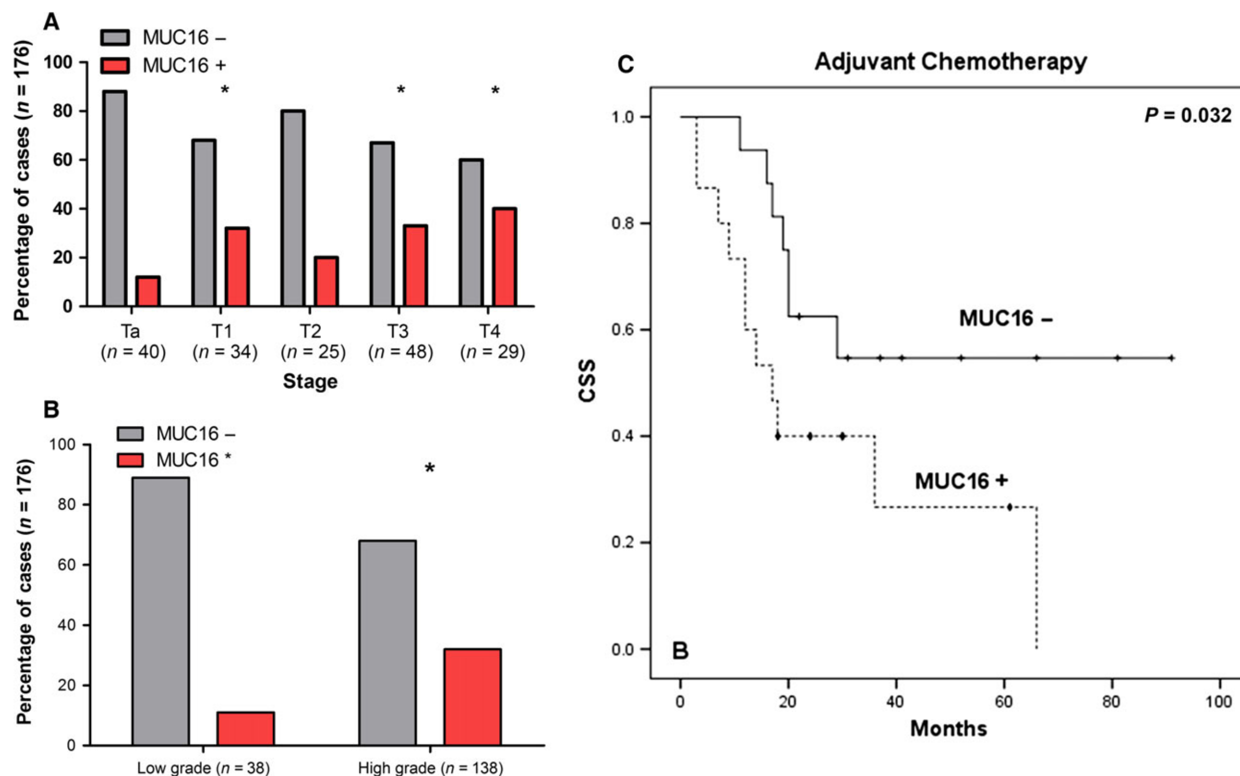


Fig. 6. (A) Associations of MUC16 with the stage; (B) grade of the disease; and (C) decreased overall survival in patients with MIBC subjected to cisplatin-based chemotherapy. Accordingly, MUC16 was associated with more aggressive bladder tumours, namely advanced stages and grade of the disease. Moreover, its presence in MIBC associates with decreased survival in MIBC subjected to chemotherapy. For (A) and (B), comparisons between data sets were made by chi-square test (**P* < 0.05); in (C), a log-rank test was performed, *P* = 0.032. + censored MUC16-negative tumours; ♦ censored MUC16-positive tumours.

future glycomics and glycoproteomics studies and for designing specific ligands against bladder cancer cells. Moreover, we have highlighted a significant increase in *O*-6 sialylation in bladder tumours, particularly the STn antigen. Finally, we have mined the glycoproteome of advanced bladder tumours for STn-expressing glycoproteins. This resulted in the identification of MUC16 as a novel biomarker for a subset of bladder tumours presenting poor prognosis. It also highlighted a molecular link between bladder and ovarian cancer, where abnormally glycosylated MUC16 plays a key role in disease progression and dissemination. Future studies should now be focusing on the biological role of this glycoprotein in bladder cancer. Our findings also reinforce the need to comprehensively address the CA125 antigen in the sera and, possibly, also urine of patients with bladder cancer. Furthermore, we augment that a careful mapping of MUC16 and other cancer-associated glycoproteins may provide the necessary structural information for highly specific biomarkers and targeted therapeutics.

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Author contributions

LLS and JAF conceived and designed the project; RC and LLS provided the samples; SC, RA, CG, DF, AP, EF, MN, DN, AT, MR and AMNS acquired the data; SC, RA, DF, TA, LL, RC, AMNS, LLS and JAF analysed and interpreted the data; RA and JAF wrote the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Schematic representation protein *O*-GalNAc glycosylation biosynthesis evidencing the cancer-associated short-chain glycans explored in this study.

Fig. S2. Schematic representation of the analytical strategy for S6T and S3T evaluation by immunohistochemistry.

Fig. S3. Analytical workflow for (A) whole proteome analysis starting from FFPE tissues and (B) identification of STn expressing glycoproteins in bladder tumours.

Fig. S4. Proteins isolated from FFPE muscle-invasive bladder tumours distributed according to cellular localization (A), molecular (B) and cell functions (C) based on gene ontology analysis.

Fig. S5. (A) Western blot for glycoproteins expressing the STn antigen in advanced bladder tumours. (B) Identification of STn glycoforms in CD44 and ITGB1 glycoproteins isolated from advanced bladder tumours by immunoprecipitation. (C) Immunohistochemistry

and PLA for CD44, ITGB1 and STn in bladder tumours.

Fig. S6. Annotated nanoLC-ESI-LTQ-orbitrap-CID-MS/MS spectra for a MUC16 glycopeptide substituted with a HexNAc and HexNAc-Hex residues evidencing the specific glycosites (highlighted in the assignment table below).

Fig. S7. Association between MUC16 classification by immunohistochemistry in FFPE cancer tissues (IHC; negative vs positive) and *MUC16* expression.

Table S1. Proteins identified with high confidence level in Tn-negative, blood group A negative, STn-positive tumour samples recovered from formalin-fixed paraffin embedded tissues.

Table S2. Identified membrane glycoproteins from Tn-negative, blood group A negative, STn-positive MIBC, with O-HexNAc as posttranslational modifications after neuraminiase treatment.

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ANEXO D

Abnormal Protein Glycosylation and Activated PI3K/Akt/mTOR Pathway: Role in Bladder Cancer Prognosis and Targeted Therapeutics

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RESEARCH ARTICLE

Abnormal Protein Glycosylation and Activated PI3K/Akt/mTOR Pathway: Role in Bladder Cancer Prognosis and Targeted Therapeutics

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Abstract

Muscle invasive bladder cancer (MIBC, stage ≥T2) is generally associated with poor prognosis, constituting the second most common cause of death among genitourinary tumours. Due to high molecular heterogeneity significant variations in the natural history and disease outcome have been observed. This has also delayed the introduction of personalized therapeutics, making advanced stage bladder cancer almost an orphan disease in terms of treatment. Altered protein glycosylation translated by the expression of the sialyl-Tn antigen (STn) and its precursor Tn as well as the activation of the PI3K/Akt/mTOR pathway are cancer-associated events that may hold potential for patient stratification and guided therapy. Therefore, a retrospective design, 96 bladder tumours of different stages (Ta, T1-T4) was screened for STn and phosphorylated forms of Akt (pAkt), mTOR (pmTOR), S6 (pS6) and PTEN, related with the activation of the PI3K/Akt/mTOR pathway. In our series the expression of Tn was residual and was not linked to stage or outcome, while STn was statically higher in MIBC when compared to non-muscle invasive tumours (p = 0.001) and associated decreased cancer-specific survival (log rank p = 0.024). Conversely, PI3K/Akt/mTOR pathway intermediates showed an equal distribution between non-muscle invasive bladder cancer (NMIBC) and MIBC and did not associate with cancer-specif survival (CSS) in any of

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these groups. However, the overexpression of pAKT, pmTOR and/or pS6 allowed discriminating STn-positive advanced stage bladder tumours facing worst CSS (p = 0.027). Furthermore, multivariate Cox regression analysis revealed that overexpression of PI3K/Akt/mTOR pathway proteins in STn+ MIBC was independently associated with approximately 6-fold risk of death by cancer (p = 0.039). Mice bearing advanced stage chemically-induced bladder tumours mimicking the histological and molecular nature of human tumours were then administrated with mTOR-pathway inhibitor sirolimus (rapamycin). This decreased the number of invasive lesions and, concomitantly, the expression of STn and also pS6, the downstream effector of the PI3K/Akt/mTOR pathway. In conclusion, STn was found to be marker of poor prognosis in bladder cancer and, in combination with PI3K/Akt/mTOR pathway evaluation, holds potential to improve the stratification of stage disease. Animal experiments suggest that mTOR pathway inhibition could be a potential therapeutic approach for this specific subtype of MIBC.

Introduction

Bladder cancer is the second most deadly genitourinary tumour and presents significantly worse prognosis upon *muscularis propria* invasion [1]. Approximately 20–30% of the newly diagnosed cases are muscle invasive bladder cancers (MIBC; T2-T4 stages), while 50% are non-muscle invasive bladder tumours (NMIBC) with high potential to progress to invasion. MIBC treatment includes cystectomy and (neo)adjuvant cisplatin-based chemotherapy regimens [2]. However, significant variations in the natural history of the disease and responses to treatment can be observed between tumours with identical histological features, reflecting their high molecular heterogeneity [3]. Furthermore, approximately 50% of cases develop metastasis within 5 years, urging the identification of biomarkers to assist prognostication and the development of more effective targeted therapeutics [4].

To meet this need, we have recently addressed the expression of the cancer-associated sialyl-Tn antigen (STn) on a small prospective series of unselected bladder cancer patients [5]. STn is an abnormal post-translational modification that results from a premature stop in cell-membrane proteins *O*-glycosylation by sialylation of the Tn antigen (Fig 1A). In bladder tumours, STn it was mainly present in advanced stage cases, while absent from most low-grade NMIBC [5]. Moreover, it was not expressed by the normal urothelium, denoting a cancer-specific nature [5]. Studies *in vitro* showed that STn expression endowed bladder cancer cells with high invasion capability [5] and an immunotolerogenic phenotype, potentially favoring disease dissemination [6]. Alterations in cell-surface protein glycosylation have be implicated in the activation of intracellular oncogenic signalling pathways [7], including the phosphoinositide-3 kinase (PI3K)/Akt signalling pathway [8] which is thought to play a critical role in bladder cancer development. These preliminary observations support the hypothesis that STn expression may play a key role in disease outcome, which warrants a deeper investigation. Several studies also suggest that Tn antigen, which is a precursor of STn, may be also implicated in oncogenic events [7]; however nothing is known about the expression of this glycan in bladder tumours.

The phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) pathways are interconnected signaling cascades essential for bladder cell growth and survival (Fig 1B). The PI3K/Akt/mTOR or mTOR pathway integrates a multiplicity of extracellular signals to regulate downstream signaling and protein synthesis, which ultimately leads to

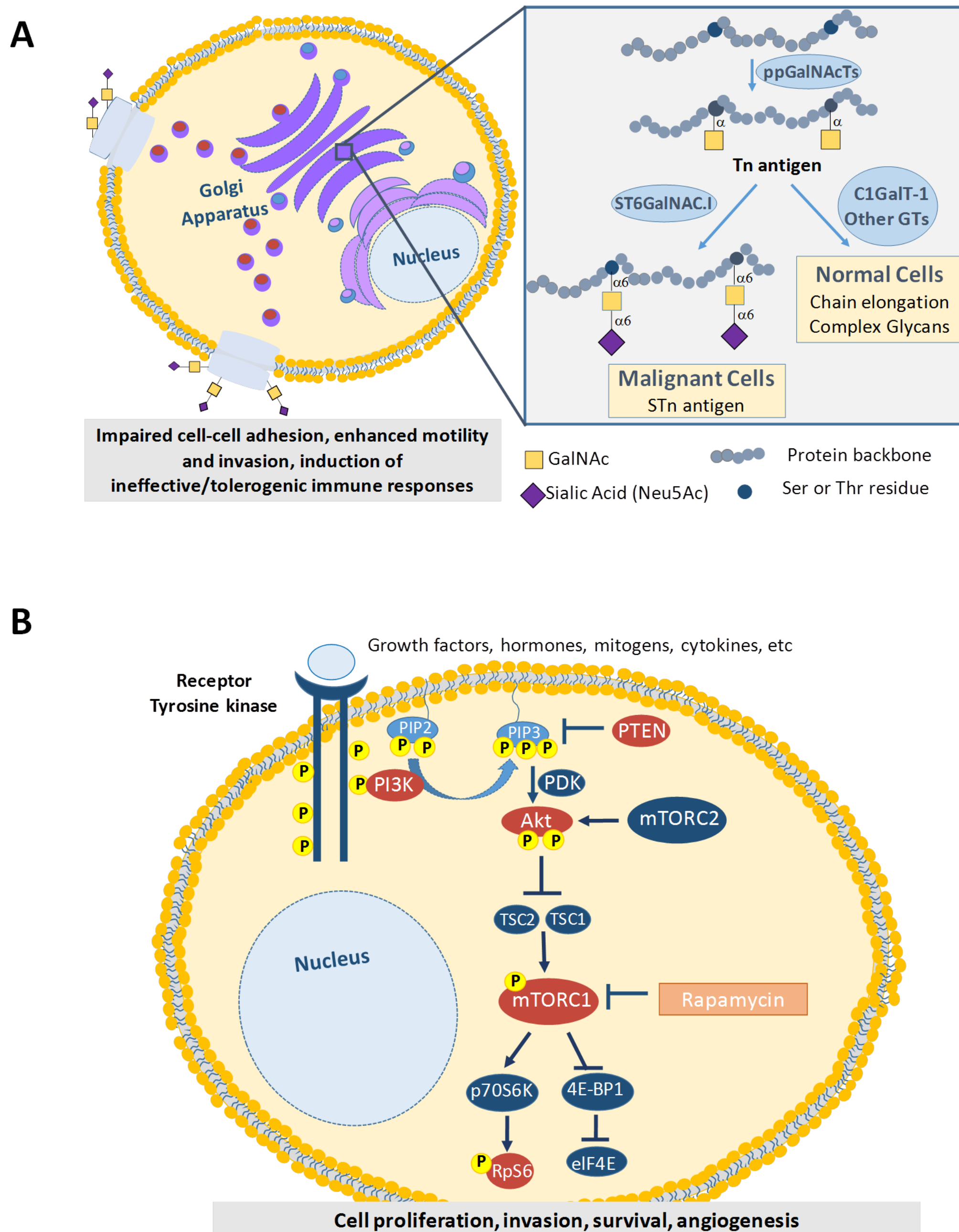

Fig 1. Schematic representation of membrane protein *O*-glycosylation and the PI3K/Akt/mTOR pathways. A) Representation of membrane protein *O*-glycosylation with emphasis on the STn expression by cancer cells. This is a highly regulated process of critical importance for protein stability and function. Briefly, newly synthesized proteins are *O*-glycosylated in the Golgi apparatus by the ppGalNAcTs-mediated addition of GalNAc moiety to Ser/Thr residues. This originates the Tn antigen (GalNAc-O-Ser/Thr-protein backbone), which is the simplest O-glycan. In normal cells these chains are extended through the sequential addition of other sugars first by CGALT-1 and then other enzymes. This culminates in highly complex, heterogeneous and elongated glycans often terminated by ABO or Lewis blood group related antigens (left drawing). In cancer cells the Tn antigen is immediately sialylated by ST6GalNAc.I, originating the STn antigen (Neu5Ac-GalNAc-O-Ser/Thr-protein backbone), thereby inhibiting further chain elongation (right drawing). The expression of STn at the cell surface influences cell-cell adhesion and cancer cell recognition, favouring motility, invasion and immune escape. B) Schematic representation of the PI3K/Akt/mTOR pathway, which is ubiquitously activated in bladder tumours. This is a highly conserved pathway regulated mainly by a wide variety of extracellular signals, including mitogenic growth factors, hormones, nutrients, cellular energy levels, and stress conditions. These signals activate tyrosine receptor kinases that recruit PI3K, which catalyses the conversion of membrane-bound PIP2 to PIP3. Then Akt and PDK-1 are activated through binding to PIP3. PTEN preferentially dephosphorylates PIP3, inhibiting signalling progression. Full Akt activation requires double phosphorylation by PDK-1 itself and PDK-2 (not shown). Akt phosphorylates mTOR directly or may also inactivate TSC1/TSC2 complex, inhibiting mTOR inactivation. mTORC1 triggers cell growth and proliferation by phosphorylating eukaryotic translation regulators, among these p70S6 kinase (p70S6K or S6K1) that, in turn, phosphorylates the

ribosomal protein S6 (pS6), and the eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1). For the protein mTOR to activate its signalling cascade, it must form the rapamycin-sensitive ternary complex mTORC1. Key PI3K/Akt/mTOR-pathway proteins pAkt, pmTOR and pS6 explored in this studied are highlighted by orange circles.

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a competitive growth advantage, metastatic competence, angiogenesis, and therapy resistance [9]. The signaling cascade begins with PI3K activation in the cell membrane followed by serine/threonine kinase Akt cell membrane translocation and activation. The best studied downstream substrate of Akt is the serine/threonine kinase mTOR, whose downstream effector is S6 kinase-1 (S6K1). In particular, a subset of mTOR pathway alterations have been shown to occur in bladder cancer, such as mutations in *PIK3CA* gene, which culminates with increased mTOR signaling and bladder cancer cells resistance to apoptosis [10]. Moreover, the pharmacological or biochemical inhibition of the PI3K pathway drastically reduced the invasive capacity of bladder cancer cell lines. Furthermore, over half of primary human bladder tumours present high Akt phosphorylation and the aberrant activation of this pathway has been suggested to contribute to invasion [11]. Another event influencing mTOR pathway activation in bladder tumours involves the loss of tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten) function [12]. PTEN normally suppresses activation of the PI3K/Akt/mTOR pathway antagonizing PI3K and preventing activation of Akt and PDK-1. PTEN also functions to regulate chemotaxis and cell motility, thereby promoting tumor invasion [13]. In summary, there are evidences that a comprehensive evaluation of PI3K/Akt/mTOR pathway associated proteins may hold significant potential for value for patient stratification. Moreover, many preclinical and clinical studies support that mTOR inhibitors, such as sirolimus (rapamycin) and their derivatives may improve cancer treatment [13,14].

Based on these observations we hypothesize that Tn and/or STn may act synergistically with the mTOR pathway to drive bladder cancer progression. As such, we have devoted to evaluating the expression of STn and proteins associated with the activation of the PI3K/Akt/mTOR pathway activation in bladder tumours at different stages. We anticipate that the combination of extracellular and intracellular oncogenic events may improve patient stratification and provide insights for novel therapeutics. Furthermore we have estimated the impact of sirolimus in chemically-induced urothelial tumours in mice, envisaging the creation of a rationale for more effective bladder cancer therapeutics.

Materials and Methods

Ethics Statement

This work involves experiences in tumour samples of patients diagnosed with bladder cancer in the Portuguese Insitute of Oncology of Porto. All procedures were performed after patient's written informed consent and approved by the Ethics Committee of Portuguese Institute of Oncology—Porto. All clinicopathological information was obtained from patients' clinical records.

It also involves animal experiments. All procedures involving animals were performed in accordance with the European Directive 2010/63/EU. During the course of this study, the animals were fed *ad libitum* with standardized food (Tecklad Global Diet, Harlan, Spain). The following protocol was approved by the Portuguese Ethics Committee for Animal Experimentation (Direção Geral de Veterinária, Approval no. 520/000/000/2003). All mice used in the experiment were acclimatized for one week under routine laboratory conditions before starting the experiments. They were housed randomly in groups of 4–5 in plastic cages, with hard wood chips for bedding. The animals were maintained in a room with a controlled

temperature of 23±2°C, a 12-hour light/dark cycle and 55±5% humidity. The animals' drinking solutions were changed once a week or earlier if necessary, and the volume drunk was recorded. Weekly food intake was also noted. All mice were monitored throughout the experiment for signs of distress and loss of body weight. The animals were sacrificed with 0.4% sodium pentobarbital (1 ml/Kg, intraperitoneal).

Population

This study was performed in a retrospective series of 96 formalin-fixed paraffin-embedded bladder tumours obtained from archived paraffin blocks at the Portuguese Institute of Oncology—Porto (IPOP), Portugal. Bladder tumours were extracted from 82 men and 14 women, ranging in age from 38 to 92 years (median of 69.5 years), admitted and treated at the IPOP between 2005 and 2007. Forty seven of the examined tumours were histologically classified as NMBIC (Ta and T1) and 49 as invasive lesions (T2-T4). Sixteen were low grade and 80 were high grade tumours, according to the 2004 WHO grading criteria. Furthermore, carcinoma *in situ* (CIS) was found concomitantly in 20.8% of the patients. The average follow up time period was 45 months (1–134 months). Cystectomy was performed in 64 patients (66.7%) while the other 32 (33.3%) were submitted to transurethral resection. Lymphadenectomy was performed in approximately 47% of the patients and from those 37% presented metastasis. Fifty four (56.3%) tumours were primary and 42 (43.7%) were recurrent tumors. From the recurrent tumours, 38% had no prior treatment, 27% were treated with Mitomicin C, 11% with BCG and 19% were submitted to both treatments. Moreover 5% of these patients were treated with neoadjuvant chemotherapy prior to the cystectomy. Table 1 summarizes the clinicopathological information.

Cancer-specific survival (CSS) was defined as the period between the tumour removal by surgery and either patient death by cancer or the last follow-up information. All procedures were performed after patient's informed consent and approved by the Ethics Committee of IPO-Porto. All clinicopathological information was obtained from patients' clinical records.

Immunohistochemistry

The expressions of STn antigen, its precursor Tn, and phosphorylated forms of Akt (pAkt), mTOR (pmTOR), S6 (pS6) and PTEN in bladder tumours were accessed by immunohistochemistry using the avidin/streptavidin peroxidase method, as described by Ferreira et al. [5]. Information on the primary antibodies and dilutions used in this study are summarized in Table 2. Immunoreactivity was revealed using diaminobenzidine (DAB, Thermo Scientific LabVision) as chromogen and sections were counterstained with Harris's hematoxylin. Negative controls were performed by replacing the primary antibody with 5% bovine serum albumin (BSA). Positive controls were known positive tissues for the antigens under study.

Immunohistochemistry scoring of human tumours

The immunostained sections were assessed double-blindly by light microscopy by two independent observers (CC and SP) and validated by an experienced pathologist (TA). Disaccording readings were re-analyzed using a double-headed microscope (Olympus BX46; Olympus Corporation), and consensus was reached. A semi-quantitative approach was established to score the immunohistochemical labeling based on the extent and intensity of the staining.

Given the absence of Tn and STn in the healthy urothelium [5], tumours were classified as positive for these antigens when membrane and/or cytoplasmic immunoreactivity were observed in more than 5% of the tumour, as described by Ferreira et al. [5,15]. pAkt, pmTOR, pS6 and PTEN expressions were scored according to the staining intensity (weak-1 point;

Table 1. Clinical-pathological data of the studied sample (*n* = 96).

Age, *years*		
	median [min—max]	69.5 [38–92]
Gender, *n* (%)		
	Male	82 (85.4%)
	Female	14 (14.6%)
Stage, *n* (%)		
	Ta	27 (28.1%)
	T1	20 (20.8%)
	T2	9 (9.4%)
	T3	20 (20.8%)
	T4	20 (20.8%)
Grade, n (%)		
	Low	16 (16.7%)
	High	80 (83.3%)
Recurrence status, n(%)		
	Primary	54 (56.3%)
	Recurrent	42 (43.7%)
Associated Cis, n(%)		
	No	76 (79.2%)
	Yes	20 (20.8%)
Metastasis, n(%)		
	No	19 (63.3%)
	Yes	11 (36.7%)
Follow-up, *n (%)*		
	Alive, lost or death from other causes	67 (69.8%)
	Death from cancer	29 (30.2%)

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moderate-2 points; strong-3 points) multiplied by the percentage of positive cells (0–5%-0 points; >5–25%-1 point; >25–50%-2 points; >50–75%-3 points; >75–100%-4 points). Based on the classification proposed by Nishikawa et al. [16], tumours with a score <6 were considered negative, whereas those with a score≥6 were classified as positive (overexpression). pAkt was evaluated based on nuclear immunoreactivity, pmTOR and pS6 based on cytoplasmic expression and PTEN on both cytoplasmic and nuclear staining, as suggested by other publications [17,18].

Animal experiments with sirolimus and immunohistochemistry scoring

Histological sections of Imprinting Control Region (ICR) mice bearing N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced bladder lesions, resulting from our previous work on the impact of sirolimus on bladder cancer [19], were elected for this study. Briefly, four-week-old male ICR mice (25g; Harlan, Barcelona, Spain) were randomly distributed into four groups, as described in detail in a previous publication [18]. Group 1 (n = 6) included mice exposed to 0.05% BBN for 12 weeks followed by tap water for 8 weeks (total of 20 weeks). Group 2 (n = 7) included mice treated with 0.05% BBN solution for twelve weeks, maintained with normal tap water for another week, administrated intraperitoneally with mTOR-inhibitor sirolimus (1.5 mg/kg; Wyeth) for five days a week for six consecutively weeks, i.e. until the 19th week, followed by another week of tap water (total of 20 weeks). Group 3 (n = 6) included mice exposed to 0.05% BBN for 12 weeks followed by tap water for 11 weeks (total of 23 weeks). Group 4

Table 2. Antibodies used in the immunohistochemical analysis.

Antibody	Vendor	Clone	Dilution
Tn	Non-commercial Hybridoma*	IE3	1:5
STn	Non-commercial Hybridoma*	TKH2	1:20
Ki-67	Dako	MIB-1	1:100
p53	Dako	DO-7	1:100
Phos-AKT	Cell Signaling	Ser473 (736E11)	1:50
Phos-mTOR	Cell Signaling	Ser2448(49F9)	1:100
Phos-S6	Cell Signaling	Ser240/244 polyclonal	1:75
PTEN	Cell Signaling	D4.3 XP	1:50

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(n = 7) included mice treated with 0.05% BBN and sirolimus, as described for Group 2, but with an exposure to tap water afterwards of 3 weeks (total of 23 weeks). Group 3 and 4 were created to estimate the possibility of late relapse and/or molecular alterations resulting from prolonged survival. All procedures were performed in accordance with the European Directive 2010/63/EU. During the course of this study, the animals were fed *ad libitum* with standardized food (Tecklad Global Diet, Harlan, Spain). The histological changes induced by these experiments included both preneoplastic and neoplastic lesions with invasive potential and invasive tumours, as described in detail by Oliveira et al. [18]. Herein, lesions of high invasive potential and muscle invasive tumours were screened for STn and pS6 by immunohistochemistry, as described in detail for human tumours, since the antibodies used are reactive against both human and mice. Both the intensity and the extension of immunostaining were taken into consideration to score the expression of the antigens, as described in the previous section. The bladder lesions and immunostaining were assessed double-blindly by two independent observers (CC and SP) and validated by an experienced veterinary pathologist (RMGC).

Statistical analysis

Statistical data analysis was performed with IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0). Chi-square analysis was used to compare categorical variables. Kaplan-Meier survival curves were used to evaluate correlation between STn expression and cancer-specific survival (CSS) and were compared using log-rank test. Furthermore, multivariate Cox regression analysis was performed to assess the individual effect of the evaluated markers on patient's survival and adjust to potential confounders (variables that could affect CSS of NMIBC and MIBC patients). The correlation between PI3K/Akt/mTOR pathway molecules was performed using Spearman rho test.

Results

Altered protein glycosylation, translated by the expression of the STn antigen and its precursor Tn, PI3K/Akt/mTOR pathway molecules (pAkt, pmTOR, pS6), and PTEN inactivation, are salient features of bladder tumours. Herein we have devoted to a comprehensive analysis of these molecular alterations in a series of bladder cancer patients at different stages of the disease, envisaging biomarkers of poor cancer-specific survival.

Our dataset was composed by 47 NMIBC and 49 MIBC patients, as showed in Table 1. According to Fig 2, NMIBC presented a higher cancer-specific survival (CSS; mean CSS: 119 months) than MIBC patients (mean CSS: 43 months; log rank, $p<0.001$). These results

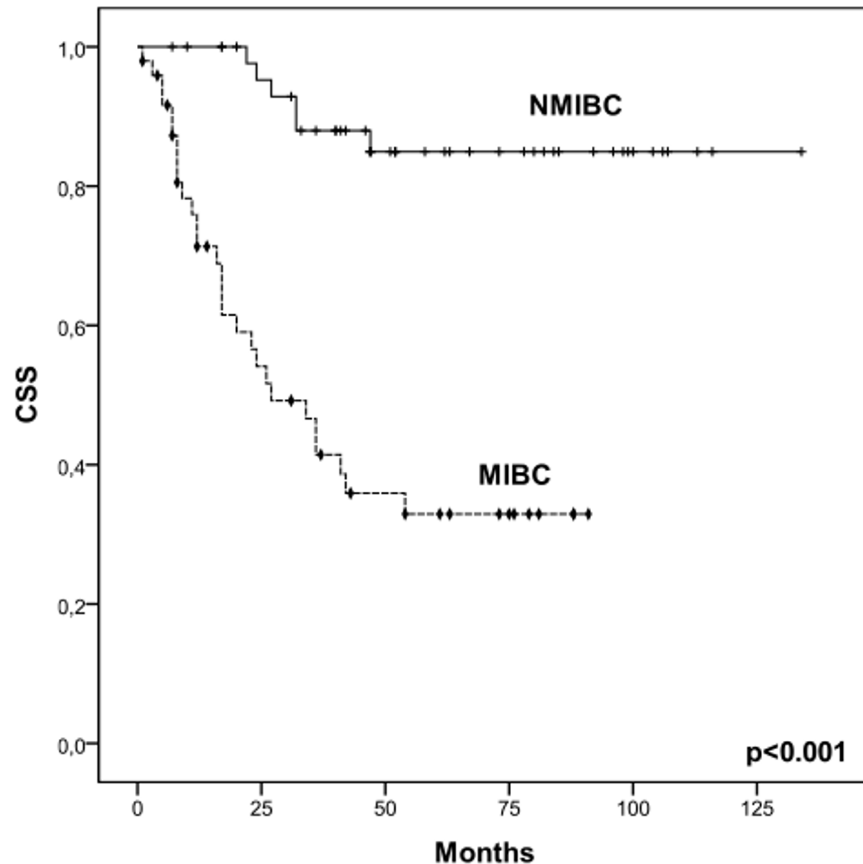

Fig 2. Association between disease groups and cancer-specific survival (CSS) in the studied patients. Kaplan-Meier analysis showing the CSS of NMIBC (Ta and T1) and s of MIBC (T2,T3 and T4). Comparison performed by log-rank test (p<0.001); + censored NMIBC patients; ♦ censored MIBC patients.

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demonstrated that our series reflected the natural course of disease and highlighted the significantly lower CSS of MIBC compared to NMIBC cases. Therefore, particular interest was set in the identification of biomarkers for late stage disease based on the comparison between NMIBC and MIBC.

Tn and STn antigen expressions in bladder cancer

The Tn antigen was observed in approximately 10% of NMBC and MIBC (Table 2) and its expression was residual, did not exceeding 5% of the tumour area and without any defined pattern. On the other hand, the STn antigen was detected in approximately 60% of the studied bladder tumours, which is in accordance with our previous findings [5]. The antigen was predominately expressed at the cell membrane, although cytoplasmic staining could also be observed. The STn antigen presented a focal expression that did not exceed 30% of the tumour area for the majority of the positive cases, irrespectively of their histological origin. STn was mainly expressed by dedifferentiated cells in tumours showing *lamina propria* (T1; 60%) and *muscularis propria* ($\geq$ T2; approximately 60–90%) invasion; conversely the percentage of positive Ta was lower than 30% ($p<0.001$; Fig 3A). Although without statistical significance, in Ta tumours STn positive cells were mainly present in superficial tumour layers away from the vessels. Conversely, STn positive cells in T1 tumours (Fig 3B) were observed accompanying and/

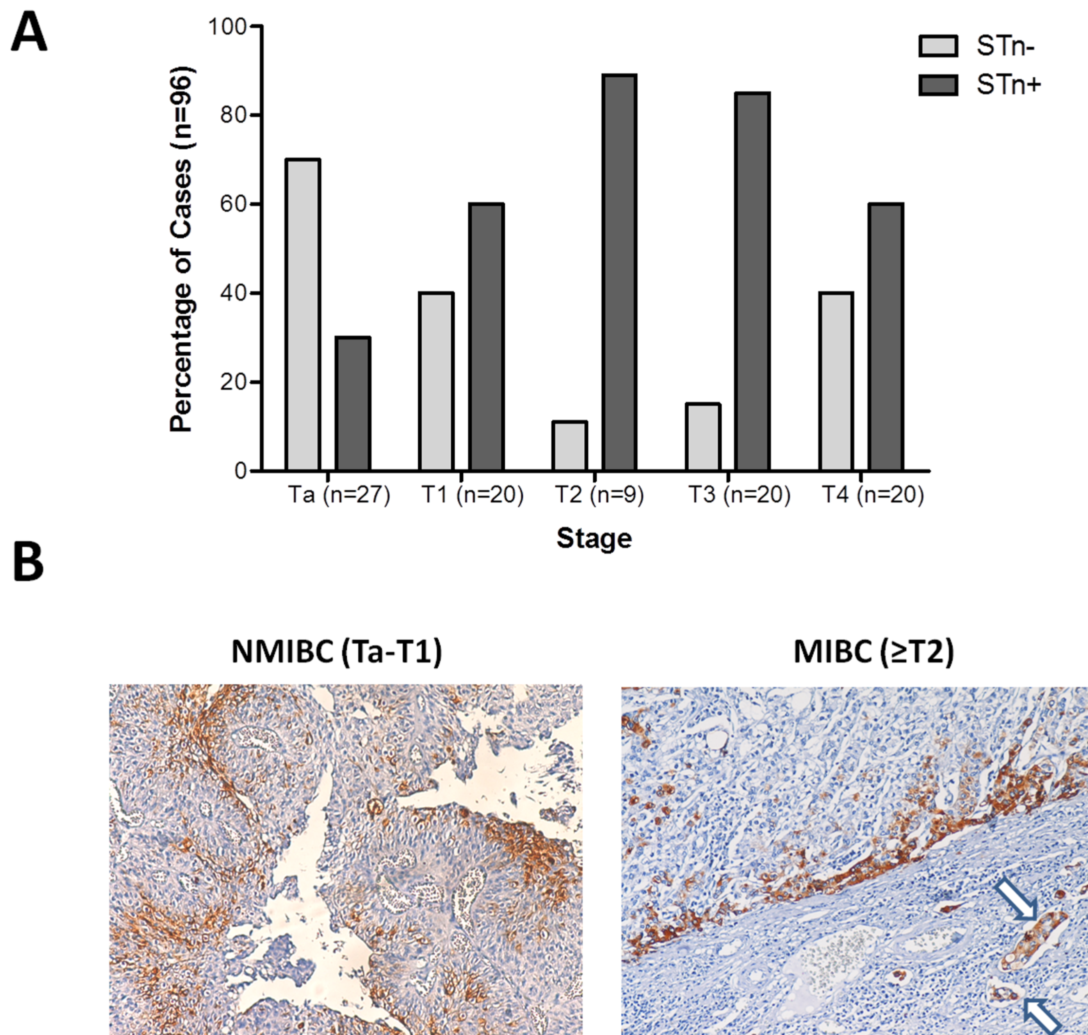

Fig 3. STn expression in different bladder tumors stages. (A) Distribution of STn negative and positive tumors along the different stages of bladder cancer; (B) Representative images of STn staining in NMIBC and MIBC. Left—NMIBC showing a predominance of STn positive cells in the superficial layers, away from the fibrovascular support; note vessels without positive cells. Right—MIBC showing the invasion front with STn positively stained cells; note positive STn urothelial cells in the vessels (arrow), suggesting possible involvement in metastasis.

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or invading the basal layer (Fig 3B), while in MIBC these cells were mostly found in the invasion fronts (Fig 3B) and invading and/or inside the vessels, which suggests a role in invasion and disease dissemination. Reinforcing these observations, the presence of STn antigen was statistically higher in MIBC when compared to NMIBC ($p = 0.001$, Table 3).

PI3K/Akt/mTOR pathway in bladder cancer

The evaluation of the PI3K/Akt/mTOR/S6 pathway was done using antibodies for active phosphorylated forms of Akt (pAkt), mTOR (pmTOR), and S6 (pS6). PTEN, that negatively regulates Akt signalling, was also evaluated.

pAkt was detected both in the cytoplasm and nucleus. In NMIBC cases several areas with different intensity of expression were observed (Fig 4A), denoting a heterogeneous pattern that was not evident in MIBC (Fig 4B). Furthermore, stromal cells of MIBC positive cases showed enhanced staining intensity mainly in the areas close to the tumour. pmTOR immunoreactivity

Table 3. Association between the evaluated markers and the stage of disease.

	Bladder Cancer		
	NMIBC n (%)	MIBC n (%)	P
Tn			
Negative	41 (87.2)	45 (91.8)	
Positive	6 (12.8)	4 (8.2)	0.461
STn			
Negative	27 (57.4)	12 (24.5)	
Positive	20 (42.6)	37 (75.5)	0.001
pAKT			
Negative	13 (28.9)	19 (38.8)	
Positive	32 (71.1)	30 (61.2)	0.312
pmTor			
Negative	30 (63.8)	33 (67.3)	
Positive	17 (36.2)	16 (32.7)	0.717
pS6			
Negative	22 (47.8)	28 (57.1)	
Positive	24 (52.2)	21 (42.9)	0.183
PTEN			
Negative	18 (38.3)	37 (82.2)	
Positive	29 (61.7)	8 (17.8)	<0.001

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was cytoplasmic and, in occasional cases, nuclear. In urothelium with apparent normal histology pmTOR expression was restricted to superficial cell layers. In NMIBC pmTOR expression was evenly distributed across the several layers of urothelial cells, although there was a more intense staining in the superficial layers (Fig 4C). Moreover, several areas with variable staining intensity were observed, denoting a heterogeneous expression. In MIBC positives cases, pmTOR expression was focal and heterogeneous (Fig 4D). pS6 immunoreactivity was predominantly cytoplasmic. In NMIBC pS6 expression was noted in all the superficial layers, both in umbrella and differentiated cells (Fig 4E). The immunoreactivity of pS6 varied across the tumour cells. In MIBC pS6 presented a diffuse expression throughout the tumour, being more present in basal and mitotic cells (Fig 4F). Several positive cases presented increased pS6 staining intensity in the invasion front as well as pS6 expression in tumour infiltrating lymphocytes and endothelial cells.

Taking into account the extension of staining and its intensity, 62/94 (66%), 33/96 (34%) and 45/95 (47%) of the bladder tumours were considered positive for pAkt, pmTOR and pS6, respectively. A Spearman rho test showed that pAkt, pmTOR, pS6 expressions were significantly correlated ($P<0.05$) irrespectively of the tumour stage, thus in accordance with a fully active pathway. Furthermore, despite histological differences, these markers presented an equal distribution among the NMIBC and MIBC and could not be associated with muscle invasion (Table 3).

On the other hand, 37/92 (40%) of the tumours were considered positive for PTEN. PTEN was expressed in the cytoplasm and nucleus of the same cells, however with lower extension of expression in MIBC (33%, Fig 4G) compared to NMIBC (83%; Fig 4H). Moreover, the PTEN-negative phenotype was significantly associated with muscle invasion (Ta and T1; $p<0.001$, Table 3), which may contribute to maintain an active PI3K/Akt/mTOR/S6 pathway in these cases.

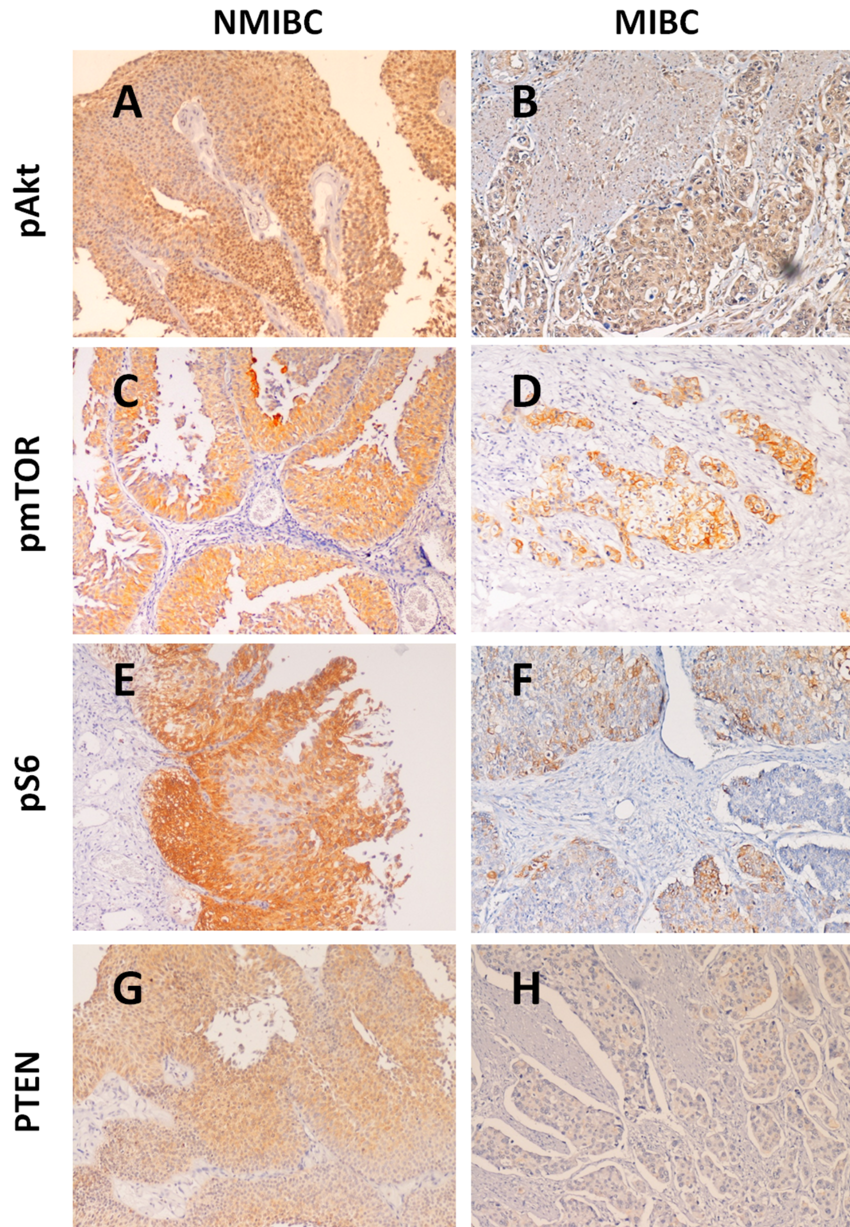

Fig 4. Expressions of pAkt, pmTOR, pS6 and PTEN in NMIBC and MIBC (40x magnification). A and B) pAKT nuclear and cytoplasmatic expression in NMIBC (A) and MIBC (B). In NMIBC cases pAkt presented a heterogeneous pattern with areas of different intensity of expression. In MIBC, stromal cells mainly in the areas close to the tumour showed higher expression. C and D) pmTOR cytoplasmic expression in NMIBC (C) and MIBC (D). In NMIBC pmTOR was expressed across several layers, although there was a more intense staining in the superficial ones. In MIBC positive cases pmTOR expression was focal. E and F) pS6 cytoplasmatic expression in NMIBC (E) and MIBC (F). In NMIBC pS6 expression was observed in all the superficial layers both in umbrella and differentiated cells. In MIBC the immunoreactivity was diffuse, however more present in basal and mitotic cells. pS6 expression was higher in the invasion front and in tumour infiltrating lymphocytes and endothelial cells. G and H) PTEN cytoplasmic and nuclear expressions in NMIBC (G) and MIBC (H). PTEN expression was higher in NMIBC compared to MIBC.

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Tn, STn, PI3K/Akt/mTOR pathway and Cancer-specific Survival

A Kaplan-Meier analysis was used to evaluate associations between the addressed biomarkers and the cancer-specific survival of patients. We observed that patients bearing STn expressing tumours had a lower CSS, irrespectively of their stage (p = 0.024; Fig 5A). This was also observed when evaluating NMIBC alone (p = 0.020; Fig 5B). More importantly, among NMIBC, STn expressing T1 tumours presented lower CSS than negative tumours ($p < 0.05$). Moreover, multivariate Cox regression analysis adjusted to potential confounders, namely age, gender, stage, grade, recurrence status, presence of concomitant CIS was performed. We found that STn is an independent prognostic marker of worst CSS (HR = 11.836; 95%CI: [1.063–131.7]; p = 0.044). Contrasting with STn, positive Tn, pAkt, pmTOR and pS6 tumours showed no differences in CSS compared to negative lesions, irrespectively of their stage. We have also observed that patients harbouring PTEN-negative tumours had lower CSS (p = 0.015, Fig 6). More studies are necessary to determine if the lack of suppressive effect of PTEN over PI3K/Akt/mTOR may account for these findings.

Based on these observations and aiming to improve the prognostic value of STn in the context of late stage disease (MIBC), we have comprehensively integrated the information from STn and PI3K/Akt/mTOR pathway biomarkers. According to Fig 7, the introduction of PI3K/Akt/mTOR pathway molecules allowed discriminating STn positive MIBC tumours with worst CSS (p = 0.027). Furthermore, multivariate Cox regression analysis (adjusted to age, stage, recurrence status, presence of concomitant CIS and metastasis) revealed that the presence of PI3K/Akt/mTOR pathway molecules in STn+ MIBC is independently associated with approximately 6-fold risk of death by cancer (HR = 5.662; 95%CI: [1.093–29.323]; p = 0.039). These observations suggest, for the first time, that the combination of STn and mTOR pathway biomarkers may hold potential to improve the stratification of advanced stage bladder tumours; however corroboration in larger series is mandatory.

Inhibition of the PI3K/Akt/mTOR pathway in animal models

BBN-induced mice bladder tumours mimicking the histology and molecular nature of human cancers [20,21], were screened for STn and pS6, the downstream effector of mTOR pathway. We observed no STn expression in the healthy mice urothelium, in accordance with previous observation for the healthy human bladder [5]. In mice healthy urothelium pS6 expression was below 20%, thus underexpressed when compared with BBN-exposed mice (Fig 8). In the control groups (Group 1 and 3, Fig 8A), the exposure to BBN led to the development of invasive tumours in 70–90% of the studied mice. Concomitantly, 83–100% of the invasive lesions overexpressed the STn antigen and all significantly overexpressed pS6 (Fig 8B). This demonstrated that BBN-induced lesions were able to recapitulate the association between altered glycosylation and an activated PI3K/Akt/mTOR pathway previously observed in advanced stage human tumours. The STn antigen was mainly found in cells adjacent to the basal layer and in those invading the stroma, as previously observed in human tumours (Fig 8B and 8C). Conversely, pS6 presented a more diffuse expression, again in accordance with the pattern observed in human lesions (Fig 8B and 8C). A comparison between groups 1 and 3 further highlighted that extended lifespan did not alter the number of invasive lesions, but significantly increased STn and pS6 overall expressions in each tumour ($p<0.05$; Fig 8B), highlighting the more aggressive nature of Group 3 lesions.

In the sirolimus-treated groups (Groups 2 and 4; Fig 8A) a smaller number of mice developed invasive tumors (20–40%) when compared to the controls (Groups 1 and 3). Moreover, only 43% of the mice treated with sirolimus overexpressed the STn antigen, irrespectively of the experience periods. Still, the extension of STn expression was significantly decreased in

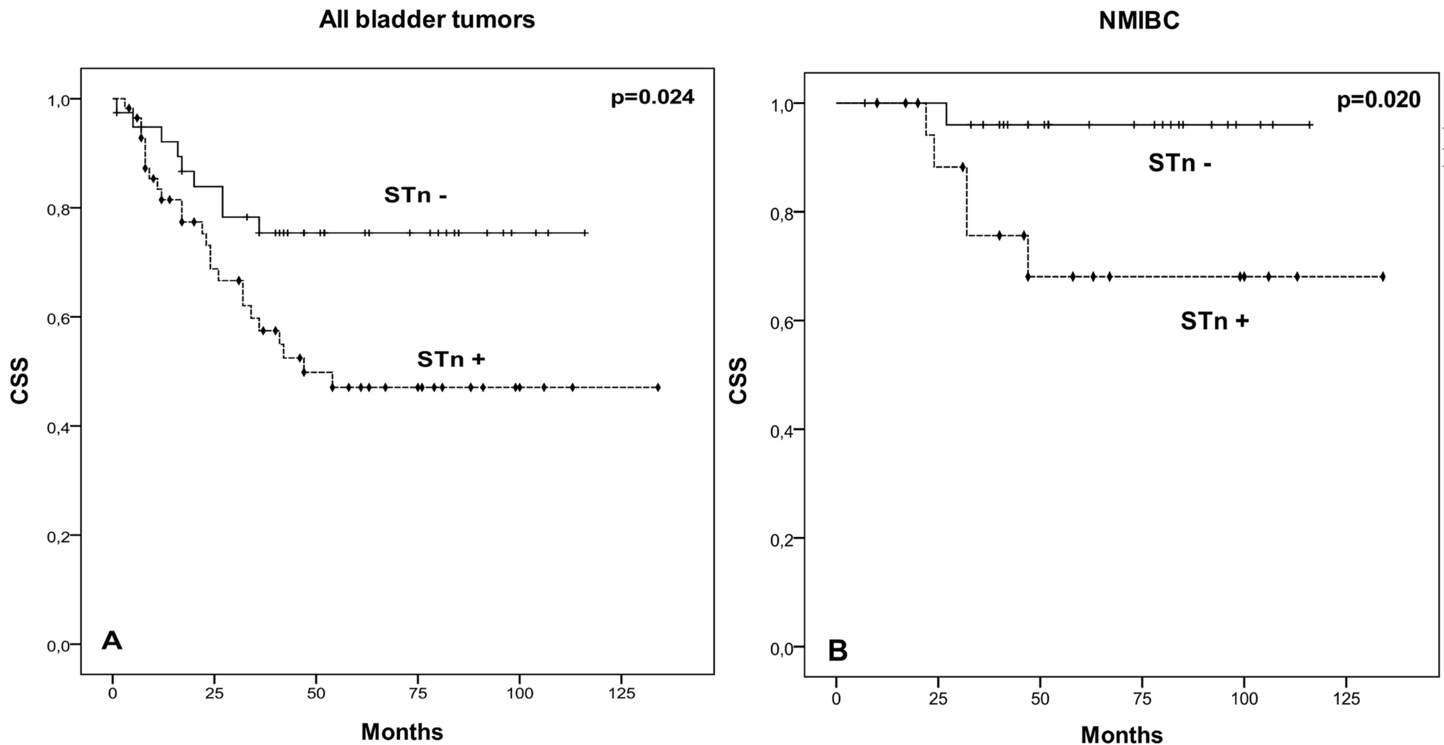

Fig 5. Effect of STn expression in cancer-specific survival (CSS). Kaplan–Meier analysis showing the association between STn and CSS in: (A) all studied bladder cancer patients; (B) NMIBC patients. Comparison performed by log-rank test (A: p = 0.024; B: p = 0.020); + censored STn negative tumours; ♦ censored STn positive tumours.

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STn-positive tumours when compared to the control groups (Fig 8B and 8C). Following the same tendency, the pS6 protein was only overexpressed in 29% of the cases in Group 2 and the extension of expression was also significantly decreased (Fig 8B and 8C). Contrastingly, the expression of pS6 in Group 4 was higher than in Group 2, again translating the higher aggressive nature of tumours obtained after longer lifespan. Despite these observations, sirolimus treatment promoted a significant reduction in the percentage of positive pS6 cells in Group 4 mice when compared to Group 3 ($p<0.05$; Fig 8B and 8C). Altogether, sirolimus administration effectively reduced tumour burden and promoted a significant reduction in the expression of STn and pS6 markers.

Discussion

Due to their high molecular heterogeneity, advanced stage bladder tumours present a significant prognostication and treatment hurdle. In this context, much controversy exists regarding the potential of conventional cancer biomarkers, urging the identification of novel molecules capable of aiding disease personalization. Furthermore, advanced stage bladder cancer remains an orphan disease in terms of therapeutics, as the only available options continue to be surgery and conventional chemotherapy [22]. The introduction of targeted therapeutics is therefore warranted.

In a previous explorative study we have observed that altered protein glycosylation translated by STn overexpression was a salient feature of a subset of advanced stage tumours [5]. Herein we have started by investigating the expression of STn precursor, the Tn antigen, in bladder tumours. We observed that this antigen presented a very low expression in bladder

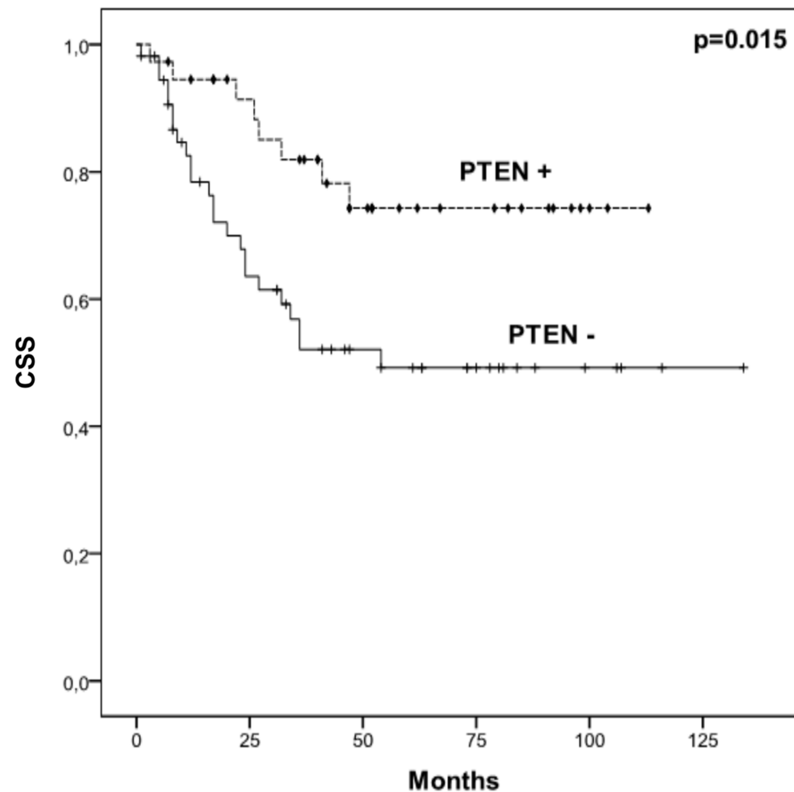

Fig 6. Effect of PTEN expression and cancer-specific survival (CSS) in the studied patients. Kaplan-Meier analysis showing the effect in CSS of PTEN expression in all studied bladder cancer patients. Comparison performed by log-rank test (p = 0.013); + censored PTEN negative tumours; ♦ censored PTEN positive tumours.

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tumours and was not associated with any particular stage of the disease. These findings suggest that the Tn antigen is rapidly sialylated or capped with more extended glycans in bladder tumours. Moreover, we have confirmed that STn expression is more associated with muscle invasive than non-muscle invasive disease in a larger patient set, suggesting that sialylation plays a key role in stopping protein glycosylation in advanced stage bladder tumours. Furthermore, we have provided new insights regarding its correlation with decreased survival, as previously observed for digestive track tumours [23–25]. Accordingly, we and other authors have shown that STn expression is responsible by the modulation of cell surface glycoprotein functions in ways that favour malignant phenotypes in gastric [26], breast [27] and bladder [5] cancers. Namely, STn expression altered the adhesive properties of cancer cells, possibly by impairing integrin function [26,27]. Furthermore, it enhanced cell motility, invasion [26,27] and epithelial-to-mesenchymal transition, a key event leading to metastasis [28]. We have also demonstrated that STn expression protects bladder cancer cells from adverse host immune responses [6]. Namely, it impaired dendritic cell maturation inducing a tolerogenic phenotype and limiting their capacity to trigger protective anti-tumour T-cell responses [6]. In resume, a significant amount of data supports a key role of STn in disease progression and dissemination, making of STn antigen, and in particular STn-glycoproteins, potential anti-cancer targets. Nevertheless, there is scarce information about the molecular nature of this subset of STn-expressing aggressive tumours and consequently about the best therapeutic options.

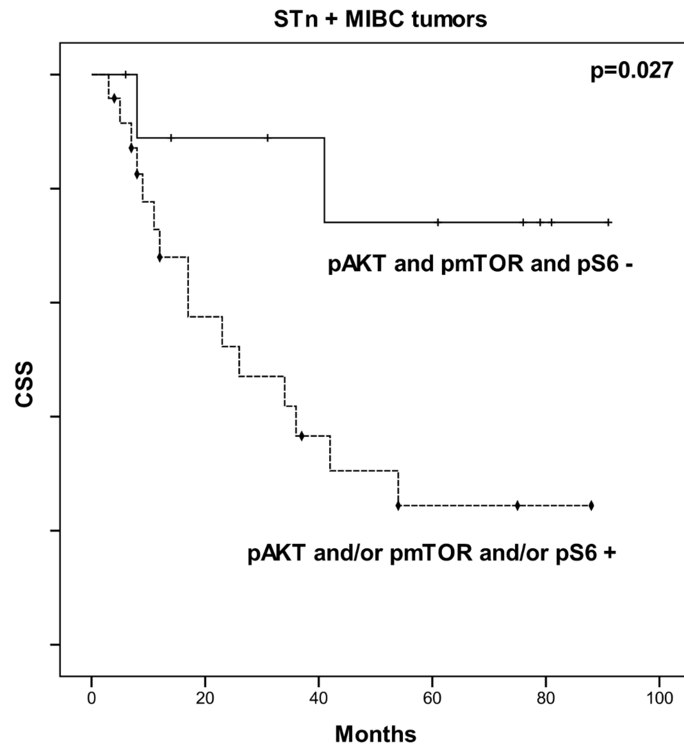

Fig 7. Effect of PI3K/Akt/mTOR pathway activation in cancer-specific survival (CSS) of patients with STn positive MIBC. Kaplan–Meier analysis showing the association between pAKT, pmTOR and pS6 expressions in the CSS of STn positive tumors MIBC: Comparison performed by log-rank test (p = 0.027); + censored pAKT and pmTOR and pS6 negative tumours; ♦ censored pAKT and/or pmTOR and/or pS6 positive tumours.

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Foreseeing a more accurate patient stratification we have also addressed the expression of PI3K/Akt/mTOR pathway markers in bladder tumours. In our series the activation of mTOR pathway proteins did not discriminate the stage of disease. Moreover it did not allow, by itself, the identification of patients facing worst prognosis, which is in accordance with recent publications [29,30]. However, we found that PTEN expression, which exerts a suppressive effect over the PI3K/Akt/mTOR pathway, was decreased in advanced stage tumours, in accordance with previous observations [31–34]. Furthermore, PTEN-negative MIBC presented worst cancer-specific survival in comparison to PTEN-positive lesions. More studies are needed to determine if the lack of suppressive effect over the PI3K/Akt/mTOR may account for poorer outcome. Interestingly, we have also observed that the overexpression of PI3K/Akt/mTOR pathway biomarkers decisively associated with worst CSS in STn positive advanced stage tumours, which currently lack effective therapeutics. These findings lead us to hypothesize that this subset of more aggressive bladder tumours may benefit from multi-targeted approaches combining mTOR-inhibitors and guided therapeutics against STn-expressing cells. However these are preliminary insights from a relatively low number of patients. More studies involving a large population are warranted to confirm these observations. It will also be important to evaluate other outcomes of aggressiveness, namely response to conventional therapeutics and metastasis development.

Our study also reinforced that bladder tumours present extensive activation of the PI3K/Akt/mTOR pathway irrespectively of their histological nature, as described in previous publications [32,35]. Such findings contribute to support the idea that most bladder tumours may be good candidates for mTOR-inhibitors therapeutics. Accordingly, mTOR-inhibitors have been

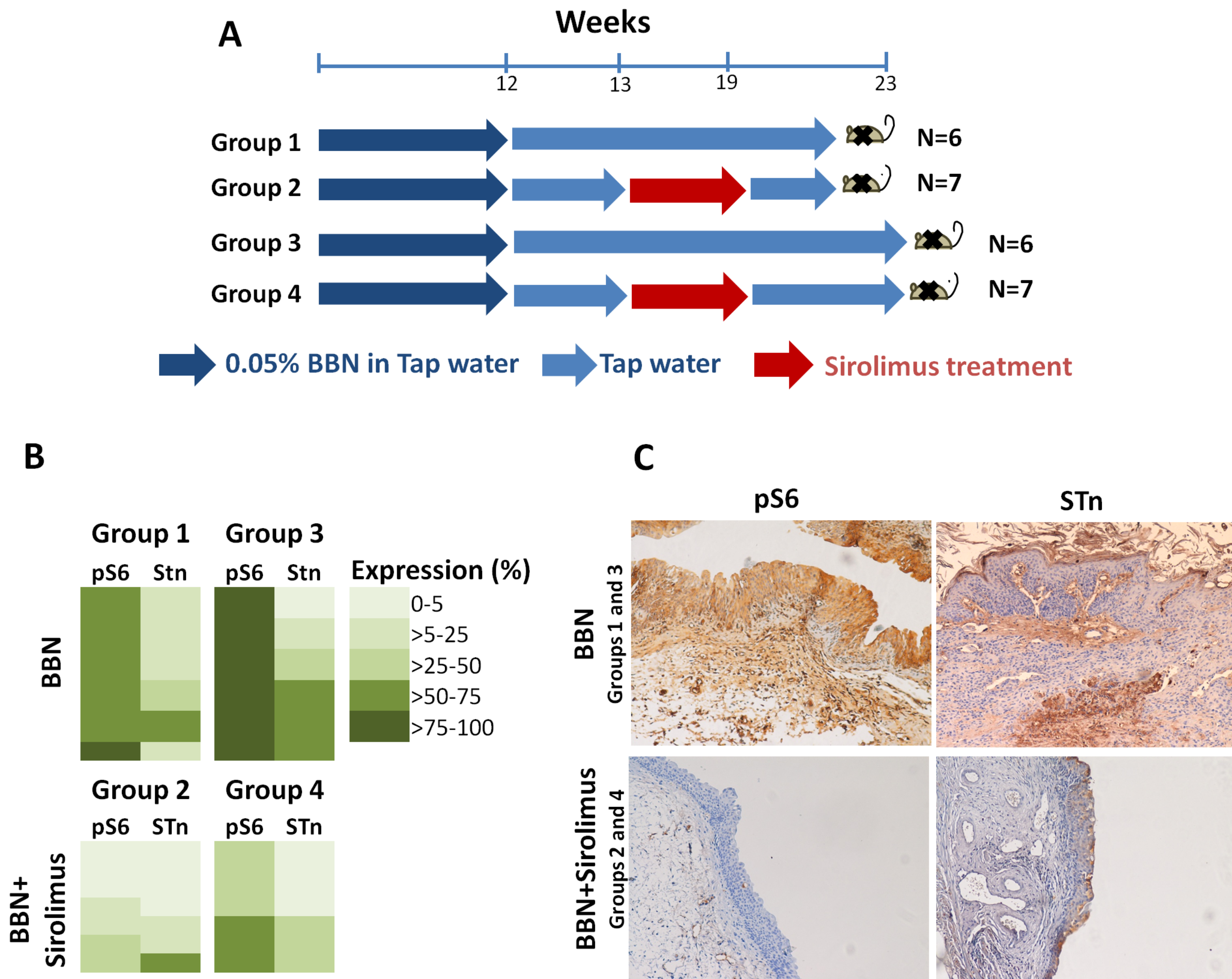

Fig 8. STn and pS6 expressions in bladder tumours from BBN-exposed male ICR mice with or without the administration of mTOR-inhibitor sirolimus (rapamycin). A) Experimental design to determine the sirolimus effect on STn and pS6 expressions in a model of urothelial carcinogenesis (male ICR mice). B) Expression of STn and pS6 in BBN-derived urothelial tumours in the presence and absence of sirolimus. BBN-induced bladder tumours (Groups 1 and 3) overexpressed STn and pS6, which was more pronounced in Group 3, after longer lifespan. Exposure to sirolimus decreased the number of invasive lesions in groups 2 and 4 (data not shown) and, concomitantly, decreased the expressions of STn and pS6. C) Histological sections showing the expressions of STn and pS6 in BBN-induced urothelial tumours before and after treatment.

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extensively explored in pre-clinical settings and two phase I/II clinical trials for bladder cancer are ongoing [36]. In particular our group has demonstrated that the combination of everolimus with cisplatin or gemcitabine decreased the proliferation of bladder cancer cell lines in comparison to the chemotherapy agent alone [14,37]. More recently we conducted studies in mice bearing chemically-induced tumours mimicking the histological and molecular nature of human tumours [20]. We concluded that administration of mTOR-pathway inhibitor sirolimus (rapamycin) effectively reduced the frequency of invasive lesions. Using the same animal model, we have now confirmed the anti-cancer activity of sirolimus in the context of aggressive bladder disease. Namely, we observed a significant reduction in tumour burden accompanied by a loss of pS6 expression, thus in accordance with the expected mechanism of action of the drug. Moreover, we are describing for the first time that chemically-induced bladder tumours expressed the

STn antigen, thereby mimicking the glycosylation pattern of human cancers. These observations are of particular importance due the lack of accurate models to access the biological role of this antigen. In fact most established cancer cell lines express residual amounts of this antigen, denoting a dependence on the tumours microenvironment. We believe that BBN-induced tumours may now constitute key models to develop successful therapeutics against STn positive bladder lesions. Moreover importantly, we have concluded that the administration of sirolimus contributed to reduce the number of STn positive cells. These observations reinforce a possible association between STn and an active PI3K/Akt/mTOR pathway in invasive tumours, as suggested upon the evaluation of human cancers. It also points out that sirolimus may constitute a valuable approach to manage STn and PI3K/Akt/mTOR-positive, which face worst OS. Still, these preliminary evidences and more in depth studies are needed before progressing to clinical phases. Namely, it will be important to support these findings in other models such as patient-derived xenografts and compare the effect of sirolimus with conventional chemotherapeutics for bladder cancer (cisplatin/gemcitabine-based regimens).

In resume, we have demonstrated that the STn antigen is a biomarker of poor prognosis, particularly in MIBC. We also suggest the existence of potentially more aggressive subgroup of STn positive MIBC characterized by an active mTOR-pathway. Such observations also provide the first link between these two apparently unrelated events in bladder cancer (altered glycosylation and the PI3K/Akt/mTOR-pathway activation). Using animal models we have also concluded that the administration of mTOR-pathway inhibitor sirolimus offers potential against these highly malignant tumours. More validation studies are now warranted to set the pace for clinical trials. Taking into consideration its cell-surface nature and key role played by STn malignancy, specific antibody-based therapeutics can also be envisaged [22,38]. The combination of these approaches may provide novel ways to improve MIBC management, which remains an orphan disease in terms of innovative treatments [22].

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Author Contributions

Conceived and designed the experiments: CC SP LL PO JAF LLS. Performed the experiments: CC SP AP EF AT DN MN CG RMGC PO. Analyzed the data: CC SP LL RMGC RC TA PO JAF LLS. Contributed reagents/materials/analysis tools: RC PO JAF LLS. Wrote the paper: LL JAF LLS PO.

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