Universidade de Aveiro Departamento de Biologia
2016

SOFIA RIBEIRO COTTON

Glycoproteomic characterization of advanced bladder cancer towards novel therapies

Caracterização glicoproteómica de cancro avançado de bexiga direcionado para novas terapias

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Glycoproteomic characterization of advanced bladder cancer towards novel therapies

Caracterização glicoproteómica de cancro avançado de bexiga direcionado para novas terapias

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Mário Jorge Verde Pereira, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro, do Doutor Luís Carlos Oliveira Lima, Investigador de Pós Doutoramento do Centro de Investigação do Instituto de Oncologia do Porto (IPO-Porto) e do Doutor José Alexandre Ferreira, Investigador de Pós-Doutoramento do Centro de Investigação do Instituto IPO- Porto.

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Talent wins games, but teamwork and intelligence wins championships. Michael Jordan

I'd like to thank Dr Mário Verde, for the help and confidence that gave me during this period.

If God closes a door he opens a window

I am also grateful for my family and my friends who always believed in me and who gave me all the support through these years, allowing me to follow my dreams, thank you.

palavras-chave

Cancro de bexiga, glicosilação, glicoproteómica, Sialil Tn, espectometria de massa, biomarcadores.

resumo

A heterogenidade da natureza molecular dos tumores de bexiga tem dificultado o estabelecimento de abordagens no campo da medicina de precisão, revelando-se a necessidade de terapias mais eficientes e novas ferramentas de detecção não-invasivas. Contudo, têm-se denotado um desenvolvimento no estudo da carcinogénese de bexiga e na progressão do tumor, acompanhado de profundas alterações na glicosilação de proteínas que, dada a sua superfície celular e a natureza secretada, apresenta um potencial elevado na melhoria da gestão da doença. Segundo esta abordagem foi efectuado um estudo sobre tumores de bexiga de diferentes naturezas clinicopatológicas para O-glicanos de cadeia curta, regularmente encontrados na maioria dos tumores sólidos, recorrendo-se à imunohistoquímica. O estudo incluiu os antígenos Tn e T e os seus homólogos sialilados sialil-Tn (STn) e sialil-T (ST), geralmente associados com um mau prognóstico. Explorou-se ainda a sialilação da natureza dos antigénios T, especificamente as sialoformas sialil-3-T (S3T) e sialil-6-T (S6T), com base em combinações de tratamentos enzimáticos. Observou-se uma predominância de sialoglicanos, em comparação com as glicoformas neutras (antígenos Tn e T) em tumores de bexiga. Em particular, o antigénio STn foi associado ao estado avançado da doença e invasão muscular. Os antígenos S3T e S6T foram detectados pela primeira vez em tumores de bexiga, estando ausentes no urotélio normal, permitindo destacar a natureza específica em tumores. Verificou-se também a sobreexpressão dos glicanos em lesões avançadas, especialmente nos casos com invasão muscular.

As análises glicoproteómicas dos tumores avançados de bexiga permitiram identificar diversas glicoproteínas-chave associadas ao cancro (MUC16, CD44, integrinas), denotando uma glicosilação alterada.As glicoformas da MUC16 STN positivas, características do cancro de ovário, encontram-se num subconjunto de tumores de bexiga em estado avançado, com um pior prognóstico. Em suma, os tumores de bexiga apresentam severas alterações no O-glicoma e no O-glicoproteoma devendo ser abordados de forma abrangente com o objectivo de desenvolver ferramentas de diagnóstico não invasivas e terapias dirigidas. As glicoformas aberrantes de MUC16 apresentam potencial como biomarcadores de mau prognóstico. Este trabalho estabeleceu um guia para a descoberta de glicobiomarcadores no cancro de bexiga, que pode ser utilizado para a estratificação dos pacientes e, por fim, levar à descoberta de novos alvos terapêuticos.

keywords Bladder tumor, glycosylation, SialylTn, glycoproteomic, mass spectometry, glycobiomarker

abstract The heterogeneous molecular nature of bladder tumours has hampered the establishment of precision medicine approaches, more efficient therapeutics and novel non-invasive detection tools. Still, it has been long described that bladder carcinogenesis and tumour progression is accompanied by profound alterations in protein glycosylation which, given its cell surface and secreted nature, holds tremendous potential for disease management improvement. Therefore, we have screened series of bladder tumours of different clinicopathological natures for short-chain *O*-glycans, found in most solid tumours, by immunohistochemistry. These included the Tn and T antigens and their sialylated counterparts sialyl-Tn (STn) and sialyl-T(ST), generally associated with poor prognosis. We have also explored the nature of T antigens 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 identified several key cancer-associated glycoproteins (MUC16, CD44, integrins) carrying altered glycosylation. Particular interest was devoted to MUC16 STn^{+}-glycoforms, characteristic of ovarian cancers, which were found in a subset of advanced stage bladder tumours facing worst prognosis. In summary, bladder tumours present severe *O*-glycome and *O*-glycoproteome alterations that should be comprehensively addressed envisaging novel non-invasive diagnostic tools and targeted therapeutics. Furthermore, abnormal MUC16 glycoforms holds potential as surrogate biomarkers of poor prognosis. Finally, this work established a roadmap for glycobiomarker discovery in bladder cancer, which may be used for patient stratification and ultimately lead to novel therapeutic targets.

A autora declara que participou na concepção e na execução do trabalho experimental, bem como na interpretação dos resultados e na redação do seguinte trabalho submetido a uma revista internacional de índole científica (Molecular Oncology), o qual faz parte integrante desta Dissertação:

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*Equal contribution.

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ABBREVIATIONS

Asn	Asparagine
ASR	Age Standardized Rate
AGC	Automatic Gain Control
BC	Bladder Cancer
BCG	Bacillus Calmette-Guerin
CID	Collision Induced Dissociation
CMP-Neu5Ac	Cytidine-5′-Monophospho-N-Acetylneuraminic Acid
CSS	Cancer-Specific Survival
CT	Computed Tomography
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DAPI	4',6-Diamidino-2-Phenylindole
dST	Disialylated Sialyl T
EGFR	Epidermal Growth Factor Receptor
ER	Endothelium Reticulum
FFPE	Formalin-Fixed Paraffin Embedded
FGFR	Fibroblast Growth Factor Receptors
FT-ICR	Fourier Transform Ion Cyclotron Resonance
Fuc	Fucose
GAG	Glycosaminoglycans
Gal	Galactose
GalNac	N- Acetylgalactosamine
Glc	Glucose
GlcA	Glucuronic Acid
GlcNac	N- Acetylglucosamine
GPI	Glycosylphosphatidyllinositol

HRP	Horseradish Peroxidase
IARC	International Agency for Research on Cancer
IdoA	Iduronic Acid
LIT	Linear Ion Trap
LRC	Advanced Laparoscopic
M	Metastasis to The Adjacent Tissues
Man	Mannose
MIBC	Muscle-Invasive Bladder Cancers
N	Lymph Node Invasion
Neu5Ac	5-N-Acetilneuraminic
NMIBC	Non-Muscle Invasive Bladder Cancer
ORC	Open Radical Cystectomy
PAHs	Polycyclic Aromatic Hydrocarbons
PD-1	Programmed Cell Death Protein 1
PDD	Photodynamic Diagnosis/Blue-Light Cystoscopy
PD-L1	Programmed Cell Death Ligand 1
ppGalNAc-Ts	Polypeptide N-Acetylgalactosaminyltransferases
PLA	Proximity Ligation Assay
PUNLMP	Papillary Urothelial Neoplasm of Low Malignant Potential
RARC	Robot-Assisted Radical Cystectomy
RTK	Receptor Tyrosine Kinases
Ser	Serine
SLe^x	Sialyl Lewis-X
ST6GalNAc-1	Alpha-N-Acetylgalactosaminide Alpha-2,6-Sialyltransferase 1
ST	Sialyl T
S3T	Sialyl-3-T
S6T	Sialyl-6-T

STn	Sialyl Tn
Thr	Threonine
VVA	*Vicia Villosa* Agglutinin
T	Thomsen-Friedenreich Antigen
Tn	Tn Antigen
TNM system	Tumour–Node–Metastasis System
TOF	Time of Flight
TUR	Transurethral Resection
TURBT	Transurethral Resection of Bladder Tumor
UDP-GalNAc	Uridine Diphosphate N-Acetylgalactosamine
VEGFR	Vascular Endothelial Growth Factor Receptors
Xyl	Xylose

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1. Clinical guidelines for bladder cancer

1.1.1. Epidemiology and ethology perspective

Bladder cancer (BC) is the 9th most common and the 13th deathliest cancer worldwide, with 430 000 new cases emerging in 2012 [1] [2]. Of note, three-quarters of all bladder cancer cases occur in men, especially after the age of 50 [3]. Particularly, muscle invasive bladder cancer (MIBC) is one of the most commonly lethal genitourinary cancers [4], currently managed through radical cystectomy with pelvic lymphadenectomy and (neo)adjuvant cisplatin-based chemotherapy regimens. The available therapy fails to avoid tumour relapse and disease progression, since almost 50% of cases relapse after radical cystectomy and the five-year overall survival does not exceed 25% [5]. Moreover, many patients die prematurely from adverse drug reactions, urging for effective and safe targeted therapeutics [6]. Notwithstanding, tremendous efforts have been put in the development of biomarker panels for early diagnosis, follow-up, patient stratification, prognosis, accurate treatment selection and development of targeted therapeutics [7]. However, the heterogenous molecular nature of bladder cancer has hampered true progresses in this field [8].

Bladder cancer incidence is higher in developed countries [4], putting Europe in the first place in terms of BC prevalence, followed by America, Asia, Oceania and Africa (Figure 1) [9]. In Portugal, BC is the eighth most common cancer and it is estimated about 3000 cases and 900 deaths in 2015 [1]. In Western world, cigarette smoking remains the most relevant risk factor, accounting for approximately 50% of bladder cancer cases [4, 10]. Furthermore, environmental and occupational exposures to aromatic amines, polycyclic aromatic hydrocarbons (PAHs), and infection with *Schistosoma hematobium* are also well-defined risks factors [11]. To a lesser extent, alcohol and coffee consumption, pollutants in drinking water, low fruit and vegetable ingestion, reduced selenium and vitamin E intake and

some medical treatments comprise additional risk factors for bladder carcinogenesis [11, 12]. Genetic factors have also been described to decisively contribute to increase the risk of bladder cancer development [13].

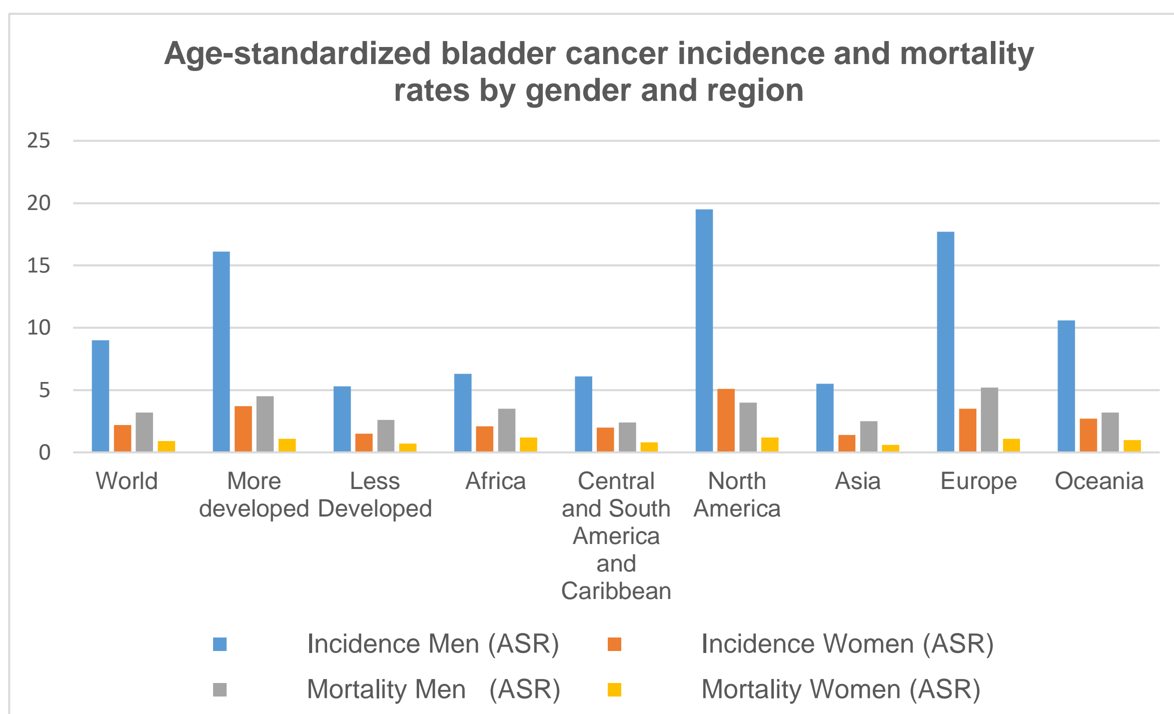

Figure 1. Age-standardized bladder cancer incidence and mortality rates by gender and region. *ASR- Age Standardized Rate. Adapted from Antoni S. et al., 2016 [9]

1.1.2. Bladder Cancer classification

According to the WHO/ISUP classification system of urothelial neoplasms, the histological features of urothelial lesions are divided into the following terms: papilloma, PUNLMP (papillary urothelial neoplasm of low malignant potential), low-grade carcinoma

and high-grade carcinoma [14] (Figure 2). In turn, the Tumour–Node–Metastasis system (TNM system) contemplates the depth of tumour invasion (T), the degree of lymph node invasion (N), and metastization to the adjacent tissues (M) [15]. Regarding the extension of muscle invasion, bladder tumours are classified as Tis, Ta, T1 (NMIBC), T2a, T2b, T3 and T4 (MIBC) (Figure 2). Namely, stage Ta corresponds to a non-invasive papillary carcinoma; Tis is a carcinoma in situ, also called "flat tumour"; T1 is a tumour invading sub-epithelial connective tissue; T2 corresponds to a tumour invading muscle, with T2a invading superficial muscle and T2b invading deep muscle; thereafter T3 invades perivesical tissue; and finally, T4 invades any of the following organs: prostate, uterus, vagina, pelvic wall or abdominal wall.

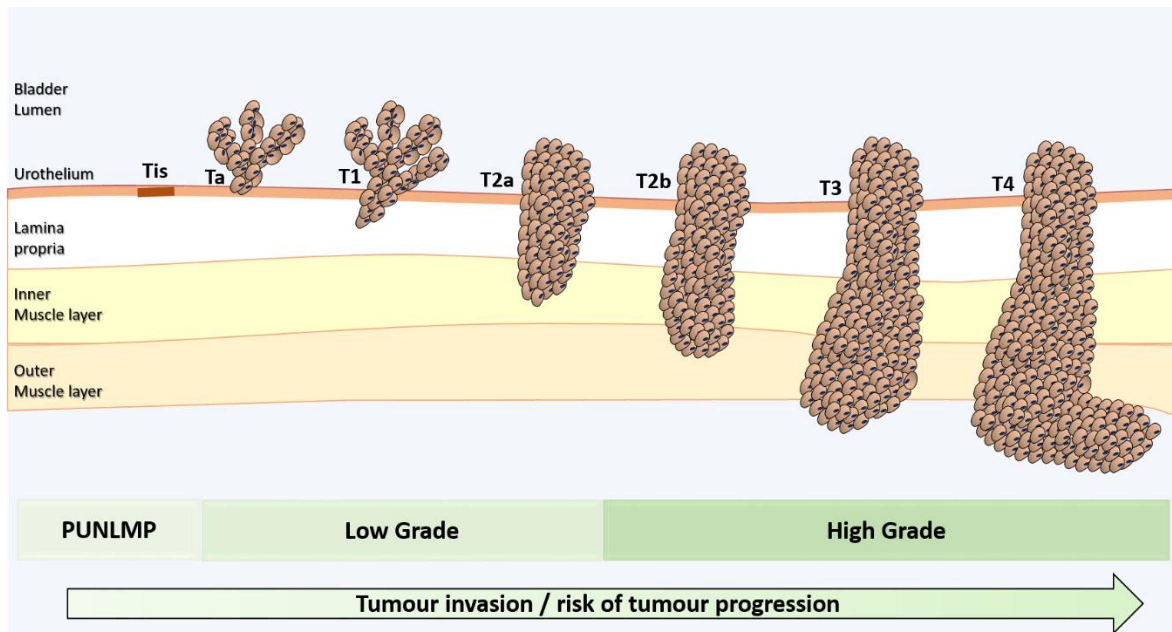

Figure 2. **Schematic representation of bladder cancer stage and grade**. The stage of the primary tumour (T) is based on the extent of penetration or invasion into the bladder wall [16] Regarding tumour grading, bladder lesions can be classified as urothelial papilloma (a benign lesion), papillary urothelial 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.

Approximately 90% of diagnosed bladder tumours are urothelial cell carcinomas (UCC), 5% are squamous cell carcinomas, and less than 2% are adenocarcinomas [4]. Seventy percent of all newly diagnosed UCC are non-muscle invasive tumours (NMIBC, Tis, pTa or pT1) [17]; however 50-70% of these will recur after treatment and roughly 10-20% will progress to muscle invasive disease (T2-T4) usually with metastasis as well as localized persistent disease within a median of 2 years [18]. About 30% of urothelial cancer cases present muscle invasion (MIBC) at the time of diagnosis, with approximately half of patients relapsing after radical cystectomy, depending on the pathological stage of the primary tumour and the presence of loco-regional or distant metastasis. Local recurrence accounts for approximately 30% of relapses, whereas distant metastasis are more common. Moreover, 10-15% of patients are already metastatic at diagnosis [19]. High grade invasive carcinomas are associated with poor prognosis, recurrence, metastasis and increased mortality rates [20].

1.1.3. Clinical manifestation, diagnosis and prognosis of Bladder Cancer

The clinical diagnose includes careful evaluation of the patient's background, including exposure to associated risk factors and family history [4, 21]. At diagnosis, over 80% of the patients present macroscopic urine haematuria, with urine examination generally excluding parasite and microorganisms infections [22]. Moreover, bladder cytoscopic examination and urine cytology are part of the routine diagnosis and commonly used as surveillance tools [23]. The first provides information about the tumour location, appearance, and size; however, it has suboptimal sensitivity for low-grade papillary tumours (Ta and T1) and high-grade Tis lesions [24]. Regarding the urine cytology, this method has reduced accuracy, especially for low-grade and low-stage tumours [23]. To overcome these limitations several molecular non-invasive urine-based clinical tests were approved by Food and Drug Administration (FDA) agency. Namely, immunoassays to detect urinary proteins,

including bladder cancer-associated antigens (BTA STAT®, BTA TRAK®), the nuclear matrix protein NMP22 immunocytofluorescence-based test (ImmunoCyt®/uCytt®); and the fluorescence *in situ* hybridization-based assay (UroVysion®) [23]. Still, the performance of these tests varies significantly and is not always reproducible in independent patient populations [25, 26]. Also, currently, no serum-based tests are available for bladder cancer detection and surveillance in clinical practice. Therefore, the search for novel secreted biomarkers is warranted for a highly specific, sensitive and non-invasive bladder cancer detection.

The election treatment for patients with NMIBC comprises TURBT (transurethral resection of bladder tumour). This may be followed by the administration of mitomycin C or *Bacillus Calmette-Guérin* (BCG) intravesically in patients presenting an intermediate or high risk of relapse upon TURBT. Concerning MIBC, radical cystectomy and (neo)adjuvant chemotherapy (cisplatin-based regimes: methotrexate, vinblastine, cisplatin and doxorubicin - MVAC, or gemcitabine and cisplatin - GC) are the mainstay of available therapeutic options [27]. Radical cystectomy with pelvic lymph node resection is the treatment of choice for non-metastatic patients. Patients with T2 or T3 tumours may also be treated before radical cystectomy with cisplatin based neoadjuvant chemotherapy [28, 29]. On the other hand, metastatic patients are treated with radical cystectomy combined with cisplatin regimes, yet they present a low survival rate of approximately 15 months [30]. Taken together, for MIBC patients undergoing these therapies, the relapse rate is over 50% depending on the stage of the primary tumour and the presence of metastasis, also the overall survival after chemotherapy does not exceed 25% [5, 31]. Several antibody-based targeted therapeutics currently in clinical trials for advanced stage bladder tumours, including anti-PD1/PDL1 and CTL4 immunotherapy, are expected to improve the management of these tumours in the future [32].

In resume, despite several efforts in the establishment of new therapeutic options, advanced stage disease still presents low survival rates. Therefore, new targets must be identified envisaging the design of new therapeutic options. Moreover, early diagnosis,

patient stratification and prognostication represents a critical step in the management of bladder cancer patients, specially MIBC. The expression of novel markers located in tumour cells have the potential to aid BC patient management [33]. Tumour cells recurrently present glycosylation alterations, often translated by increased levels of simple-mucin type O-GalNAc glycans and sialylated antigens. Advances on the glycobiology field allowed new approaches in cancer models studies. Therefore, in an effort to improve current clinical practices, bladder tumours of different stages have been extensively screened for disease-specific glycosidic signatures capable of improve diagnosis, surveillance, prognosis and offer novel therapeutic options.

1.2. Glycosylation in bladder cancer

1.2.1. General features of glycosylation

Glycosylation is the most frequent posttranslational modification (PTM) of membrane-bound and secreted proteins, resulting from the coordinated action of nucleotide sugar transporters, glycosyltransferases and glycosidases in the endoplasmic reticulum and Golgi apparatus of mammalian cells. Glycans play a major part in protein folding, trafficking and stability, also acting as mediators of cell-cell adhesion, cell differentiation, migration, cell signaling pathways, host-pathogen interaction and immune escape.[10, 13, 17, 22, 34, 35]. Particularly, three major classes of glycoconjugates are involved in the modulation of the previously mentioned biological checkpoints; namely proteoglycans, glycosphingolipids and glycoproteins (Figure 3). The proteoglycan class includes glycosaminoglycans (GAG) and hyaluronan [36]. In turn, glycophingolipids represent a glycoconjugate class where glycans bind a lipid ceramide (galactosylceramide or glucosylceramide) giving raise to gangliosides and neutral core structures [36]. Glycosylation increases the diversity of the proteome, lipidome, glycosaminoglycome and proteoglycome to a level unmatched by any other PTM, due to the diversity in sugar composition, glycosidic linkages, anomeric state, branching, chain length and substitution patterns [36]. Regarding the most common types of glycosidic

linkages, it includes N-, O- and C-linked glycosylation, glypiation (GPI anchor attachment) and phosphoglycosylation [37-41]. Furthermore, the different types of glycosylation depend on multiple factors such as enzyme availability, amino acid sequence in case of protein glycosylation and macromolecular conformation of the glycoconjugates [42].

Focusing on the most common types of glycosylation, two main classes of glycans can be found at the cell surface, namely N-glycans and O-glycans. N-glycans are covalently attached to protein asparagine residues by N-glycosidic bonds, which GlcNAcβ1-Asn being the most common. Precursor N-glycan synthesis begins on the cytosolic face of the ER and is further elongated after the structure is flipped into the ER lumen. In proteins, the candidates for receiving an N-glycan are called Asn-X-Ser/Thr “sequons”, with “X” being any amino acid residue except proline [43-45]. Oligosaccharide transferase (OSTase) scans the nascent protein polypeptides for this consensus sequence and then transfers the precursor glycan (Glc3Man9GlcNac2-) from dolichol pyrophosphate to the Asn residue. To this point, all N-linked glycoproteins have the same precursor glycan structure. Glycan processing to diversify the glycans on individual glycoproteins occurs in the Golgi and combines both trimming and adding sugars to the structures in a step-wise fashion [46]. Mature N-glycans chains can be modified by the action of fucosyl and sialyltransferases, yielding sialic acids, Lewis (Le) blood group related antigens (Le^a, SLe^a, Le^x, SLe^x, Le^b and Le^y) or ABO(H) blood group determinants as terminal structures. Other sugar modification 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.

The second most common form of glycosylation is O-glycosylation which often occurs in glycoproteins previously N-glycosylated in the ER. O-glycosylation occurs post-translationally by covalently α-linking a GalNAc moiety from a sugar donor UDP-GalNAc to protein serine or threonine residues and is controlled by a family of 20 membrane-bound enzymes denominated UDP-GalNAc: polypeptide glycosyltransferases (ppGalNAc-Ts), which have distinct but overlapping specificities, allowing a fine-tuned control of the initiation of this process. As opposed to N-glycosylation there is no consensus sequence for the

activity of N-acetylgalactosamine transferase, and following the first sugar addition a highly variable number of sugars are consecutively added to the growing glycan chain such as galactose, N-acetylglucosamine, fucose or sialic acid but not mannose, glucose or xylose residues [42]. A second level of complexity in O-glycosylation is the processing of carbohydrate chains by other glycosyltransferases. After the first glycan (GalNAc) is added, forming the Tn antigen, the core 1 structure is synthesised by Gal-transferase (β (1-3)-galactosyltransferase, C1Gal-T1 or T-synthase), in a Cosmc chaperone dependent manner, which adds Gal to GalNAc. The core 1 structure may be also termed T antigen or Thomsen-Friedenreich antigen (Galβ1-3GalNAcα-O-Ser/Thr). Alternatively, Tn and T antigens can be sialylated by sialyltransferases forming the sialyl-Tn, sialyl-T and disialyl-T antigens. Of note, the formation of the sialyl-Tn antigen stops any further processing of the oligosaccharide chain. Core 1 may function as a precursor of other core structures (from core 2 to 8), by the addition of different monosaccharides, such as galactose, N-acetylgalactosamine, N-acetylglucosamine and sialic acids. Furthermore, cores 1-4 are the most common in humans [42]. The extension of core units provides a vast array of glycan structures, and is catalysed by N-β3/4-acetylglucosaminyltransferases (β3/4 Gn-Ts) and/or β3/4-galactosyltransferases (β3/4 Gal-Ts), leading to the formation of side chains designated type-1 (Galβ1-3GlcNAc-R) and type-2 (Galβ1-4GlcNAc-R) chains. These chains present a ubiquitous expression, and therefore are widely expressed among epithelial tissues. Mature O-glycans may present terminal structures similar to the ones found in N-glycans [42]. This type of glycosylation is particularly found and modulates the biological role of mucins, a family of high molecular weight glycoproteins rich in repetitive sequences of serine and threonine residues, termed tandem repeat domains (VNTR), which are ubiquitously present in mucous secretions, on cell surfaces as transmembrane glycoproteins, and in bodily fluids [47]. Mucins that span the plasma membrane are known to be involved in signal transduction, to mediate cell-cell adhesion or to have an anti-adhesive function [48]. These glycoproteins have also been shown to have roles in fertilization and immune responses [49]. Their presence shields the epithelial surfaces against physical and chemical damage, protecting against infection by pathogens [50]. The expression of mucin genes is regulated by a large number of cytokines

and growth factors, differentiation factors and bacterial products [49]. Moreover, secreted and transmembrane mucins are involved in inflammation and cancer, being strongly associated to tumour progression [51] and high grade muscle invasive bladder cancer [51, 52].

Aberrant glycosylation in cell surface glycolipids, membrane-associated and secreted glycoproteins occurs in essentially all types of human cancers, and many glycans constitute tumour-associated antigens [53]. Generally, the most frequently described cancer related changes in glycosylation patterns include synthesis of highly branched and heavily sialylated glycans, the premature termination of biosynthesis, resulting in truncated glycan forms, and the expression of glycosidic antigens of foetal type [54]. Frequently, the formation of these aberrant structures results from the altered regulation of one or more key glycosyltransferases [55, 56], decreased glycosyltransferases chaperone function [57], glycosyltransferases misslocation in secreting organelles [58], and availability of sugar nucleotide donors and cofactors [59]. Moreover, as previously suggested, glycans and glycoconjugates play a major role in biological processes and clinical outcomes. For instance, the success of blood transfusion or organ transplantation is directly influenced by the glycosidic patterns of cell surface macromolecules [34]. Cell signalling, inflammation and metastasis also constitute mechanisms where glycoconjugates composed by N-linked, O-linked glycans and glycosphingolipids interact with carbohydrates linked to proteins and define clinical outcome [60]. Biomedical research is daily challenged to gather knowledge on neoplastic diseases [34], as cancer remains a massive health problem worldwide. Nowadays glycoproteomics and glycomics strategies appear as strong solutions, allowing a comprehensive evaluation of systems biology and mirroring cellular statues, envisaging novel cancer biomarkers.

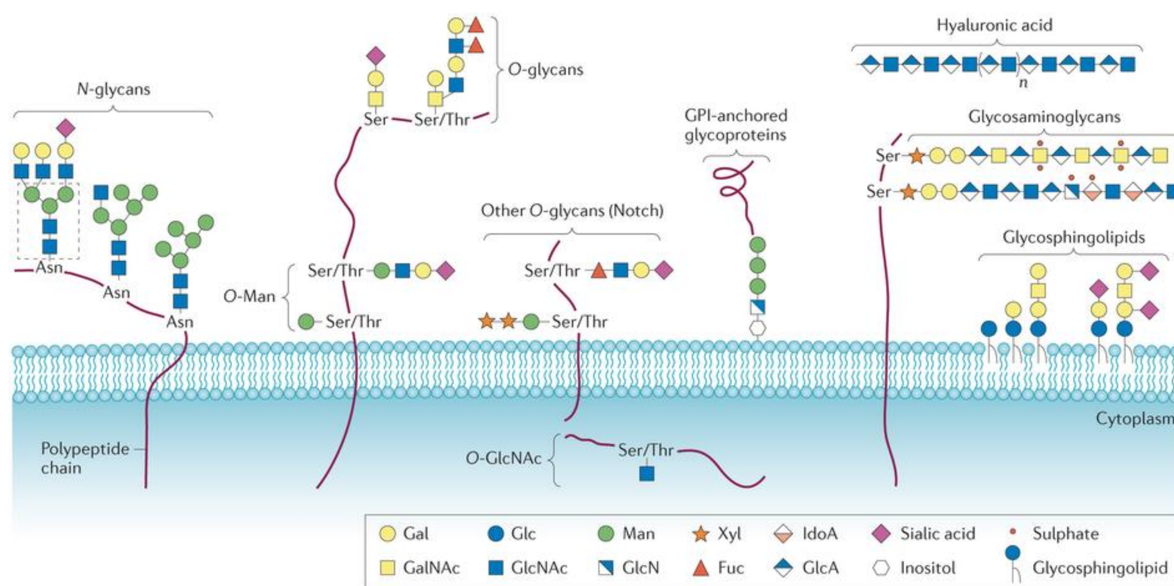

Figure 3. Common classes of glycoconjugates in mammalian cells. Glycans can be found in various types of macromolecules. Glycosphingolipids are major components of the outer leaflet of the cell plasma membrane. O-glycans can be extended, producing various 'cores' and different terminal structures that are usually fucosylated and sialylated. Other types of O-glycans include the O-mannose (O-Man), O-fucose (O-Fuc), O-galactose (O-Gal) and nucleocytoplasmic O-linked β-N-acetylglucosamine (O-GlcNAc). N-glycans share a common pentasaccharide core region that can be further diversified into oligomannose, hybrid or complex types and further modified by the terminal structures GlcNAc, Gal and sialic acid. Some glycoproteins can also be found in the outer leaflet of the plasma membrane linked to a phosphatidylinositol called glycosylphosphatidylinositol (GPI)-anchored proteins. Glycosaminoglycans are linear co-polymers of acidic disaccharide repeating units mostly found attached to the so-called proteoglycans. An exception is hyaluronic acid, which is a glycosaminoglycan found free in the extracellular matrix. *Adapted from Pinho et al., 2015* [36].

1.2.2. Abnormal glycosylation in bladder cancer

Alterations in glycosylation of bladder tumours relate to the loss of ABO blood group determinants in advanced stage carcinomas of secretor individuals (the Se- secretor-locus dictates the capability of an individual to secrete glycoproteins carrying blood group antigens in saliva and other tissues) [61, 62]. Concomitantly, changes in Lewis antigens patterns, and over-expression of simple mucin type O-GalNAc glycans have also been reported [63].

Studies have shown that loss of tissue ABH (O) antigens in the initial biopsy of bladder carcinomas is predictive of a much greater chance of subsequent invasion than in those tumours in which the ABH antigens are detectable [64]. However, a significant number of patients whose initial tumours were reported as blood group antigen negative failed to develop an invasive tumour. It is possible that these conflicting results may, at least in part, be explained by differences in methodology or interpretation, or both. These antigens are present on normal bladder epithelium but not on some low-grade and early- stage papillary transitional cell carcinomas of the bladder. In bladder urothelium the most well examined change has been the deletion of blood group A antigens from A individuals and H antigen from O individuals. Moreover, it has been reported the disappearance of the activity of the A and B gene-encoded transferases in bladder tumours from blood group A and B individuals which explains the deletion of these antigens in bladder tumours [65]. The A, B, H antigens have biosynthetic and structural similarities with the Lewis antigens, including the type 1 Lewis^a and type 2 Lewis^x antigens. Several authors have associated the Lewis^a expression patterns with malignant transformations of the bladder and invasion [66]. The sialylated form of Lewis^a, the SLe^a antigen, has been observed in bladder dysplasia, CIS, non-invasive and invasive carcinomas of the bladder [67]. Nevertheless, no correlation was found with invasive or metastatic potential. Altogether, one can associate the expression of Lewis an antigen with malignant bladder cancer cellular phenotype [68]. In turn, up to 90% of advanced bladder tumours express Le^x, contrasting with no expression in health urothelium. This strongly suggests association between Le^x expression and malignant transformation [69]. Moreover, the sialylated forms of Lewis^x antigens have been closely linked to the invasive and metastatic potential of bladder cancer [70]. In turn, the expression of GnT-V and consequently of β1-6 branched N-linked oligosaccharides was closely related to low malignant potential in bladder cancer, a finding that could be applied to risk stratification [71]. Furthermore, hyaluronic acid synthase 1 (HAS1) expression in tumour tissues is a predictor of bladder cancer recurrence and treatment failure. HA promotes tumour metastasis and is an accurate diagnostic marker for bladder cancer. Moreover, the measurement of hyaluronic acid and hyaluronidase (the HA-HA test) has been applied to

the screening of bladder cancer. It has been shown recently that HAS1 regulates bladder cancer growth and progression by modulating HA synthesis and HA receptor levels [72]. Additionally, overexpression of galectins, a N-glycosylated proteins, and it association in development of cancer is due to their interaction with poly-*N*-acetylglucosamines on matrix proteins, which as the biological function of supporting cellular invasion [73]. Galectins increased expression are also correlated with bladder cancer [74]. In comparison to normal and low-grade bladder tumours, mRNA levels of galectin-1 were overexpressed in majority of high-grade cases [75, 76]. Parallel results were also found in protein level of high grade bladder cancers, by western blot and immunohistochemistry [77]. Similarly to galectin-1, galectin-3 demonstrates increased expression in most tumours compared to normal urothelium [75, 76, 78]. Successively, also for bladder tumours, galectin-7 was namely a potential chemosensitivity marker to cisplatin, however this fact has still to be verified [79]. Concerning to glycosphingolipids, biological functions includes cell-cell adhesion, proliferation, differentiation, apoptosis, motility and immune recognition and has also an important role in fluid membrane regulation [80-82]. Thus, these molecules have the potential of becoming biomarkers and pharmacological targets for bladder tumors. Ganglioside GM3 is a glycosphingolipid overexpressed in tissue of human bladder cancer [83], related to GM3 synthase increased expression and a decreased regulation in Gb3 and GD3 synthase [83]. Furthermore, increased levels of GM3 are correlated to low invasive potential [83], proliferation, motility, tumour development and upper apoptosis [84]. Still, exogenous GM3 may become a therapy for bladder cancer, inhibiting the proliferation and adhesion of cancer cell lines, besides inhibits tumour growth in orthotopic models [85]. Finally, GM2/GM3 complexes also inhibits cell motility and growth in bladder cancer [86].

Finally, increased levels of simple-mucin type *O*-GalNAc glycans have also been observed in bladder cancer. Several reports associate the presence of Tn and T antigens with recurrence and metastasis suggesting these antigens may be surrogate markers of profound cellular alterations [87][74]. There is also growing evidences linking the overexpression of the sialyl-T antigen and ST3Gal.I, the enzyme responsible by T antigen sialylation, with bladder cancer aggressiveness and recurrence [88]. In turn, T antigen may

origin ST by ST3Gal1 or core 2 carried out by C2GalT1 [89-91]. The addition of sialic acid to T or Tn antigen, form sialylated structures which inhibits further chain extension the sialyl T or sialyl Tn antigen respectively, except the likely addition of a second sialic acid to ST converting into a disialylated structure. STn antigen, is a Tn antigen attached to a sialic acid (Neu5Ac) on carbon 6, sialylated by ST6GalNAc-I. On the other hand, *ST3Gal-1* sialylates T antigen to a Neu5Ac on carbon 3, forming sialyl-3-T (S3T) [90], or it may also remain sialylated twice by ST3Gal1 and thereafter by ST6GalNAc-I creating Disialyl-T antigen (dST) [89-91]. In summary, Tn antigen may be converted in STn, T antigen (or Core 1) and Core 3 structures, by ST6GalNAc-I, T- synthase (or C1GnT) or C3GnT, respectively. Successively, T antigen may origin ST antigen by ST3Gal1 and Core 2 by enzymatic action of C2GnT. Furthermore, ST can be sialylated by ST6GalNAc-I originating dST (Figure 4). Several types of cancer demonstrate this abnormal sialylation which is related to poor prognosis and worse response to the disease. There is important to highlight the increased expression of Tn and STn observed in malignant cancer cells [92-94].

Sialylated glycan terminal structures and simple-mucin-type carbohydrate antigens have their expression modified and amplified. Tumour progression, proliferation, invasion, metastasis and angiogenesis are various mechanisms affect by aberrant function of glycans expression, which makes them key mediators in these processes [36]. Thus, alterations of glycosylation are associated with oncogenic transformation leading to molecular heterogeneity [36]. As previously reported, alterations of O-glycosylation have the potential to be applied in screening, patient’s prognosis, pointers to therapy response and other clinical settings [96]. The knowledge of these specific glycosylation alterations associated to development cancer cascade have major significance in the improvement of cancer research and consequently clinic outcomes [96].

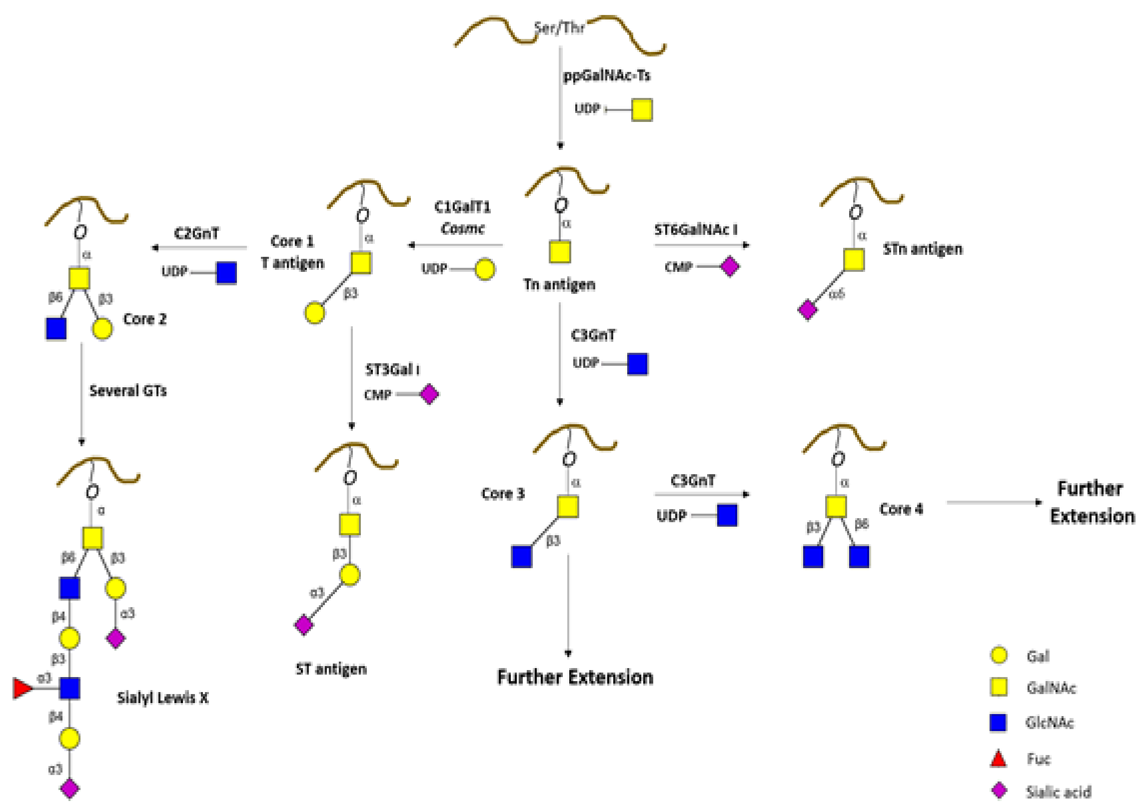

Figure 4. Schematic representation of simple mucin-type O-glycan biosynthesis. *O*-glycan extension begins with the addition of a GalNAc monosaccharide to a serine or threonine residue of the protein backbone. This reaction is catalyzed by polypeptide *N*-Acetylgalactosamine transferases (ppGalNAcTs), which constitute a superfamily of 20 enzymes, and gives rise to the Tn antigen. To this follows the synthesis of the core 1 structure by the β(1-3)-galactosyltransferase, C1Gal-T1, which requires the molecular chaperone COSMC. Core 1 may function as a precursor of other core structures. In turn, the extension and termination of core units provides a vast array of glycan structures, catalyzed by N-acetylglucosamine (GlcNAc) transferases (GnTs; as C2GnT, C3GnT), sialyltransferases like α2,3-sialyltransferases (ST3Gal-Ts) and α2,6-sialyltransferase I (ST6GalNAc-I) and fucosyltransferases. The early sialylation of Tn and T antigens form the sialyl-Tn (STn), and sialyl-T (ST) antigens, and the formation of the sialyl-Tn antigen stops any further processing of the oligosaccharide chain. Of note, further core elongation can lead to the formation of side chains that can be modified by the action of fucosyl and sialyltransferases, yielding Lewis blood group related antigens, such as Sialyl-Lewis^{x} (SLe^{x}), which function as terminal structures and stop chain elongation. *Adapted from Ferreira et al., 2015 [95]*

1.2.3. Regulation, biological and clinical significance of STn expression in bladder cancer

One of the most common cancer-associated simple mucin-type O-glycans is the sialyl-Tn antigen (STn, Neu5Acα2-6GalNAcα-*O*-Ser/Thr), which is neo- or overexpressed in more than 80% of human carcinomas [97]. Since the expression of STn in normal tissues is rare and low when compared to cancer tissues, STn can be considered a pan-carcinoma antigen and a good tumour marker of carcinogenesis. The Sialyl-Tn antigen is synthetized by the alpha-N-acetylgalactosamine alpha-2,6-sialyltransferase 1 (ST6GalNAc-1) glycosyltransferase, which adds a sialic acid from Cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac) to the Tn antigen. Commonly, STn overexpression stems from the incapability of the cell glycosylation machinery to produce more elongated glycans. Namely, several reports attribute its expression to a disorganisation of secretory organelles (ER and Golgi) in cancer cells, and absence or altered expression and/or activity of glycosyltransferases responsible for the synthesis of glycan core structures [98]. In particular, the overexpression of ST6GalNac.I has been found to promote the premature sialylation of the Tn antigen, giving raise to STn. Mutations on *Cosmc*, a molecular chaperone essential for T-synthase function has been found to promote the accumulation of Tn and STn antigens in neoplastic lesions [99]. Ferreira et al. has recently demonstrated that over 70% of high-grade NMIBC and MIBC expressed the STn antigen, whereas 80% of low-grade NMIBC and healthy urothelium do not. STn was mostly expressed by cells in non-proliferative tumour areas, known for their high resistance to cytostatic agents currently used in advanced stage bladder cancer patients treatment [100]. *In vitro* studies have also demonstrated that STn plays a role in bladder cancer cells invasion an migration [100]. Other studies in STn-expressing bladder cancer cells shown that STn has the ability to down-regulate the anti-cancer immune-response through different mechanisms. First, it hinders the expression of MHC-II and co-stimulatory molecules by dendritic cells (DCs), resulting in impaired ability to present cancer-associated antigens to T cells and making DCs unresponsive to successive activation stimuli. Second, it hinders the expression of

inflammatory, Th1-inducing cytokines in DCs, which may result in an attenuation of the Th1 microenvironment and reduced ability to activate and polarize T cells towards the Th1 phenotype. Altogether, these results highlight the expression of STn by cancer cells as a crucial event in the establishment of the tolerogenic microenvironment which allows cancers to escape from the attack of innate and adaptive immunity [101]. More recently, our group has demonstrated for the first time that hypoxia, a highly relevant feature of advanced stage bladder tumours, promotes STn antigen overexpression in bladder cancer cell lines, enhancing the migration and invasion capability of those presenting more mesenchymal characteristics, in an HIF-1α-dependent manner. As such, STn overexpression may, in part, result from a HIF-1α mediated cell-survival strategy to adapt to the hypoxic challenge, favoring cell invasion. In addition, targeting STn-expressing glycoproteins may offer potential to treat tumour hypoxic niches harboring more malignant cells [102]. Other authors have also associated STn expression with loss of differentiation [101], cellular recognition [103], actin cytoskeleton dynamics, cell-cell aggregation, migration, metastization [99, 104], and cancer progression [101]. As such, STn expression vows for more aggressive cellular phenotypes, having a key role in bladder cancer progression and dissemination. Given its association with key neoplastic transformation checkpoints, urges to explore STn expression as a possible theragnostic biomarker capable of improving bladder cancer patient management and overall survival.

1.3. Mining STn-glycoproteome envisaging novel biomarkers and targeted therapeutics

1.3.1. Current and emerging targets for bladder cancer therapeutics

Modest disease control rates, with sporadic marked chemotherapy responses has led to the investigation of biomarkers for assessment of postoperative prognosis and the potential value of perioperative chemotherapy, and as predictors of response to chemotherapy or its monitoring. Most biomarkers are associated with tumour angiogenesis.

Small studies, usually retrospective, have investigated microvessel density, altered p53 tumour expression, serum vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), urinary and tissue basic fibroblast growth factor, urinary (wild-type and mutant) and tissue fibroblast growth factor receptor-3, and more recently, thrombospondin-1, circulating tumour cells, and multidrug resistance gene expression [105]. Particularly, fibroblast growth factor receptors (FGFRs) and vascular endothelial growth factor receptors (VEGFRs) are tyrosine kinase receptors (RTK) playing major roles in cellular function [105]. Growth factors such as hormones and cytokines activate tyrosine kinase receptors thereby, influencing cellular proliferation and growth [106]. Tyrosine kinase receptors are often overexpressed or mutated in bladder cancer, leading to an overestimation of downstream pathways [105]. Furthermore, targeting signaling transduction pathways proteins (MAP Kinases, PI3 kinases and GTPases), cell cycle checkpoints, apoptosis pathways, translation related proteins and cellular metabolism constitute novel bladder cancer therapies [107, 108]. In addition, several studies have been exploring immune checkpoints such as CTLA-4 (Cytotoxic T-Lymphocyte Associated Protein 4), PD-1 (Programmed cell death protein 1) and PD-L1 (Programmed death-ligand 1), as well as oncolytic adenoviruses [105].

In resume, bladder cancer patient management remains a significant burden to both patients and health care systems. The need for new and effective therapies has driven remarkable research efforts during the past decades, leading to the genetic, epigenetic and proteomic characterization of bladder tumours. However, despite promising pre-clinical and clinical studies, few novel therapeutics have transposed beyond phases I and II of clinical trials. Of note, the most promising results were observed in multi-targeted approached. As such, future studies should continue to assess the benefit of simultaneous inhibition of multiple targets.

1.3.2. Analytical approaches for the characterization of oligosaccharides

Combining molecular information from glycan and peptide moieties holds tremendous potential for designing highly specific targeted therapeutics. However, the fact that glycan structures do not obey to a predefined template, but are rather the result of the highly regulated action of several glycosyltransferases rapidly responding to microenvironmental and physiological stimuli, presents a significant analytical hurdle. Nevertheless, glycomics-based chromatography and mass spectrometry (MS) methods have reached a standardization stage, providing highly sensitive analytical tools for precise mapping of the glycome [109, 110]. Proteomic approaches enable the identification of proteins, fragments of proteins and peptides, allowing the production of extensive datasets [111]. Proteomics might also determine proteins abundance, intermolecular interactions and post-translational modifications [112-115]. Bodily fluids such as urine and blood, as well as tissues, have been screened through proteomic approaches in order to identify disease-associated biomarkers [111].

In the past decade, mass spectrometry has emerged as a core analytical technology for high-throughput protein analysis, mainly due to a rapid advance in the resolution, mass accuracy, sensitivity, and scan rate of mass spectrometers, and the introduction of hybrid mass analyzers. The hybrid mass analyzers result from combinations of ion sources with several mass analyzers such as LIT (linear ion trap), Orbitrap, FT-ICR (Fourier transform ion cyclotron resonance), Quadrupole and TOF (time of flight) [116]. Also, the combination of a high performance separation technique such as high performance liquid chromatography (HPLC) and mass spectrometry (MS) become the solution for on-line combination of separation technique, reducing the time consumption. This coupling was possible with the improvement of soft ionization technique such as electrospray ionization (ESI) and the development of adapted HPLC plumbing systems with reduced flow rate such as vented columns and nano-liquid chromatography (nano-LC) [117]. Therefore, the current standards for glycans and glycomolecules analysis involves liquid chromatography coupled with an electrospray tandem mass spectrometer (LC-ESI-MS^2) or a matrix-assisted laser

desorption/ionization time-of-flight mass spectrometer (MALDI-TOF/TOF MS). Yet, the unambiguous identification of structural iso(mer/bar)ic species solely by tandem mass spectrometry, however, continues to be a challenging task. Also, as analytical technologies advance, data analysis and annotation also have become highly complex.

Despite the possibilities of mass spectrometry exploring glycans and glycomolecules, few studies were conducted in bladder cancer for this aim using this technology. Yet, such studies that conducted a precise analysis of cancer cells glycoproteome already give a glimpse of the presence of unique subsets of abnormally glycosylated proteins [118]. Recently, a preliminary study has demonstrated that bladder cancer cells express abnormally O-glycosylated proteins expressing STn antigen [100]. Glycans profiling by mass spectrometry can clarify structural features of these molecules, such as STn, our goal molecule. Ricardo S. *et al.*, find aberrant glycoforms of mucins (T, STn and Tn) in ovarium tumours and studies in gastric cancer revealed similar features for this type of cancer. Both studies concluded that STn glycoforms is a candidate with biomarker potential [119, 120]. Knowing that there are no confirmed predictive molecular markers to guide clinical management of bladder cancer patients and also due to the high expression of STn in malignant cells of advanced bladder cancer, proteomics approaches may help to identify a target that could have a significant role in management of patients with this disease [7, 101, 111].

Despite all the current knowledge on glycobiology, the emerge use of proteomic approaches and the evidences pointing at glycans as possible useful cancer biomarkers, several factors have been hampering glycobiomarkers implementation in clinical practice. Particularly, there are conflicting study results due to variations in cohort size, ethnicity, type, stage and grade of bladder cancer, biological sample type and samples processing, as well as lack of endpoints standardization. Moreover, there is a lack of studies inferring on the biological impact or on the prognostic potential of glycans. As such, there is a need for standardized studies validating glycobiomarkers in larger series for prospective use, comparing current clinicopathological parameters for risk assessment and including the

impact of glycobiomarkers in therapeutic outcome. The application of proteomic and mass spectrometry analytical platforms to well characterized clinical samples is expected to translate into highly cancer-specific glycobiomarkers with potential of detecting early diagnosis and promising therapies [101]. Guided by the clinical needs of patients with advanced bladder cancer, the focus of this thesis is studding STn-glycoproteome envisaging proteomic biomarkers of primary and recurrent bladder cancer.

CHAPTER 2

AIMS AND SCOPES

AIMS AND SCOPES

Muscle invasive bladder cancer (MIBC) is considered a neglected neoplasia, nevertheless, over 54,000 deaths have been reported in Europe and around 165,100 deaths worldwide in 2012, placing it amongst the deadliest genitourinary cancers [1]. Cisplatin-based regimens are the only available therapeutics for invasive and metastatic cases, nevertheless, due to treatment failure, the five-year overall survival does not exceed 25% and many patients die prematurely from adverse drug reactions, urging for effective and safe targeted therapeutics. Alteration of sialylated antigens are strongly associated with progression and invasiveness of the bladder tumour advanced stages [36, 101]. Particularly, the STn is highly associated with malignant phenotypes, increased metastatic potential, poor prognosis and decrease overall survival [33, 121, 122]. Glycomics and glycoproteomics methods have reached a standardization stage, providing highly sensitive analytical tools for precise mapping of the glycome and for studying single glycan modifications. Therefore, these methods allow the identification of STn-associated proteins.

In this context, the engendered information leads us to the broad objective of this study that is to gain further knowledge on bladder cancer cell sialylation, envisaging targeted therapeutic approaches for key cancer STn-associated proteins.

This will be approached through the specific aims:

(i) To screen series of bladder tumours with different clinicopathological nature for cancer-associated short-chain O-glycans (Tn and STn; T and ST: S6T and S3T)

(ii) To profile the O-glycans in bladder tumours, by the nanosystem liquid chromatography electrospray ionization mass spectrometry (nanoLC-ESI-LTQ-Orbitrap-CID-MS/MS) method;

(iii) To identify potential glycoprotein biomarkers and evaluate their role in bladder cancer prognosis

CHAPTER 3

MATERIAL AND METHODS

MATERIAL AND METHODS

3.1. Patient and sampling

The screening of cancer-associated short-chain *O*-glycans (Tn and ST; T and ST, S6T and S3T) was performed on 47 formalin-fixed, paraffin embedded tissue sections (FFPE) 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 [123], 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 [37-84]. The male/female gender ratio in MIBC was of 9:1 and the median age was 71 years [38-84]. Forty cases were considered stage Ta, 34 stage T1, 25 stage T2, 48 stage T3, and 29 stage T4 (for further analysis T1-T4 staged tumours were compared against Ta staged tumours). All MIBC patient were treated with cystectomy, 27 of which were also treated with adjuvant chemotherapy (cisplatin+gemcitabine). All tumour samples were revised by a pathologist according 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

institutions Ethics Committee of IPO-Porto after informed patient's consent. All clinicopathological information was obtained from patient´s clinical records.

3.2. 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.* [100]. The expression of the Tn, sialyl-Tn and T antigens was directly evaluated using in-house mouse monoclonal antibodies 1E3, TKH2 and 3C9, respectively [124-126]. 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, Missouri, 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) according to Figure S1A-Supporting Information. 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) according to Figure S1B-Supporting Information. 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. Bladder tumours and metastasis were also screened for MUC16 using rabbit anti-Human CA-125 monoclonal antibody EPR1020 (Abcam, Cambridge UK; 1:200 in PBS) at room temperature for 1 hour. In addition, prior to glycoproteomics studies, FFPE tissues were screened for blood group A determinants using mouse monoclonal anti-human blood group A antigen antibody HE-195 (Thermofisher, Massachusetts, USA; 1:100 in PBS) after 1h 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). Disaccording readings were re-analyzed 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 labeling based on the percentage of positively stained cells. For glycans evaluation 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.

3.3. Glycoprotein extraction and enrichment

Proteins were extracted from FFPE bladder tumours from male MIBC patients conserving STn expression, using Qproteome FFPE tissue kit (Qiagen, Hilden, Germany) according to the supplier's instruction. The amount of protein in each extract was estimated with RC DC protein assay kit (BioRad, California, USA). The samples were blotted for STn as previously described [102] and five tumours presenting a similar blotting pattern were pooled together. Thirty micrograms of protein were then separated by 4–16% gradient SDS–PAGE under reducing conditions, bands excised from the gels and proteins reduced, alkylated and digested *in situ* for MS analysis as described by Ferreira *et al.* [127] (according to Figure S3A-Supporting Information). In addition, approximately 1mg of total protein resulted from pooling extracts from 5 tumours of male MIBC patients showing negative Tn and blood group A antigens were screened for STn-expressing glycoproteins. The procedures were conducted as previously described [102]. Briefly, the protein pool was subjected to neuraminidase treatment (10 U *Clostridium perfringens* neuraminidase Type VI (Sigma-Aldrich, Missouri, USA)) before being loaded on 300 µl of agarose-bound *Vicia villosa* agglutinin (Vector laboratories, California, USA) 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 to Sigma-Aldrich, Missouri, USA). The glycoproteins were then eluted by 4x 500 µl 0.05% RapiGest (Waters, Massachusetts, USA) with heating to 90°C for 10 min. The glycoprotein

fraction was then directly reduced with 5 mM DTT (Sigma-Aldrich, Missouri, USA) for 40 min at 60˚C, alkylated with 10 mM iodoacetamide (Sigma-Aldrich, Missouri, USA) for 45 min, and digested with trypsin (Promega, Wisconsin, USA) (according to Figure S3B-Supporting Information).

3.4. nanoLC-ESI-LTQ-Orbitrap-CID-MS/MS and data mining

A nanoLC system (Dionex, 3000 Ultimate nano-LC) was coupled on-line to a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Massachusetts, USA) equipped with a nano-electrospray ion source (EASY-Spray source; Thermo Scientific, Massachusetts, USA). 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 µl/min. After 3 minutes, the flux was redirected to the analytical column (EASY-Spray C18 PepMap, 100 Å, 150 mm x 75µm ID and 3 µm particle size) at a flow rate of 0.3 µl/min. Column temperature was set at 35º C. Peptide separation occurred using a linear gradient of 5–40% eluent B over 117 min., 50–90% eluent B over 5 min. and 5 min. with 90% eluent B. In order to favor the separation and identification of peptides presenting high hydrophobicity, samples were also analyzed 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 6 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 (AGC) 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.

3.5. MS/MS data curation

Data were analyzed automatically using the SequestHT search engine with the Percolator algorithm for validation of protein identifications (Proteome Discoverer 1.4; Thermo Scientific, Massachusetts, USA). 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 2 missed cleavage sites, a precursor ion mass tolerance of 10 ppm, 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 Xcorr ≥ 1.0 and ΔCn ≤ 0.05. Cytoplasm membrane proteins with at least 1 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/) to generate the final protein list. Protein molecular and biological functions were interpreted using Panther [128].

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

The simultaneous detection of mucin-16 (MUC16) STn^{+}-glycoforms and ITGB1 plus CD44 was done by *in situ* proximity ligation (PLA) assays 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 [119, 120]. Briefly, FFPE tissues were deparaffinized, rehydrated and submitted to acid and heat-induced antigen retrieval, followed by incubation with 3% hydrogen peroxide and blocking solution in a humidity chamber, as previously described [100]. MUC16 was detected by direct PLA assay using monoclonal antibody CA125 (Clone M11; DAKO, California, USA) conjugated with PLA probe PLUS (concentration 0.005 mg/ml) and B72.3 monoclonal antibody against STn, which showed similar recognition but lower background when compared with TKH2, with

PLA probe MINUS (concentration 5 ng/ml). Antibodies were conjugated following the instructions of Duolink *in situ* Probemaker and were hybridized for 1 h at 37ºC. Next, ligation was performed for 30 min at 37ºC and amplification was carried out for 120 min at 37ºC to produce rolling circle products, followed by incubation with horseradish peroxidase (HRP) labelled probes and addition of the chromogen. Finally, sections were counterstained using hematoxylin, dehydrated, cleared and mounted for optical microscope analysis. Regarding the indirect PLA assays for ITGB1 and CD44, FFPE tissues were incubated with anti-CD44 (EPR1013Y; Abcam, Cambridge UK) and anti-ITGB1 (A-4 clone, Santa Cruz Biotechnology, California, USA) overnight at 4ºC in a humidity chamber. Then, the PLA probes anti-rabbit MINUS and anti-mouse PLUS were both added and sections were incubated at 37ºC for 1 hour. 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 (DAPI) for 10 minutes 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 [129] and sequential ovarian cancer tissue sections showing MUC16 and STn co-localization by immunohistochemistry [119].

3.7. MUC16 transcription in bladder tumours

RNA was isolated from FFPE tissue samples using the Absolutely RNA FFPE Kit (Stratagene, California, USA), as previously described [130]. Up to 2 mg of total RNA was reverse transcribed with random primers, using the "High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems, California, USA). Real-time PCR amplification of cDNA samples was performed in a StepOne Real-Time PCR System (Applied Biosystems, California, USA) using TaqMan Gene Expression Master Mix, primers, and probes provided by Applied Biosystems, California, USA. MUC16 expression was measured with TaqMan expression assay (ID: Hs01065189_m1) from Applied Biosystems, California, USA. The raw $-\Delta Ct$ was used to analyze MUC16 expression and therefore used as an estimate of the mRNA relative levels. ΔCt stands for the difference between the cycle threshold (Ct) 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.

3.8. Immunoprecipitation for CD44 and ITGB1

CD44 and ITGB1 were immunoprecipitated from total protein extracts (IP) with anti-CD44 (EPR1013Y; Abcam, Cambridge UK) and anti-ITGB1 (A-4 clone, Santa Cruz Biotechnology, California,USA) 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, Missouri, USA) were used as controls.

3.9. Statistical methods

Statistical data analysis 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. 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.

CHAPTER 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1. 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* [121] and more recently in superficial bladder tumours [104]. Table 1 summarizes the expression of these glycans in the studied samples according to their disease subtype.

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%

4.1.1. Expression of non-sialylated short-chain *O*-glycans (Tn and T antigens)

Table 1 highlights that Tn and T antigens are poorly expressed in bladder tumours (approximately 20-50% of total cases) in comparison to their sialylated counterparts (62% and 100%, respectively; highlighted in Figure S4-Supporting Information). More importantly, these antigens are mostly found in high-grade tumours, irrespectively of the degree of invasion. Nevertheless, the number of T antigen positive cases largely exceeds the Tn positive (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. Accordingly, 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* [102]. 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 6 studied healthy urothelia cases, demonstrating the malignant nature of these molecular alterations.

4.1.2. 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 irrespectively of their grade and degree of invasion (62-100%; Table 1; Figure S4). In agreement with previous studies [33, 101], 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 (Figure 5A); yet in tumour areas presenting extensive staining (>50%) (Figure 5B), it could also be detected in papillary urothelium and invasive fronts (Figure 5B). 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 [33, 101]. Hence, cells neighboring 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 Figure 6). However, a significant increase in the extension of sialylated T antigen could be observed in more advanced cases, suggesting an over-expression and/or increased activity of sialyltransferases (Figure 6). In agreement with these observations, it has been

demonstrated that advanced stage bladder tumour overexpress ST3Gal.I [88], the glycosyltransferase responsible by 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 [33, 100, 131, 132].

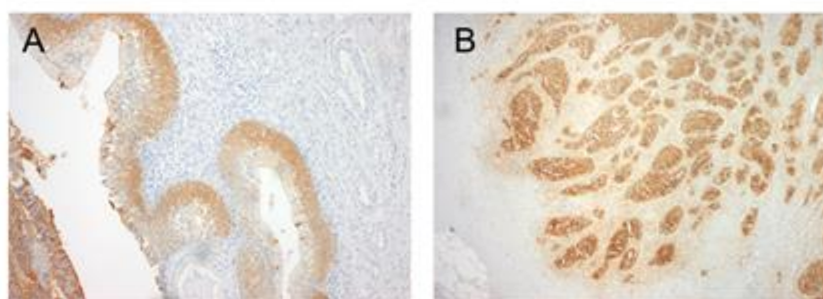

Figure 5. Immunohistochemistry for sialyl-Tn (STn) antigen evidencing A) expression in cells longing and invading the basal layer in high-grade NMIBC and B) extensive staining including in cells invading the muscle layer in MIBC.

4.1.3. 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 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 removing

of □-3 linked Gal residues from S6T antigens exposing STn antigens for recognition (Figure S2A). Accordingly, we observed positive staining after enzymatic treatment in STn negative tumours (Figure S2A), as well as 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 Figure 6). 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 of more aggressive NMIBC, suggesting that *O*-6 sialylation plays a key role in *bacillus* binding to the epithelium [104]. 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 [133] in advanced stage bladder tumours [100]. 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 [42], gaining more insights on the biological mechanisms underlying these molecular alterations and its clinical relevance.

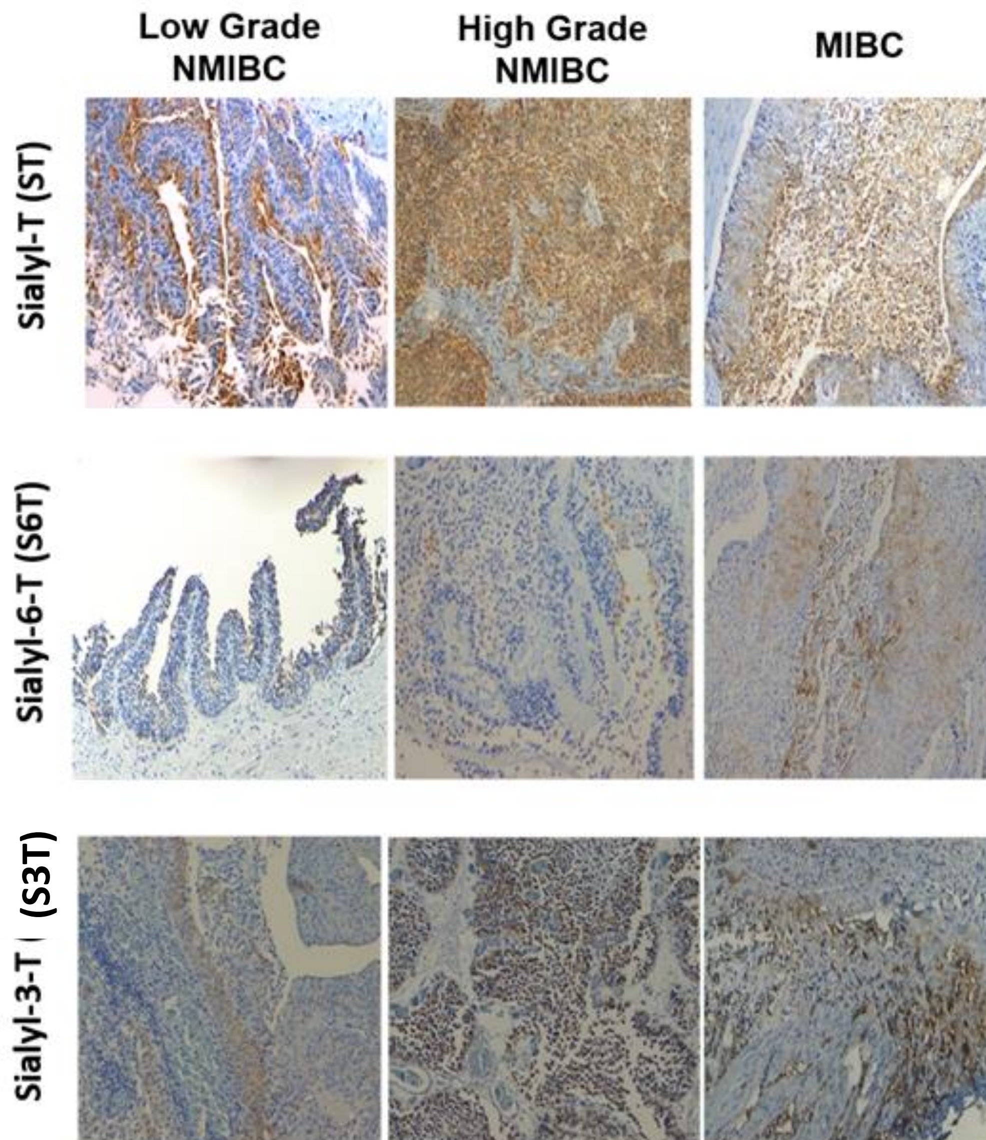

Figure 6. Immunohistochemistry for sialylated T antigens (ST: corresponding to mono and di-sialylated T glycoforms; S3T and S6T) for low and high grade superficial papillary muscle invasive bladder tumours. The Figure highlights the increase in T sialylation with the severity of the lesions. Since the S6T antigen was determined based on comparisons with STn expression after β-(1,3)-galactosidase digestion, only negative STn negative tumour lesions are being presented in this figure. Moreover, since 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.,

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 (Figure 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; 67% 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 by T antigen *O*-3 sialylation, in high grade tumours [88]. 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 tumours that co-overexpress non-sialylated 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 etiologies [33, 100, 102, 104, 131,

132, 134]. 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 to understand the molecular mechanisms underlying glycomic alterations, including: i) the events modulating the expression and activity of glycosyltransferases and glycosidases in bladder tumours; ii) access the distribution of glycosyltransferases throughout the secretory organelles and pathways; iii) explore mutations in key enzymes involved in *O*-glycans biosynthesis and its functional impact. Such information will be crucial to access the biological and clinical significance of altered *O*-glycosylation in bladder cancer, provide relevant insights for glycoproteomics studies and ultimately the design of novel and more effective therapeutics [95].

4.2. 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 [33, 100, 102, 104, 132]. Moreover, we have observed that STn expression significantly favors cell motility and capacity to invade [100, 102] as well as immune escape [101]. Therefore, mapping the STn-glycoproteome is crucial to develop highly specific targeted therapeutics against advanced bladder tumours. However, while the majority of glycoproteomics studies presented so far have focused mostly on bodily fluids and, to lower extent, human tissues, none has attempted to address protein glycosylation in FFPE tissues. Herein, we extracted

proteins from 5 MIBC tumours and screened the samples for STn expression by western blot, which retrieved similar expressions patterns (Figure S5A-Supporting Information). These samples were then pooled and analyzed by a conventional gel-based and nanoLC-MS/MS proteomics approach (Figure S3A-Supporting Information), which allowed the identification of 2578 peptides, corresponding to 294 of the most abundant proteins in these tumours. This illustrated the feasibility of using FFPE has starting material for retrospective proteomic studies on clinical samples. Gene ontology interpretation of the results using Panther highlighted the presence of proteins from all cell compartments, including plasma membrane proteins (4%; Figure S4A-Supporting Information); nevertheless, an overrepresentation of cytoplasmic and cytoskeleton proteins could be observed (Figure S4A). The main represented molecular functions included binding, structural and catalytic activities, whereas the main biological functions were set on metabolic and cellular processes (Figures S4B and C). Protein extracts were then digested with a α-neuraminidase to remove sialic acids from STn-expressing glycoproteins exposing the Tn antigen. This allowed the introduction of an enrichment step based on affinity to *Vicia villosa* agglutinin (VVA) lectin that selectively binds terminal GalNAc residues. The absence of Tn and blood group A determinants in these chosen cases ensured the specificity of the enrichment for STn-expressing proteins (Figure S3B). Subsequent nanoLC-MS/MS analysis led to the identification of over 400 *O*-glycosites and 143 membrane glycoproteins putatively expressing the STn antigen, 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 Figure 7. In particular, STn-expressing proteins mostly mediate

binding to other proteins and have hydrolase catalytic activities. They also mediate cell-cell communication and signaling, as well as 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 [135] (Figure 8). This list included CD44, a typical bladder cancer stem-cell associated glycoprotein also associated with drug resistant phenotypes and poor prognosis [136], and several integrins, in accordance with previous observations [102]. For validation purposes, we have immunoprecipitated CD44 and ITGB1 in these samples and confirmed the expression of STn by western blot (Figure S5B) and PLA (Figure S5C). In addition to these glycoproteins, we have also identified, for the first time, MUC16 and abnormal MUC16 glycoforms in bladder tumours. Interestingly, these heavy 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) [137-139]. Again, we have confirmed the presence of STn in MUC16 derived glycopeptides based on characteristic CID-MS/MS fragmentation spectra (Figure 9A). Moreover, we found glycopeptides carrying both GalNAc and Gal-GalNAc substituents, highlighting the complex antigenic glycoarray presented by bladder cancer associated glycoproteins (Figure S6-Supporting Information). In addition, the analysis of consecutive bladder tumour sections revealed that MUC16 expression is associated and co-localized with STn expression in 90% of the cases, irrespectively of their

histological classification (Figure 9B). Moreover, PLA that allows the simultaneous detection of the protein and the glycan whenever there is close proximity, confirmed the presence of MUC16 STn^{+}-glycoforms in clinical samples (Figure 9C). 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 to lower stage disease, suggesting that this antigen may predict advanced bladder cancer [139, 140].

Furthermore, abnormal CA125 levels have been observed in patients with unresectable tumours, again reinforcing its association with worse prognosis [139], reinforcing the need to address the biological and clinical relevance of this molecule in future studies.

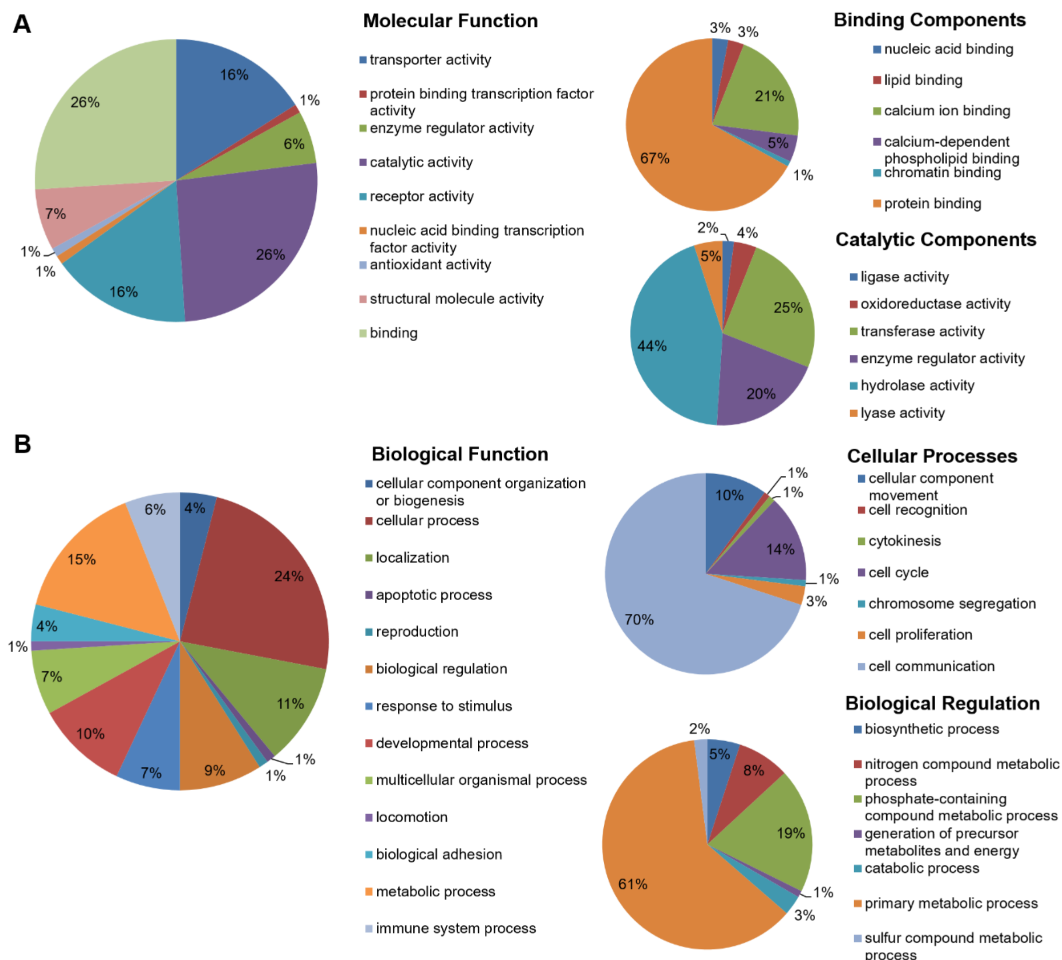

Figure 7. Distribution of candidate STn-expressing glycoproteins in muscle-invasive bladder tumours comprehensively integrated according to cellular localization (A), molecular (B) and cell functions (C) based on gene ontology analysis by Panther bioinformatics tool. STn-expressing proteins were found 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 9 main classes of molecular functions, with an overrepresentation of catalytic activities (Hydrolase, lysase and transferase activities) and protein biding 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.

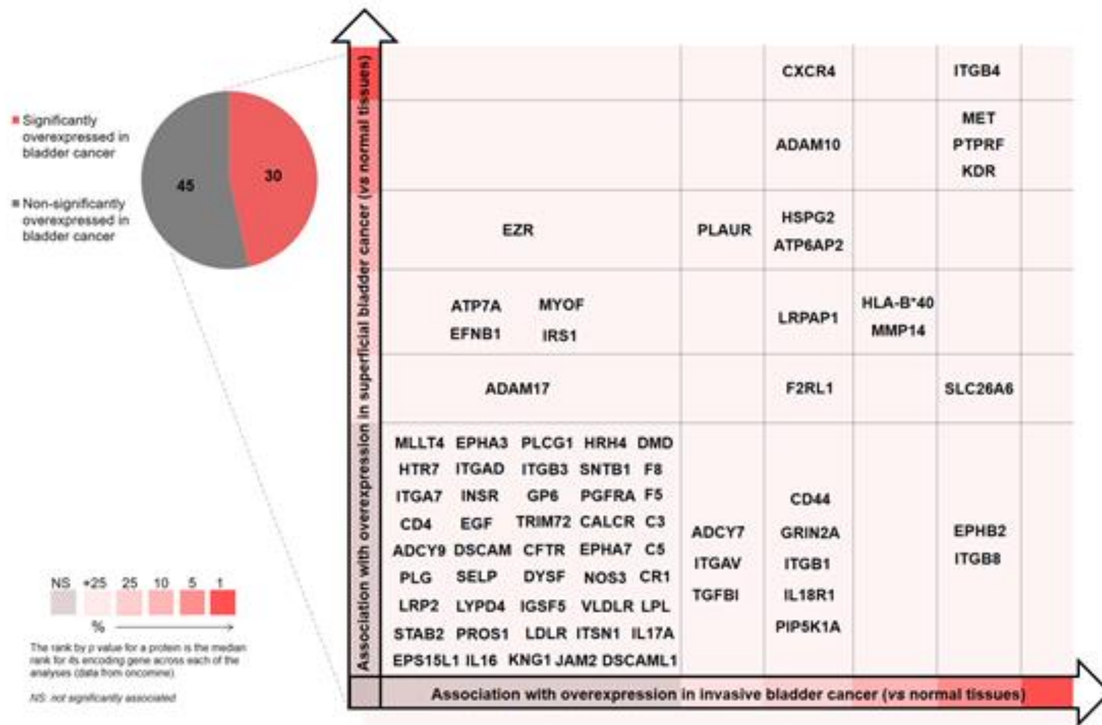

Figure 8. Candidate STn-expressing glycoproteins in muscle-invasive bladder tumours comprehensively distributed according to its association with the severity of the lesions. Briefly, the identified glycoproteins were distributed according to associations with the type of lesion based on an *in silico* 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.

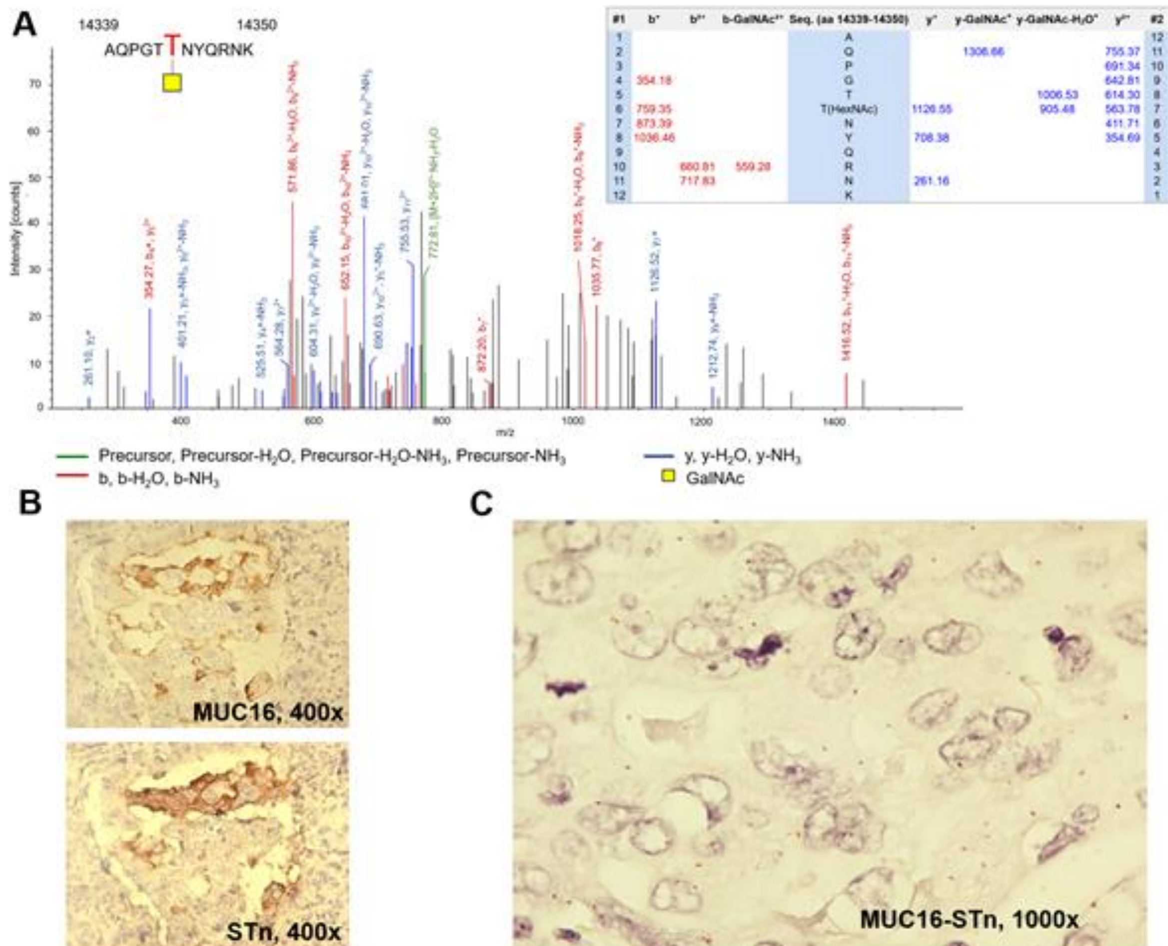

Figure 9. A) Exemplificative annotated nanoLC-ESI-LTQ-orbitrap-CID-MS/MS spectra for a MUC16 glycopeptide substituted with a HexNAc residue evidencing the specific glycosite; B) Co-localization of MUC16 and STn in bladder tumours by immunohistochemistry; C) Expression of MUC16 STn-glycoforms in bladder 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 co-localization of MUC16 and STn (B) in bladder tumours also reinforce this hypothesis. Finally, the red dots on the PLA image (C) in areas of co-localization, result from the simultaneous detection of both antigens, reinforcing this evidence.

4.3. Clinical significance of MUC16 expression in bladder cancer

Given the key role of MUC16 in ovarian cancer [119, 138], 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 tumours cells for the majority of the positive cases (Figure 9B), irrespectively 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% (figure 10A; $p<0.005$). Concerning WHO criteria, MUC16 positive cells were mostly observed in the high grade cases ($p=0.008$; figure 10B), 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 (Figure S7-Supporting Information, $p=0.005$). Moreover, we found that MUC16 expression associates with lower cancer specific survival (CSS) in MIBC patients treated with cisplatin and gemcitabine, suggesting a possible role in drug resistance that is being currently evaluated. These observations are in agreement with

findings from serological CA125 evaluation [138, 141] and strongly support the need for a deeper investigation on the biological and clinical significance of MUC16 in bladder cancer.

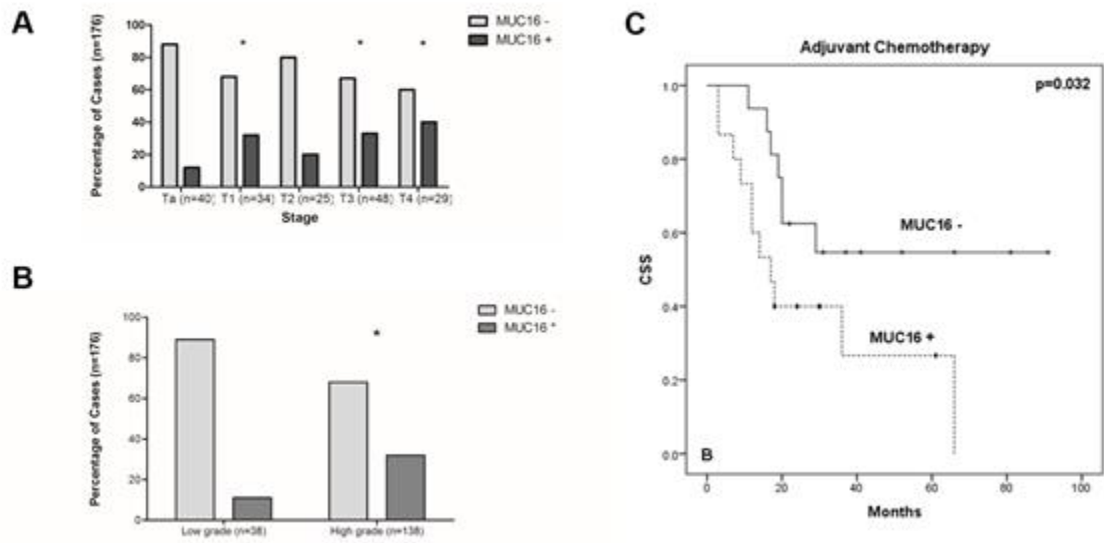

Figure 10. A) Associations of MUC16 with the stage and B) grade of the disease and C) decreased overall survival in MIBC patients subjected to cisplatin-based chemotherapy. Accordingly, MUC16 was associated with more aggressive bladder tumours, namely advanced stages and grade of the disease. Moreover, its presence in MIBC associates with decreased survival in MIBC submitted to chemotherapy.

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVE

CONCLUSIONS AND FUTURE PERSPECTIVE

It has been long known that advanced bladder tumours present significant alterations in glycosylation that relate with 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 future glycomics and glycoproteomics studies and to the design of 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 bladder cancer patients. 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. More importantly, we have highlighted the enormous potential of glycoproteomics as an essential tool in the context of precision oncology for the identification of patient subsets and of novel and highly specific therapeutic targets.

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APPENDIX

APPENDIX

A. Supplementary figures

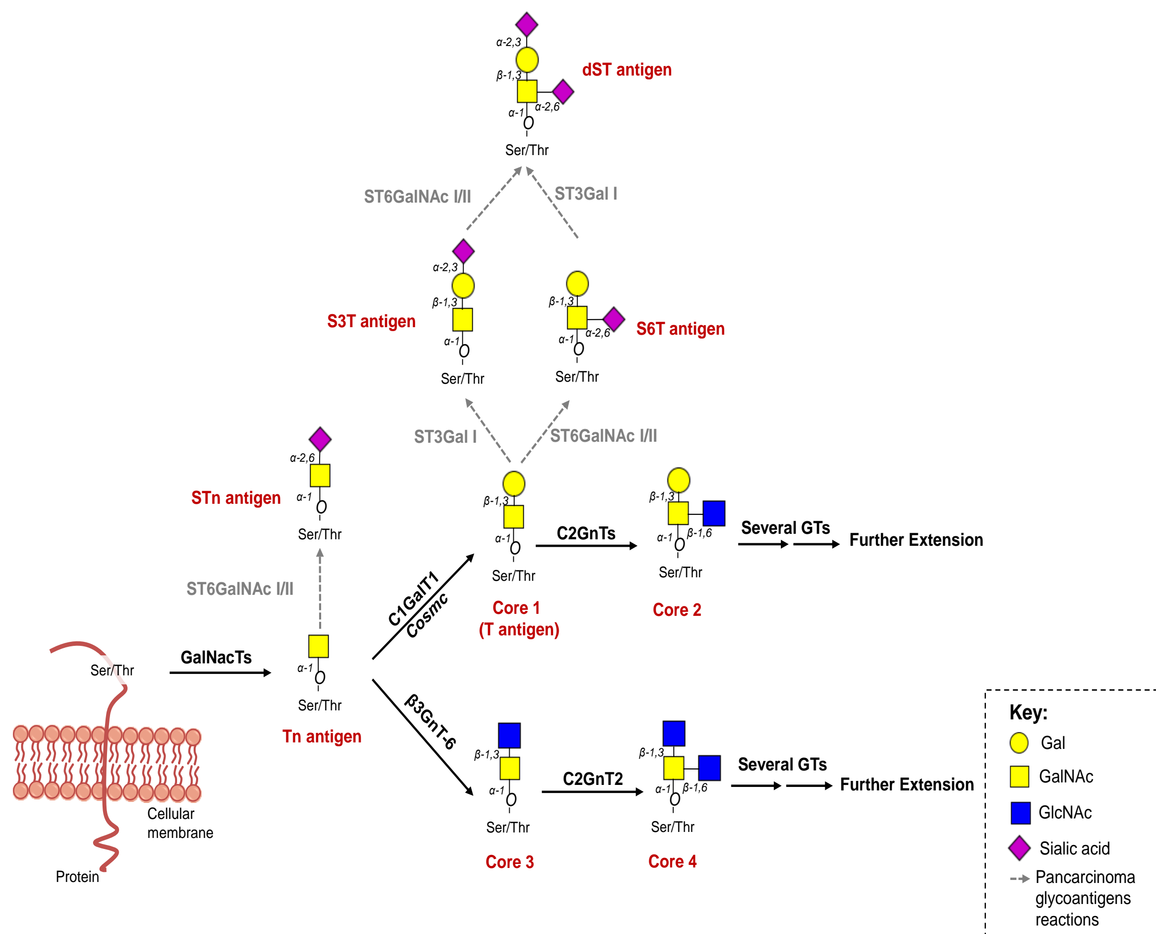


Figure S1. Schematic representation protein O-GalNAc glycosylation biosynthesis evidencing the cancer-associated short-chain glycans explored in this study. O-GalNAc glycosylation is a posttranslational modification commonly found in membrane glycoproteins extracellular domains and secreted glycoproteins. It can also be observed in secretory organelles inside the cell. This type of O-glycosylation plays a key role in the definition of protein conformation and key biological functions including: i) cell-cell and cell-extracellular matrix adhesion; ii) recognition by the immune system, pathogens and virus; iii)

protection of glycoproteins against proteolysis; iv) regulation of key intracellular signalling pathways through the modulation of cell receptors activities, amongst other roles. Glycoprotein *O*-GalNac glycosylation initiates in the endoplasmatic reticulum and its further elongated into more structurally complex structures in the golgi. Briefly, O-glycosylation begins with the addition of GalNAc to a serine or threonine residue of a given protein backbone originating the simplest form of O-glycosylation, the Tn antigen. The reaction is catalyzed by polypeptide N-Acetylgalactosamine transferases (ppGalNAcTs), a superfamily of 20 enzymes with high substrate and tissue specificity, whose expression is regulated at cellular and tissue levels. The coordinated action of these glycosyltransferases determines the density and distribution of glycosylation sites on a given glycoprotein. Frequently, β-(1-3)-galactosyltransferase, C1Gal-T1 and its molecular chaperone COSMC, elongates the Tn antigen by adding a Gal residue to the O-3 GalNAc residue. This originates the core 1 structure, also designated T antigens, which functions as precursor of more elongated core structures by coordinated action of several glycosyltransferases, as depicted in more detail in this Figure. Of note, mature O-glycans may present ABO and Lewis blood group related antigens that decisively contribute to the definition of the antigenic profile of a given cell. However, in cancer cells, early sialylation of Tn and T antigens form the sialyl-Tn (STn), and sialyl-T (ST) antigens, whose biosynthesis is highlighted in detail in the Figure. Accordingly, ST6GalNAcs may promote the O-6 sialylation of the Tn antigen but also T, originating S6T. On the other hand, ST3Gal.I promote the O-3 sialylation of the T antigen originating S3T. The coordinated action of these enzymes give rise to the disialylated T glycoforms. These event stop further glycan elongation and may dramatically change the conformational and functional properties of a given glycoprotein, favouring cell migration, immune escape and activation of key oncogenic pathways.

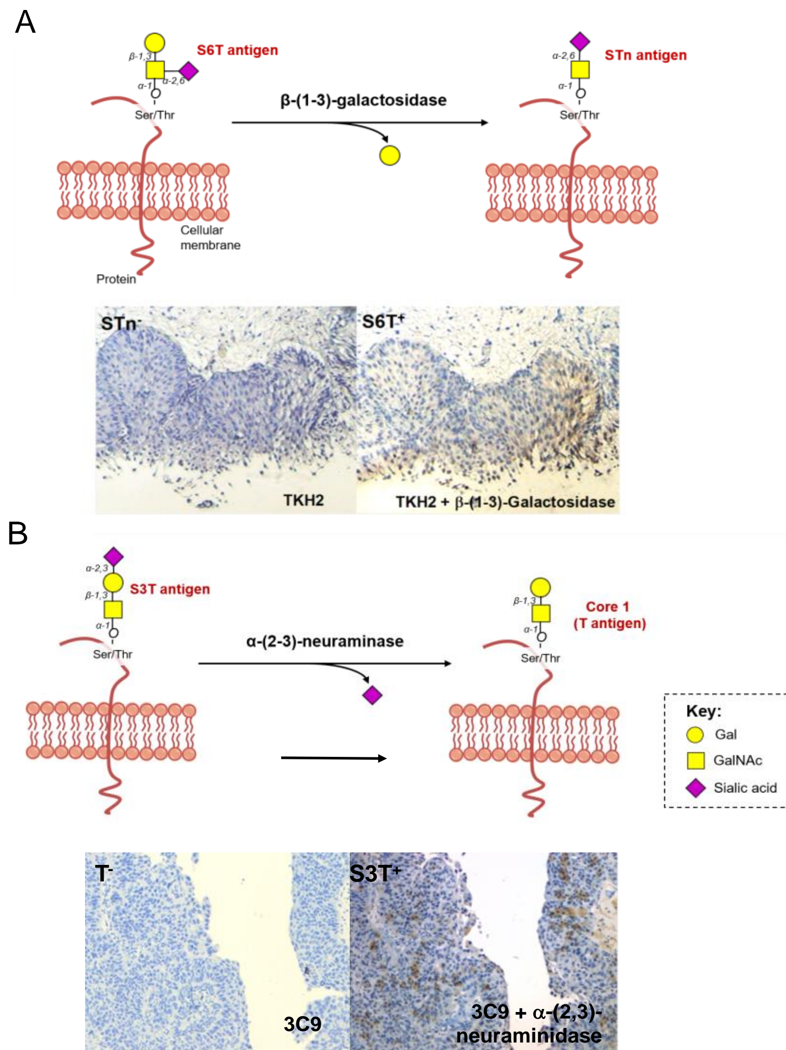
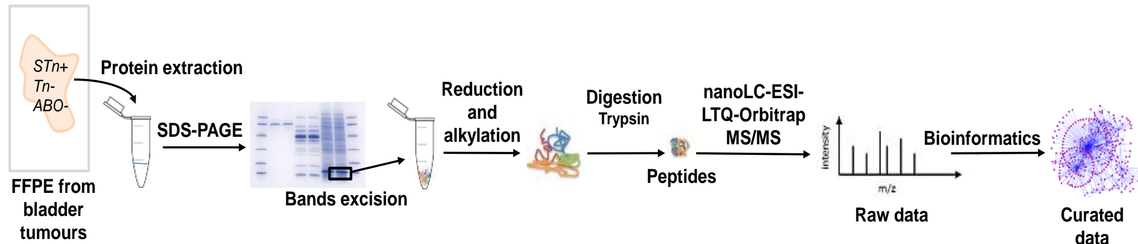


Figure S2. Schematic representation of the analytical strategy for S6T and S3T evaluation by immunohistochemistry. A) The S6T antigen may be considered structurally related with STn, since it presents an O-6 sialylation of its GalNAc residue. As such we have used a β-(1,3)-galactosidase to remove the Gal residue exposing an STn antigen for recognition by TKH2 monoclonal antibody. This allowed the detection of STn in previously negative STn tumours sections, strongly suggesting the expression of S6T. In addition, there was a significant increase in staining in several STn positive tumours after enzymatic digestion (not shown). B) The S3T antigen presents an O-3 Gal sialylation and after α-(2,3)-neuraminidase digestion exposes 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 tumours sections prior and after enzymatic digestion.

A. Tumour proteome analysis



B. Tumour *O*-glycoproteome analysis

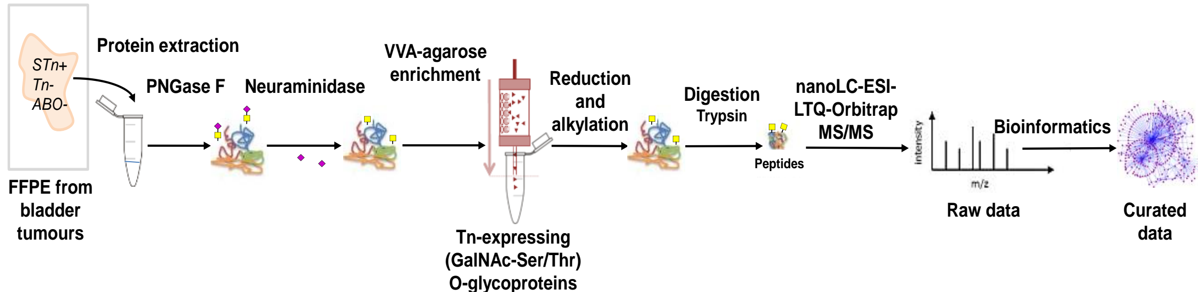


Figure S3. Analytical workflow for A) whole proteome analysis starting from FFPE tissues and B) identification of STn expressing glycoproteins in bladder tumours. A) **Analytical workflow to access the proteome.** Briefly, the proteins were extracted from FFPE muscle-invasive tumours isolated from male patients using the Qproteome FFPE tissue kit (Qiagen, Hilden, Germany) according to the vendor instructions. The proteins were pooled and then separated on 4-20% SDS-PAGE gels, excised, digested with trypsin and analyzed by nanoLC-ESI-LTQ-orbit-MS/MS. Data was comprehensively interpreted using Phanter. B) Analytical workflow used to access putative STn-expressing glycoproteins in a pool of proteins extracted from tumours of five male patients with muscle invasive bladder tumours. Glycoproteins modified with the STn antigen were identified by nanoLC-ESI-LTQ-obritrap-CID-MS/MS after □-neuraminidase digestion followed by enrichment for GalNAc-expressing protein glycoforms. Enrichment was done based on affinity for the VVA lectin, which selectively bindings glycans with terminal GalNAc residues. To decrease the possibility of false positives only Tn and blood group A negative tumours the mentioned structural feature were excluded from analysis. After enrichment the glycoproteins were reduced, alkylated, digested with PnGAse F for N-deglycosylation (removal of N-glycans that could interfere with the analysis) and finally trypsin to yield peptides for nanoLC-ESI-LTQ-orbitrap-CID-MS/MS (orbitrap MS) analysis. The final protein list included only membrane glycoproteins with putative *O*-glycosylation domains as determined by the NetOGlyc 4.0 software [ref] and/or exhibiting a glycopeptide with at least one HexNAc substituent.

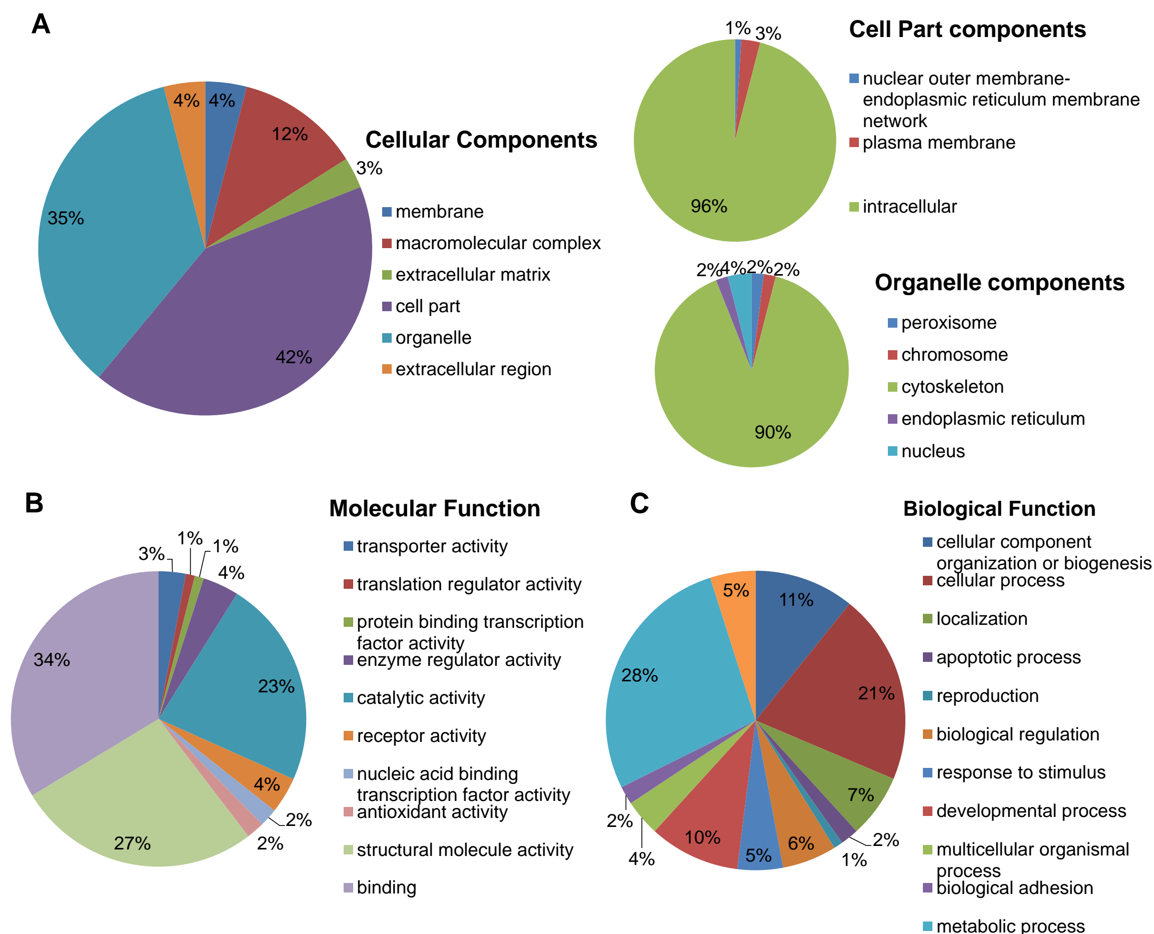


Figure S4. Distribution of proteins isolated from a pool of FFPE tissues of patients with muscle-invasive bladder tumours according to cellular localization (A), molecular (B) and cell functions (C) based on gene ontology analysis by Panther software[ref]. Briefly, SDS-PAGE-nanoLC-ESI-MS/MS analysis of a pool of advanced bladder tumours from male patients identified 294 of the most abundant proteins. Gene ontology analysis using Panther highlighted the presence of proteins from all cell compartments, mostly cytoplasmatic and cytoskeleton proteins; nevertheless, low percentages of plasma membrane proteins (4%) and extracellular matrix (3%) could also be observed. The main represented molecular functions included binding, structural and catalytic activities, whereas main molecular functions were set on metabolic and cellular processes. Nevertheless, a wide array of proteins from different cellular components, involved in different molecular and biological functions could be identified. This demonstrates the possibility of performing proteomics-based analysis in proteins recovered from challenging matrixes such as FFPE tissues.

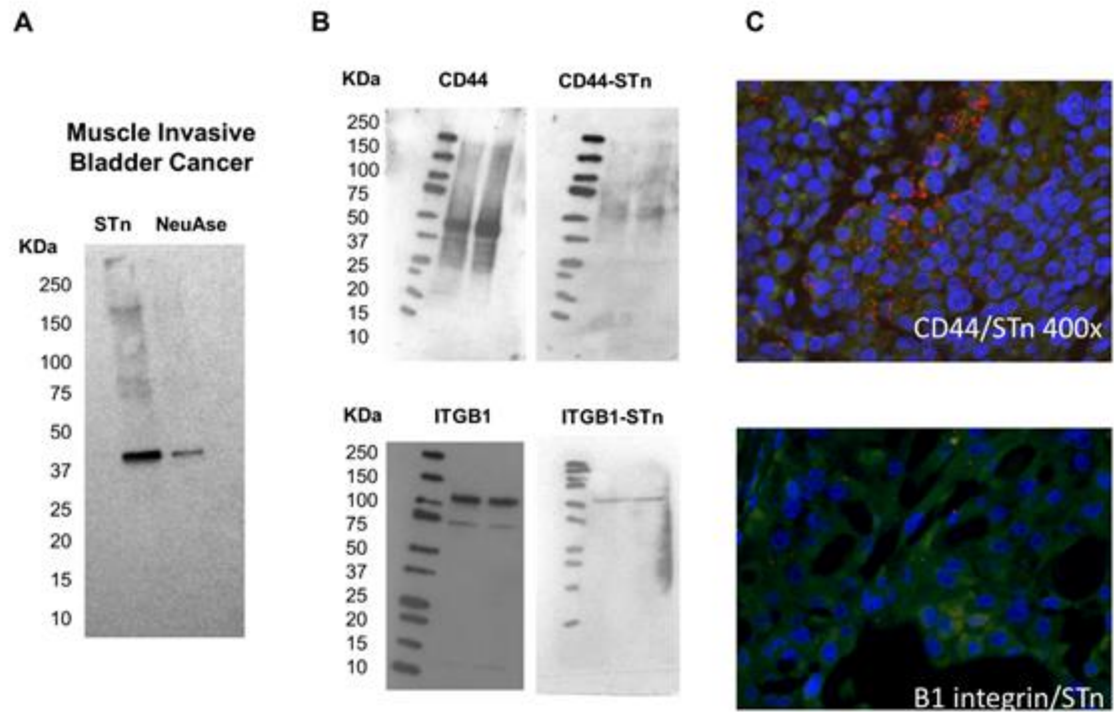
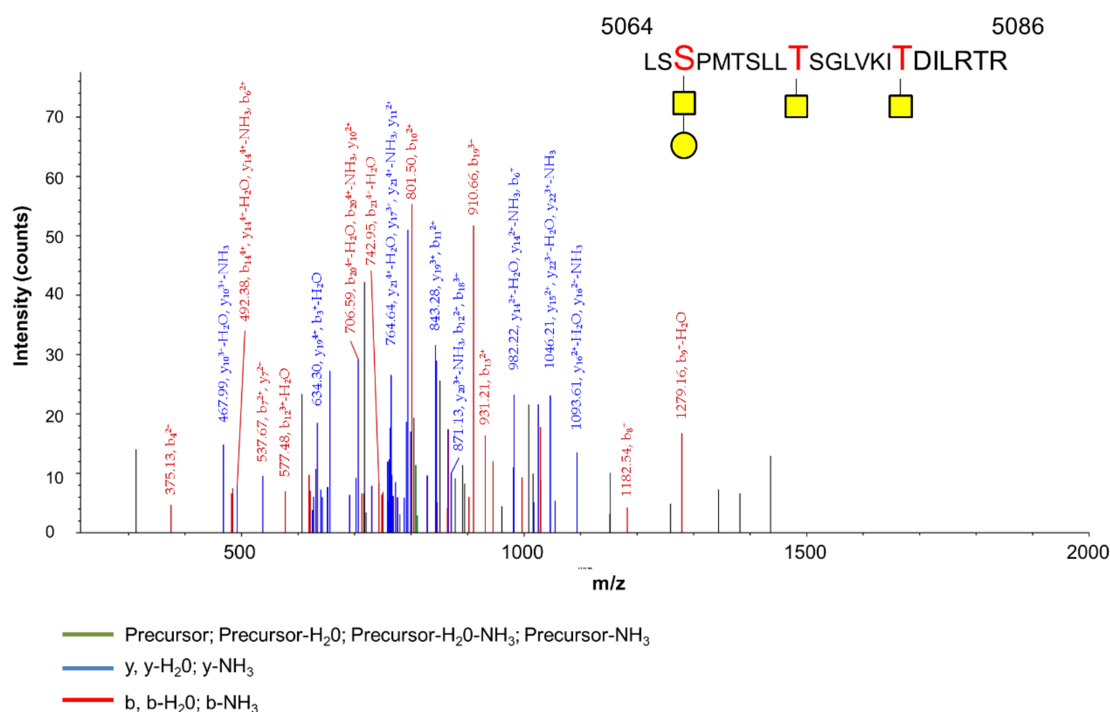


Figure S5. A) Glycoproteins modified with the STn antigen in advanced bladder tumours. All studied tumours (n=5) presented a western blot pattern similar to the one presented. This that was sensitive to neuraminidase treatment confirming the specificity of the signal. **B) Identification of STn glycoforms in CD44 and ITGB1 glycoproteins isolated from advanced bladder tumours by immunoprecipitation.** CD44 and ITGB1 were immunoprecipitated from protein extracts using antibody-immobilized agarose beads and blotted for CD44 and ITGB1 and STn thereafter. This was a neuramidase-sensitive signal (data not shown), confirming the presence of the antigen. Of note, the smear in CD44 blots results from the existence of several splice variants as well as glycoforms of this glycoprotein. Alltogther these findings confirm that CD44 and ITGB1 are substituted with the STn antigen in advanced bladder tumours, in aggrement with glycoproteomics analysis. **C) PLA for CD44 and ITGB1 and STn in tumours positive for both the glycoprotein and the glycan.** The red dots in the Figures are generated from positive PLA for CD44-STn and ITGB1-STn, confirming western blot analysis.



#1	b^{+}	b^{2+}	b^{3+}	b^{4+}	AA Seq. (5064-5066)	y^{+}	y^{2+}	y^{3+}	y^{4+}	#2
1					L					23
2					S			1054.22	790.92	22
3	653.29				S-HexHexNAc			1025.21	769.16	21
4	750.34	375.67			P				656.16	20
5					M			842.13	631.85	19
6	982.43	491.72			T			798.45		18
7		535.23			S			764.77		17
8	1182.54				L					16
9					L		1046.59			15
10		800.38			T-HexNAc					14
11		843.89			S					13
12		872.41			G		794.48			12
13		928.95	619.64		L		765.96			11
14			652.66	489.75	V		709.42			10
15					K					9
16			733.05		I					8
17				626.07	T-HexNAc		539.29			7
18			872.77	654.83	D	773.46				6
19			910.46		I	658.44				5
20				711.37	L					4
21				750.39	R					3
22				775.66	T					2
23					R					1

Figure 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 bellow).

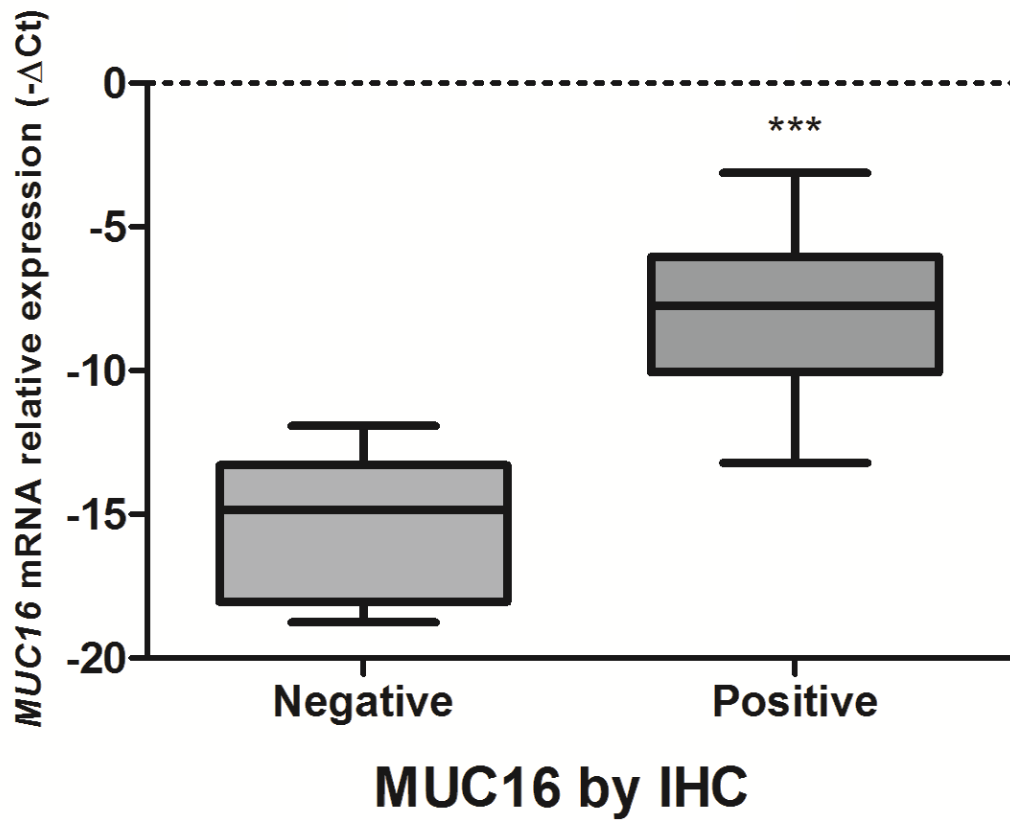


Figure S7. Association between MUC16 classification by immunohistochemistry in FFPE cancer tissues (IHC; negative vs positive) and *MUC16* expression. The graph clearly demonstrates an overexpression of *MUC16* in tumours considered positive for this glycoprotein.

B. Scientific Articles

Exploring increased sialylation by targeted *O*-glycoproteomics identifies MUC16 as a poor prognosis biomarker in advanced stage bladder tumours

Sofia Cotton[1*], Rita Azevedo[1,2*], Cristiana Gaiteiro[1], Dylan, Ferreira[1], Luís Lima[1,3], Andreia Peixoto[1,2,3], Elisabete Fernandes[1,2,3], Manuel Neves[1], Diogo Neves[1], Teresina Amaro[4], Ricardo Cruz[5], Ana Tavares[1,6], Maria Rangel[7], André M. N. Silva[8], Lúcio Lara Santos[1,9,10] and José Alexandre Ferreira[1,2,3,11] *Equal contribution

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Keywords: bladder cancer; glycosylation; sialic acids; precision medicine; glycoproteomics; MUC16

Running title: Targeted *O*-glycoproteomics in bladder cancer

Hypoxia enhances the malignant nature of bladder cancer cells and concomitantly antagonizes protein O-glycosylation extension

Andreia Peixoto1,2,3,4,*, Elisabete Fernandes1,3,4,5,*, Cristiana Gaiteiro1,*, Luís Lima1,3,6, Rita Azevedo1,4, Janine Soares1, Sofia Cotton1, Beatriz Parreira1, Manuel Neves1,4, Teresina Amaro7, Ana Tavares1,7, Filipe Teixeira8, Carlos Palmeira1,9, Maria Rangel10, André M. N. Silva11, Celso A. Reis3,4,6,12, Lúcio Lara Santos1,9,13, Maria José Oliveira2,3, José Alexandre Ferreira1,3,4,6,14 1

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Keywords: glycosylation, bladder cancer, hypoxia, invasion, sialyl-Tn

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C. Congresses and Workshops

CONTROVÉRSIAS
E DESENVOLVIMENTO EM
CANCRO GÁSTRICO
CERTIFICADO
Sofia Cotton
Participou na Reunião
CONTROVÉRSIAS E DESENVOLVIMENTO EM CANCRO GÁSTRICO,
que decorreu no IPO-PORTO, no dia 30 de Setembro de 2015.
Prof. Dr. Lúcio Lara Santos
Coordenador