UNIVERSIDADE NOVA DE LISBOA
FACULDADE DE CIÊNCIAS MÉDICAS

ALMA MATER STUDIORUM UNIVERSITÀ DI BOLOGNA
DIPARTIMENTO DI MEDICINA SPECIALISTICA,
DIAGNOSTICA E SPERIMENTALE

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# THOMSEN-FRIEDENREICH ANTIGENS IN BLADDER CANCER: EVALUATION OF THEIR PROGNOSTIC VALUE

**PAULO FILIPE SEVERINO**
**Thesis submitted for the Degree of Doctor in Life Sciences**
**in the Specialty of Immunology**
**in the Faculdade de Ciências Médicas**
**and for the Degree of Doctor in Oncology and Experimental Pathology**
**in the Specialty of Experimental Pathology**
**in the Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale**

**October 2014**

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*à minha Família*
*e a Valentina Aversa*

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David Mitchell, *Cloud Atlas*

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# Antigénios Thomsen-Friedenreich em cancro de bexiga: avaliação do seu valor prognóstico


## Resumo

**Introdução.** O cancro de bexiga é uma patologia comum que representa o 6° e o 5° cancro mais incidente em Portugal e na Itália, respetivamente. Em mais de metade dos casos ocorre reincidência durante o primeiro ano, requerendo acompanhamento clínico ao longo da vida. A instilação intravesical de Bacillus Calmette-Guérin (BCG) (uma estirpe atenuada do *Mycobacterium bovis*) representa uma imunoterapia eficaz no combate ao cancro de bexiga, no entanto, muitos aspetos da interação de BCG com as células tumorais bem como com as células do sistema imunitário permanecem por desvendar. As células tumorais de bexiga expressam frequentemente as formas sialiladas dos antigénios de Thomsen-Friedenreich (TF), *i.e.*, sialil-T (sT) e sialil-Tn (sTn). Contudo ainda se desconhece o significado da sua expressão na malignidade tumoral e se afeta a eficácia da terapêutica BCG. **Objetivo do estudo.** Investigar o papel dos antigénios sT e sTn no fenótipo maligno de células de cancro de bexiga bem como na resposta mediada pelo sistema imunitário à terapia com BCG. **Metodologia.** Para tal, foram utilizadas as linhas celulares de cancro da bexiga HT1376 e MCR, geneticamente modificadas por transdução com vetores codificantes para as sialiltransferases *ST3GAL1* ou *ST6GALNAC1*, de forma a expressar homogeneamente os antigénios sT ou sTn respetivamente. Estes modelos celulares foram estudados após confronto com BCG. O nível de BCG internalizado foi avaliado por citometria de fluxo. O perfil global de expressão genética dos modelos celulares antes e após incubação com BCG foi analisado pela tecnologia de *microarray*. O perfil de citocinas secretadas pelos modelos celulares após incubação com BCG, bem como de macrófagos estimulados pelo secretoma de células de cancro de bexiga que por sua vez foram estimuladas previamente por BCG, foi estudado pelo sistema *multiplex* de “imuno-esferas”. **Resultados.** A análise do transcritoma dos modelos celulares revelou que grupos de genes envolvidos em funções específicas foram modulados em paralelo nos dois modelos celulares, após transdução, independentemente da sialiltransferase expressa. Ou seja, em células que expressavam a sialiltransferase *ST3GAL1* ou *ST6GALNAC1*, os genes envolvidos na regulação da

segregação cromossómica e na reparação do DNA foram consistentemente regulados negativamente. Genes descritos na literatura como marcadores para o cancro de bexiga foram também modulados. A incubação com BCG resultou numa tendência ao aumento da expressão de genes relevantes na preservação e estabilidade genómica e menor malignidade, no entanto, apenas em células que expressavam sT ou sTn. Entre as dez citocinas testadas, apenas a IL-6 e IL-8 foram expressas pelas linhas celulares de cancro da bexiga, com indução destas após estimulação com BCG, e principalmente em células que expressavam *ST3GAL1* ou *ST6GALNAC1*. Em macrófagos, citocinas inflamatórias, tais como IL-1β, IL-6 e TNFα, e a citocina anti-inflamatória IL-10, foram induzidas apenas pelo secretoma de células de cancro da bexiga confrontadas com BCG, com maior relevância quando estas expressavam *ST3GAL1* ou *ST6GALNAC1*, prevendo a estimulação de macrófagos semelhantes aos de tipo M1 e uma melhor resposta à terapia com BCG. **Conclusões.** O efeito geral da expressão destas sialiltransferases e dos produtos enzimáticos sT ou sTn nas células de cancro de bexiga conduz a um fenótipo de maior malignidade. Contudo, a maior avidez de estas na produção de citocinas inflamatórias após confronto com BCG, bem como a maior capacidade de estimulação de macrófagos, predirá uma resposta à terapia com BCG mais eficaz em tumores que expressem os antigénios de TF sialilados. Tais conclusões são totalmente concordantes com os nossos mais recentes dados clínicos obtidos em colaboração, que mostram que em doentes com cancro de bexiga que expressam sTn respondem melhor a terapia BCG.

# Antigeni Thomsen-Friedenreich nel cancro della vescica: valutazione del valore prognostico

## Riassunto

**Introduzione.** Il cancro della vescica è una patologia comune che rappresenta la 6ª e la 5ª neoplasia a maggiore incidenza in Portogallo ed in Italia, rispettivamente. Più della metà dei casi presentano una recidiva entro un anno, richiedendo quindi il monitoraggio a vita. L'instillazione intravescicale di Bacillus Calmette Guerin (BCG) (un ceppo attenuato di *Mycobacterium bovis*) rappresenta una immunoterapia efficace contro il cancro alla vescica, ma molti aspetti dell'interazione del BCG con le cellule tumorali e con le cellule del sistema immunitario sono ancora da comprendere. Le cellule tumorali di vescica spesso esprimono le forme sialilate degli antigeni Thomsen-Friedenreich (TF), *i.e.*, sialil-T (sT) e sialil-Tn (sTn). Però il significato biologico della loro espressione e nel determinare l'efficacia terapeutica del BCG è ancora sconosciuto. **Obiettivo dello studio.** Studiare il ruolo degli antigeni sT e sTn nel determinare il fenotipo maligno delle cellule tumorali della vescica e la risposta immunitaria alla terapia con BCG. **Metodologia.** Sono state utilizzate le linee cellulari di cancro alla vescica HT1376 e MCR, geneticamente modificate mediante trasduzione genica con vettori codificanti le sialiltrasferasi *ST3GAL1* o *ST6GALNAC1*, allo scopo di ottenere l'espressione omogenea degli antigeni sT o sTn. Questi modelli cellulari sono stati studiati dopo l'incubazione col BCG. Il livello di internalizzazione di BCG è stato valutato mediante citometria a flusso. Il profilo di espressione genica delle linee cellulari prima e dopo incubazione col BCG è stato studiato mediante tecnologia *microarray*. Mediante un sistema immunobiglie multiplex, è stato possibile studiare simultaneamente l'espressione di numerose citochine secrete o dalle cellule di cancro della vescica dopo incubazione con BCG e o dai macrofagi stimulati dal secretoma delle cellule tumorali di vescica dopo incubazione col BCG, . **Risultati.** Le analisi del trascrittoma delle linee cellulari hanno rivelato che vi erano gruppi di geni accomunati dal fatto di svolgere una analoga funzione, che venivano regolati in parallelo nelle due linee cellulari dopo trasduzione con l'una o l'altra sialiltrasferasi. In particolare, in cellule che esprimevano *ST3GAL1* o *ST6GALNAC1*, molti geni coinvolti nella corretta segregazione cromosomica

e nella riparazione del DNA erano costantemente sottoespressi. Inoltre, geni descritti in letteratura come marcatori del cancro della vescica venivano modulati nelle cellule trasdotte con l'una o l'altra sialiltrasferasi. L'incubazione col BCG induceva una tendenza alla sovraespressione di geni coinvolti nel mantenimento della stabilità genomica e nella riduzione della malignità, ma soltanto in cellule che esprimevano sT o sTn. Tra le dieci citochine analizzate, solo IL-6 e IL-8 erano secrete dalle linee cellulari. Il contatto con BCG ne stimolava la secrezione, in particolare nelle cellule che esprimevano *ST3GAL1* o *ST6GALNAC1*. Nei macrofagi, la secrezione delle citochine infiammatorie IL-1β, IL-6 e TNFα e della citochina antinfiammatoria IL-10 veniva indotta solo se il secretoma di cellule di cancro della vescica proveniva da cellule trattate con BCG, in particolare quando le cellule esprimevano gli antigeni sT o sTn, prevedendo la stimolazione dei macrofagi simili ai macrofagi di tipo M1 e una migliore risposta alla terapia con BCG. **Conclusioni.** L'espressione delle due sialiltrasferasi e dei loro prodotti, gli antigeni sT o sTn, nelle cellule tumorali della vescica sembra indurre un fenotipo più maligno. Tuttavia, la maggiore capacità delle cellule tumorali che esprimono queste sialiltrasferasi di secernere delle citochine infiammatorie dopo contatto con BCG, così come la loro maggiore capacità di stimolare i macrofagi, fa prevedere che la risposta al BCG sia più efficace nei tumori che esprimono questi antigeni TF sialilati. Ciò è perfettamente in accordo con i nostri più recenti dati clinici ottenuti in collaborazione, che dimonstrano che i pazienti con cancro della vescica che esprimono sTn rispondono meglio alla terapia con BCG.

# Thomsen-Friedenreich antigens in bladder cancer: evaluation of their prognostic value

## Abstract

**Background.** Bladder cancer is a common malignancy representing the 6th and the 5th most incident cancer in Portugal and in Italy, respectively. More than half of the cases relapse within one year, requiring though a lifelong follow-up. Intravesical instillation of Bacillus Calmette-Guérin (BCG) (an attenuated strain of *Mycobacterium bovis*) represents an effective immunotherapy of bladder cancer, although many aspects of the interaction of BCG with cancer cells and host immune cells remain obscure. Bladder cancer cells often express the sialylated forms of the Thomsen-Friedenreich (TF), *i.e.*, sialil-T (sT) e sialil-Tn (sTn). However, it's still unknown the sense of such expression in tumour malignancy and in the BCG therapy efficacy. **Aim of the study.** To investigate the role of the sT and sTn antigens on the malignant phenotype of bladder cancer cells and the immune mediated response to BCG therapy. **Experimental**. We have utilized populations of the bladder cancer cell lines HT1376 and MCR, genetically modified by transduction with the sialyltransferases *ST3GAL1* or *ST6GALNAC1* to express homogeneously sT or sTn antigens. The level of BCG internalized was assessed by flow cytometry. The whole gene expression profile of BCG-challenged or unchallenged bladder cancer cell lines was studied by microarray technology. The profile of cytokines secreted by BCG-challenged bladder cancer cells and that of macrophages challenged by the secretome of BCG-challenged bladder cancer cells was studied by multiplex immune-beads assay. **Results**. Transcriptome analysis of the sialyltransferase-transduced cells revealed that groups of genes involved in specific functions were regulated in parallel in the two cell lines, regardless the sialyltransferase expressed. Namely, in sialyltransferase-expressing cells, genes involved in the proper chromosomal segregation and in the DNA repair were consistently down-regulated, while genes reported in literature as markers for bladder cancer were modulated. BCG-challenging induced a tendency to up-regulation of the genes preserving genomic stability and reducing malignancy, but only in cells expressing either sT or sTn. Among the ten cytokines tested, only IL-6 and IL-8 were expressed by bladder cancer cell

lines and up-regulated by BCG-challenging, mainly in sialyltransferases-expressing cells. In macrophages, inflammatory cytokines, such as IL-1β, IL-6 and TNFα, and the antinflammatory IL-10 were induced only by the secretome of BCG-challenged bladder cancer cells, particularly when expressing either sialyltransferase, predicting the stimulation of M1-like macrophages and a better response to BCG therapy. **Conclusions.** The general effect of the expression of the two sialyltransferases and their products in the bladder cancer cells is toward a more malignant phenotype. However, the stronger ability of sialyltransferase expressing cells to produce inflammatory cytokines upon BCG-challenging and to stimulate macrophages predicts a more effective response to BCG in tumours expressing the sialylated TF antigens. This is fully consistent with our recent clinical data obtained in collaboration, showing that patients with bladder cancer expressing sTn respond better to BCG therapy.

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### Chapter III

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## Supplement I

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# List of Abbreviations

| | |
|---|---|
| BC | Bladder cancer |
| BCG | Bacillus Calmette-Guérin (an attenuated strain of *Mycobacterium bovis*) |
| C1GALT1 | Glycoprotein-*N*-acetylgalactosamine 3-beta-galactosyltransferase 1 (or core 1 synthase) |
| C1GALT1C1 | C1GALT1-specific chaperone 1 (or Cosmc) |
| CD4 | Cluster of differentiation 4 molecule |
| CD8 | Cluster of differentiation 8 molecule |
| Fuc | Fucose |
| Gal | Galactose |
| GalNAc | *N*-acetylgalactosamine |
| Glc | Glucose |
| GlcNAc | *N*-acetylglucosamine |
| GU | Genomic unstable bladder cancer molecular subtype |
| HT1376 | Bladder cancer cell line isolated from a 58 year old Caucasian woman with invasive TCC |
| $HT1376_{NC}$ | HT1376 cell line mock-transduced with the virapower lentiviral expression system |
| $HT1376_{ST3G1}$ | HT1376 cell line *ST3GAL1*-transduced with the virapower lentiviral expression system (heterogeneously expressing sT) |
| $HT1376_{sT}$ | HT1376 cell line *ST3GAL1*-transduced with the virapower lentiviral expression system (homogeneously expressing sT) |
| IL-1β/6/8/10 | Interleukin 1 beta/ 6 / 8 / 10 |
| INFγ | Interferon gamma |
| MAA | *Maackia amurensis* lectin |
| MCR | Bladder cancer cell line isolated from a subcutaneous metastatic lesion of a 51 year old male diagnosed with invasive TCC |
| $MCR_{NC}$ | MCR cell line mock-transduced with the virapower lentiviral expression system |
| $MCR_{ST6GN1}$ | MCR cell line *ST6GALNAC1*-transduced with the virapower lentiviral expression system (heterogeneously expressing sTn) |
| $MCR_{sTn}$ | MCR cell line *ST6GALNAC1*-transduced with the virapower lentiviral expression system (homogeneously expressing sTn) |
| MHC | Major histocompatibility complex |
| MUC | Mucin |
| NMIBC | Non-muscle invasive bladder cancer |
| PNA | Peanut lectin |
| s6T | Galβ1,3(Siaα2,6)GalNAc TF related antigen |
| SCCL | Squamous cell cancer-like bladder cancer molecular subtype |
| Sia | Sialic acid |
| SNA | *Sambucus nigra* lectin |
| sT | Sialyl-T antigen |
| ST3GAL1 | ST3 beta-galactoside alpha-2,3-sialyltransferase 1 |
| ST6GALNAC1 | ST6 (alpha-*N*-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 1 |
| sTn | Sialyl-Tn antigen |
| T | Galα1,3GalNAc-*O*-Ser/Thr TF related antigen (or core 1 structure) |
| TCC | Transitional cell carcinoma |
| TF | Thomsen Friedenreich antigens |
| Tis | Bladder carcinoma *in situ* |
| Tn | GalNAc-*O*-Ser/Thr TF related antigen |
| TNFα | Tumour necrosis factor |
| TUR | Transurethral resection |
| Uro A | Urobasal A bladder cancer molecular subtype |
| Uro B | Urobasal B bladder cancer molecular subtype |

# CHAPTER I

## *Introduction*

# *I.1 Bladder Cancer*

## Epidemiology

Bladder cancer (BC) is a common malignancy in the world (Figure 1.1a), presenting the 8th highest incidence in 2008, with more than 10/100 000 new cases. Particularly high incidence rates are observed in Southern and Western Europe (Figure 1.1b) with more than 20/100 000 new cases. In Portugal (Figure 1.1c) and in Italy (Figure 1.1d), bladder cancer represents the 6th and the 5th most incident cancer (Ferlay, Shin *et al*. 2010).

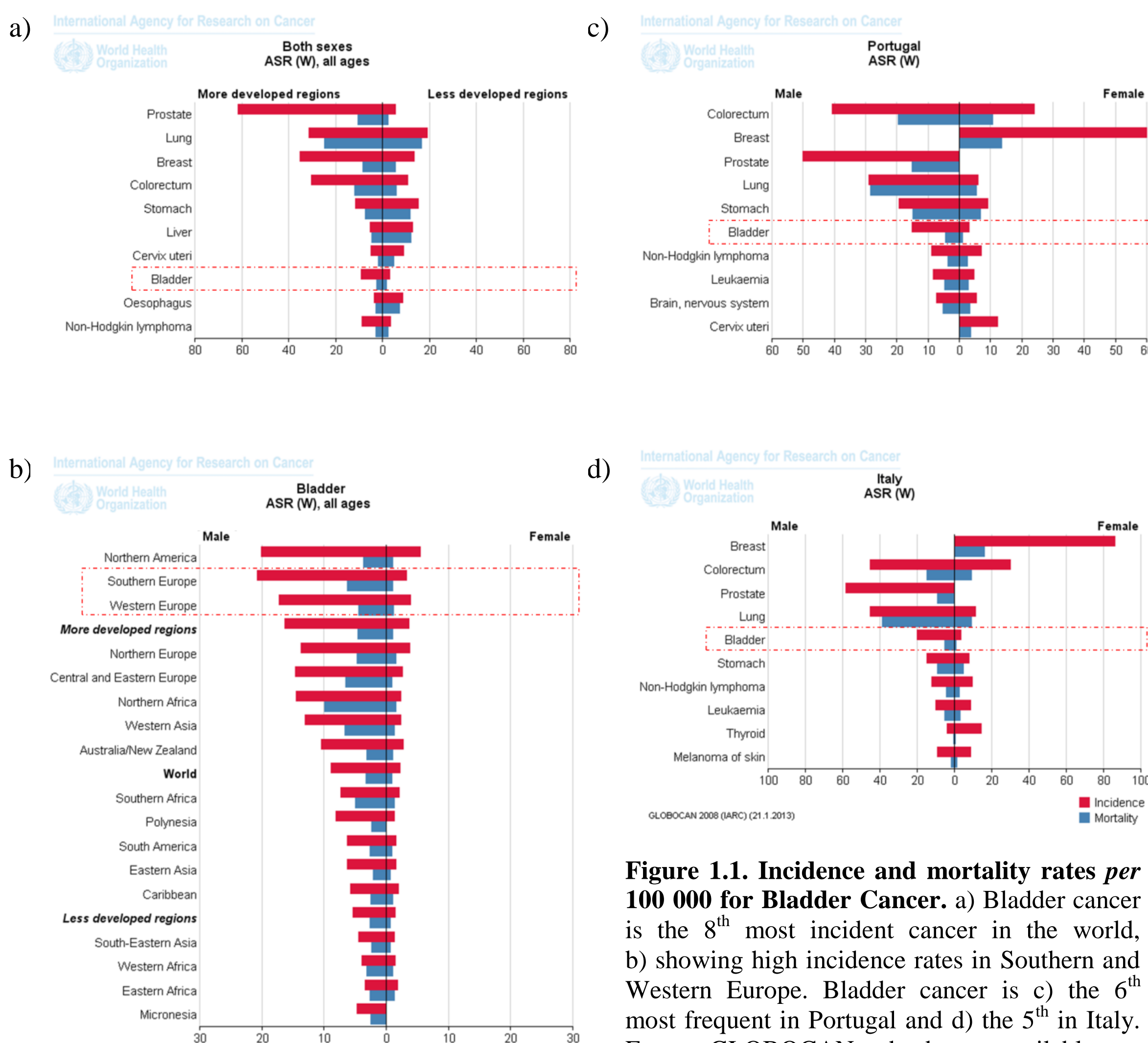



**Figure 1.1. Incidence and mortality rates *per* 100 000 for Bladder Cancer.** a) Bladder cancer is the 8th most incident cancer in the world, b) showing high incidence rates in Southern and Western Europe. Bladder cancer is c) the 6th most frequent in Portugal and d) the 5th in Italy. From GLOBOCAN database available at http://globocan.iarc.fr (Ferlay, Shin *et al*. 2010).

Women have approximately a 4-fold lower incidence and mortality rate for bladder cancer (Fajkovic, Halpern *et al*. 2011). A decreasing trend in mortality has been evident in most European countries over the last decades (Ferlay, Randi *et al*. 2008). Still, bladder cancer displays the highest recurrence rates among solid tumours with a significant percentage of progression to muscle invasion (Jacobs, Lee *et al*. 2010). More than half of the cases relapse within one year, requiring though a lifelong follow-up (Babjuk, Oosterlinck *et al*. 2011). Bladder cancer shows also the highest prevalence rates, representing the 5$^{th}$ and 4$^{th}$ most prevalent cancer in Portugal (Figure 1.2a) and in Italy (Figure 1.2b) respectively (Bray, Ren *et al*. 2012).

a) b)

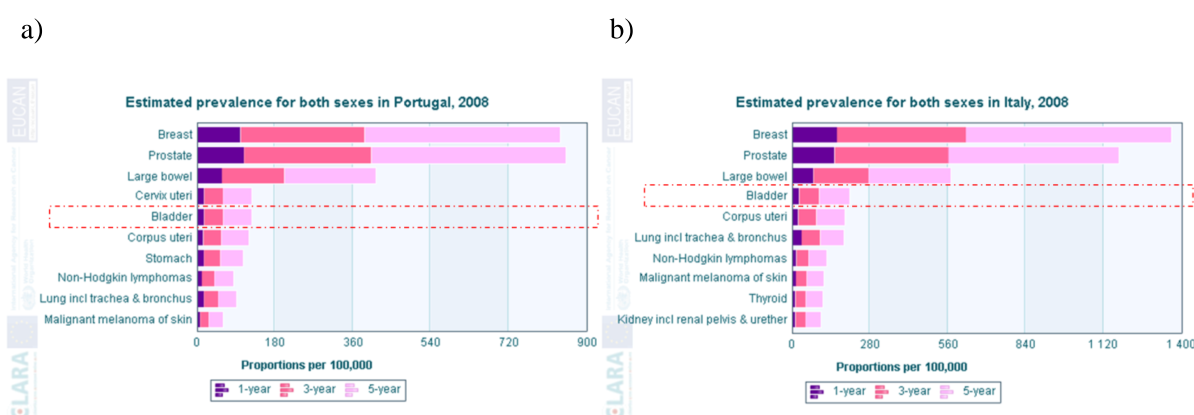



**Figure 1.2. Prevalence rates *per* 100 000 of the ten most prevalent cancers in a) Portugal, and b) in Italy.** Bladder cancer is the 5$^{th}$ most prevalent cancer in Portugal and the 4$^{th}$ In Italy. From EUCAN database available at http://eco.iarc.fr/ (Bray, Ren *et al*. 2012).

## Aetiology and risk factors

There are multiple risk factors associated with the development and progression of bladder cancer. Tobacco smoking is the principal risk factor (relative risk of about 4, according to a follow-up occurring between 1995 and 2006), being responsible for approximately 50% of death caused by bladder cancer (Freedman, Silverman *et al*. 2011). The incidence of bladder cancer is directly related to the lifetime tobacco use and the number of cigarettes smoked per day (Brennan, Bogillot *et al*. 2000). A recent study showed that bladder cancer in smokers displayed a younger age of diagnosis, larger tumours, a higher tumour stage and a higher grade than in never smokers (van Roekel, Cheng *et al*. 2013). The precise mechanism by which cigarette smoking causes bladder cancer has yet to be determined (Zeegers, Kellen *et al*. 2004). It seems most likely that

the risk of this cancer is related to the large number of chemicals present in smoke, such as 4-aminobiphenyl, polycyclic aromatic hydrocarbons (PAH), *N*-nitroso compounds, heterocyclic amines, arylamines and various epoxides agents (Zeegers, Tan *et al*. 2000). Variability in susceptibility for chemically induced urinary bladder cancer is due to the individual genetic background (Roos 2008). The initial step in chemically induced bladder carcinogenesis formation of reactive and DNA-affecting metabolites from bladder procarcinogens occurs via the catalytic activity of cytochromes P450 (CYP). For instances, cigarette smoking leads to an increase in exfoliated urothelial cells expressing *CYP1A1* cytochrome which catalyses the formation of reactive metabolites from PAH (Dorrenhaus, Muller *et al*. 2007). The induction of *CYP1A1* can be regarded as an indicator of cancer initiation. Moreover, Roos suggested that some individuals with different CYP alleles background may present different susceptibility to bladder carcinogenesis (Roos 2008). A clear polymorphism-dependent effect on bladder cancer susceptibility is known for two phase II enzymes though, namely *N*-acetyltransferase 2 (*NAT2*), which is involved in arylamine metabolism (Golka, Prior *et al*. 2002) and glutathion *S*-transferase M1 (*GSTM1*) (Dong, Potter *et al*. 2008). In addition, bladder cancer was the first cancer to be related to certain occupations. An increased risk for bladder cancer was observed among workers in the industries of dye, printing, rubber, transportation and in the electrical/gas/sanitary services. Some compounds related to professional exposure, such as *o*-toluidine, aniline, nitrobenzene, 4,4'-methylenebis(2-chloroaniline) and the aromatic amines 2-naphthylamine, 4-aminobiphenyl and benzidine (Samanic, Kogevinas *et al*. 2008, Sorahan 2008, de Vocht, Sobala *et al*. 2009, Talaska, Gaultney *et al*. 2012, Burger, Catto *et al*. 2013) are responsible for the majority of occupational bladder cancer cases. An increased risk of bladder cancer has been reported in people living in areas close to industrial waste-treatment spots (Garcia-Perez, Fernandez-Navarro *et al*. 2013). The ingestion of water with high levels of arsenic or nitrate, or the intake of some drugs, such as cyclophosphamide, analgesics containing phenacetin are also associated with the risk of developing bladder tumours (Guo, Chiang *et al*. 1997, Letasiova, Medve'ova *et al*. 2012, Wang, Fan *et al*. 2012). In addition, several studies reported a correlation between the ingestion of food such as barbecued meat, salted meat, fried eggs and fat meals, and an increased risk for bladder cancer. On the other hand, the ingestion of fruits and vegetables was reported to exert a protective effect (Steinmaus, Nunez *et al*. 2000, Balbi, Larrinaga *et al*. 2001). However, this latter conclusion was recently challenged by a

multicentre cohort study with 521468 subjects who showed no statistically significance between fruit and vegetable consumption and a lower risk for bladder cancer (Buchner, Bueno-de-Mesquita *et al*. 2011).

Gender is also a risk factor for bladder cancer. Interestingly Jiang *et al* reported in a study on 1586 women a lower risk of invasive high-grade bladder cancers in those who experienced multiple infections. This could be due to the immune response triggered by the bladder infection and/or to the infection therapy (Jiang, Castelao *et al*. 2009). For this reason, the lower risk in women for bladder cancer might be directly correlated with the higher risk in women for bladder infection. In some parts of Africa and the Middle East, higher rates of squamous cell carcinoma of the bladder, rather than TCC, were correlated though to high prevalence of the parasitic chronic infection with *Schistosoma haematobium* (IARC 1994, Michaud 2007).

As occurs for other solid tumours, the incidence of bladder cancer increases with age (Ferlay, Shin *et al*. 2010) (Figure 1.3). In fact, tumours of the bladder rarely occur before the age of 40-50, arising most commonly in the seventh decade of life (Shariat, Milowsky *et al*. 2009). Individuals older than 65 years have a 3-fold increase in the incidence of bladder cancer, and the mean age of newly diagnosed cases is approximately 66, 69, 69 and 72 years in the World, Europe, Portugal and Italy respectively (Ferlay, Shin *et al*. 2010). The relative late onset of bladder cancer has been hypothesized to be the result of the accumulation of exposures to a variety of carcinogens over time, mainly cigarette smoking and occupational exposure. However, other factors may also be involved. For instance, urothelial enzymes responsible for inactivation of carcinogens may deteriorate over time, effectively increasing exposure of the urothelium to active carcinogens, or in general physiologic and immunological changes that occur with aging (Shariat, Milowsky *et al*. 2009).

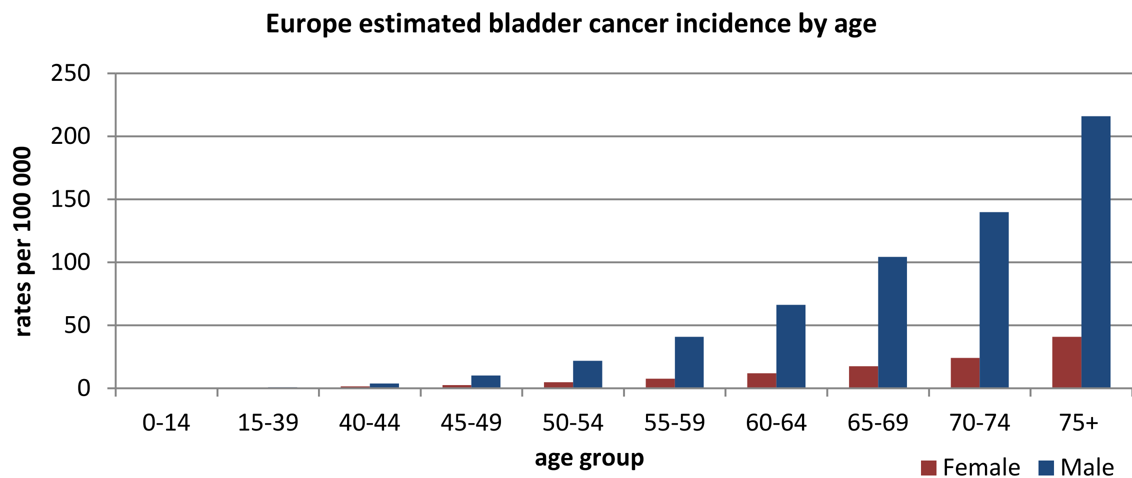



**Figure 1.3. Bladder cancer incidence rates by age in Europe according to GLOBOCAN database** (Ferlay, Shin *et al*. 2010).

## Classification and pathological staging

The most common histological types of bladder tumours (90% of cases) are originated in the urothelial epithelial cells, and are referred to as transitional cell carcinoma (TCC) (Jacobs, Lee *et al*. 2010). Approximately 5% are squamous cell carcinoma, and less than 2% are adenocarcinoma (Jacobs, Lee *et al*. 2010). Bladder cancer can be classified into different stages, depending on the level of invasion. The TNM (tumour, node, metastasis) classification system takes into consideration the depth of the bladder wall reached by the tumour (T), the invasion of lymph nodes (N) and the metastatic spread to other parts of the body (M) (Table 1.1 and Figure 1.4) (Babjuk, Oosterlinck *et al*. 2011).

**Table 1.1: 2009 TNM classification of urinary bladder cancer** (Babjuk, Oosterlinck *et al*. 2011).

| | | |
|---|---|---|
| **T – Primary tumour** | | |
| TX | Primary tumour cannot be assessed | |
| T0 | No evidence of primary tumour | |
| Ta | Non-invasive papillary carcinoma | |
| Tis | Carcinoma in situ: 'flat tumour' | |
| T1 | Tumour invades subepithelial 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 | |
| | T3a | Microscopically |
| | T3b | Macroscopically (extravesical mass) |
| T4 | Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall | |
| | T4a | Tumour invades prostate, uterus or vagina |
| | T4b | Tumour invades pelvic wall or abdominal wall |
| **N – Lymph nodes** | | |
| NX | Regional lymph nodes cannot be assessed | |
| N0 | No regional lode metastasis | |
| N1 | Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral) | |
| N2 | Metastasis in a multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral) | |
| N3 | Metastasis in common iliac lymph node(s) | |
| **M – Distant metastasis** | | |
| MX | Distant metastasis cannot be assessed | |
| M0 | No distant metastasis | |
| M1 | Distant metastasis | |

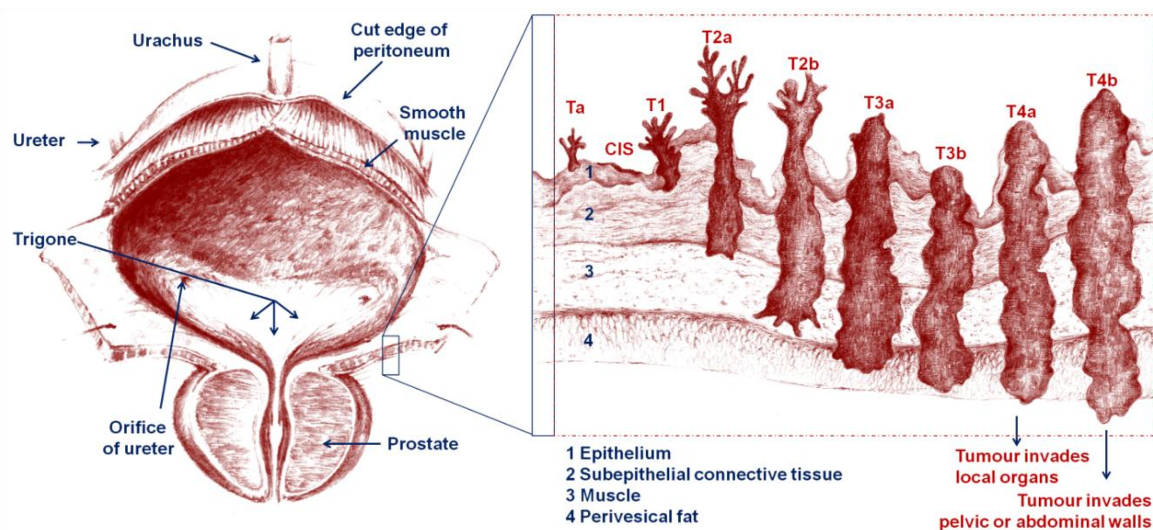



**Figure 1.4. Representation of the anatomy of a male bladder and the different stages of bladder cancer according to the TNM classification.** In the left is represented the anatomy of the human male bladder and prostate, and in the right is represented a small portion of the epithelium containing al states of bladder cancer. This figure was elaborated by both the author and the MSc Filipe Miguel Severino.

At diagnosis, more than 75% of TCC cases are non-muscle invasive (NMIBC), including those that are superficial papillary tumours (Ta), invasive tumour of the subepithelial connective tissue (T1), and carcinoma *in situ* (Tis), which are rarely lethal, but show a high recurrence rate of 50-70%. In about 10-20% of patients with Ta/T1 TCC, the disease progresses to muscle-invasion (≥T2 lesions), which can lead to metastasis and death (Jacobs, Lee *et al*. 2010). The level of differentiation (inversely related with grade) of a tumour is also an important parameter for the prognosis assessment and treatment strategies. According to the World Health Organization (WHO) 2004 bladder cancer grading, it can be distinguished in flat lesions (hyperplasia – flat lesion without atypia or papillary, reactive atypia – flat lesion with atypia, atypia of unknown significance, Urothelial dysplasia and Urothelial – Tis) and papillary lesions (urothelial papilloma – which is a completely benign lesion, papillary urothelial neoplasm of low malignant potential – PUNLMP, low-grade papillary urothelial carcinoma and high-grade papillary urothelial carcinoma) (Babjuk, Oosterlinck *et al*. 2011). Over time, great efforts have been done toward a better molecular characterization of bladder cancer (Lindgren, Sjodahl *et al*. 2012, Sjodahl, Lovgren *et al*. 2013). Recently, Sjödahl *et al* elaborated a more precise classification of bladder cancer based on several RNA and protein molecular markers. Tree major molecular subtypes of bladder cancer have been defined; urobasal A and B (Uro A and B), genomically unstable (GU), and squamous cell cancer-like (SCCL), in which Uro A tumours are associated with a good prognosis, and UroB and SCCL tumours with the worst outcome (Sjodahl, Lovgren *et al*. 2013).

### Diagnosis, prognosis and therapy

Haematuria is the most common symptom in NMIBC. Lower urinary tract symptoms may appear in patients with Tis. Diagnosis of bladder cancer depends mainly on urine cytology, cystoscopic examination of the bladder and histological evaluation of multiple tissue biopsies (Babjuk, Oosterlinck *et al*. 2011). Like in any other cancer, the identification of specific biomarkers allowing the screening of the patients has been a principal goal of several research groups (Tilki, Burger *et al*. 2011). Reliable biomarkers allow early diagnosis and help in the choice of therapeutic options. The assessment of such biomarkers is of extreme importance to establish non-invasive alternative diagnostic strategies, as well as reliable surveillance markers. Interpatient and intratumour

heterogeneity provide overwhelming odds against the existence of a single marker with the sensitivity and specificity desired for diagnosis and prognosis of bladder cancer (Stenzl, Cowan *et al*. 2009). Still, the use of multiple biomarkers can be used to improve the detection and classification of the disease (Catto, Abbod *et al*. 2010, Guo, Che *et al*. 2011). Until now, six urine markers have been approved for clinical use in the detection of bladder cancer. Based on these, commercial urine tests were developed, namely the bladder tumour antigen (BTA) stat test (which measures complement factor H and related glycoprotein), nuclear matrix protein 22 test, ImmunoCyt (detecting a high molecular weight form of carcinoembryonic antigen and two bladder tumour cell-associated mucins in exfoliated urothelial cells) and the UroVysion (a fluorescence *in situ* hybridization (FISH) assay that detects chromosomal aneuploidy and the loss of the p16 tumour suppressor gene) (Van Tilborg, Bangma *et al*. 2009, Mowatt, Zhu *et al*. 2010, Tilki, Burger *et al*. 2011). Urine markers are highly sensitive, however, less specific than urine cytology (Lokeshwar, Habuchi *et al*. 2005, van Rhijn, van der Poel *et al*. 2009).

Photodynamic diagnosis (PDD) based on blue-light cystoscopy (BLC) after intravesical instillation of 5-aminolevulinic acid or hexaminolaevulinic acid, is the most recent endoscopic technique used for detection of bladder lesions. BLC at the time of transurethral resection (TUR) facilitates a more complete resection and prolongs recurrence-free survival (Cheung, Sahai *et al*. 2013). At diagnosis, classically patients with TaT1 tumours are categorized into low-, intermediate-, and high-risk groups of both recurrence and progression according to the European Organisation for Research and Treatment of Cancer (EORTC) scoring system. Further treatment strategy of bladder cancer depends largely on its risk classification and invasive status (Figure 1.5). The primary approach to the management in cases of low risk of recurrence and progression is the TUR of the bladder tumour followed by one immediate instillation of chemotherapy. This procedure reduces the odds of recurrence when compared with TUR alone (Gudjonsson, Adell *et al*. 2009). Intravesical chemotherapy is usually approached with the instillation of mitomycin C, epirubicin or doxorubicin drugs that show equivalent efficacies (Sylvester, Oosterlinck *et al*. 2004). More recently gemcitabine has been introduced as a chemotherapeutic agent for metastatic bladder cancer (Shelley, Cleves *et al*. 2011). Owing to the likelihood of recurrence and/or progression in patients diagnosed with bladder cancer, the need for further adjuvant intravesical therapy is usually taken into account depending on the patient’s prognosis (Babjuk, Oosterlinck *et al*. 2011).

In tumours evaluated with higher risk for progression (solid lesions, positive urine cytology), other than an immediate chemotherapy intravesical instillation, a subsequent intravesical immunotherapy with the bacillus Calmette-Guérin (BCG, an attenuated strain of *Mycobacterium bovis*) is an essential treatment option (Babjuk, Oosterlinck *et al*. 2011). Invasive forms are more likely treated by cystectomy (Kaufman, Shipley *et al*. 2009).

Aggressive therapy can also be considered for those patients who are at high risk of progression and failure of BCG treatment (Raj, Herr *et al*. 2007). Therapeutic approaches in elderly have been discussed by several research groups, since the elderly and, in particular, the octogenarian have a worse tolerance to aggressive therapies (Destefanis, Bisconti *et al*. 2010). Is important to note that innate and adaptive immunity deteriorate with age (Gomez, Nomellini *et al*. 2008), resulting in a hypothetical less durable response to BCG therapy. In a retrospective analysis of 805 patients with multiple or recurrent high-grade Ta, T1, and/or Tis, it was reported that age did not affect the initial response to BCG therapy (Herr 2007) but patients older than 70 were less likely to maintain that response and remain free of tumour recurrence (Joudi, Smith *et al*. 2006). Moreover, the potential side effects or complications of intravesical BCG may not be well tolerated in elderly individuals (Herr 2007). The decision to undergo treatment for cancer is a trade off between loss of function and/or independence and extension of life. Comorbid medical conditions, functional declines and “frailty”, family dynamics, and social and psychological issues are important when deciding for the bladder cancer therapy (Shariat, Sfakianos *et al*. 2010). In general, quality of life tended to be better in patients with preservation of the bladder (Miyanaga, Akaza *et al*. 1999). Cystectomy patients reported more fatigue, appetite loss and decreased role functioning (Singer, Ziegler *et al*. 2012). In a large single centre study of 1054 patients treated uniformly with radical cystectomy and pelvic lymph node dissection for invasive bladder cancer, the recurrence-free and overall survival was 68% and 66% at 5 years and 60% and 43%, at 10 years, respectively (Stein, Lieskovsky *et al*. 2001). The 10-year disease-specific and overall survival rates in node-positive patients after cystectomy have been reported to be 28% and 21%, respectively (Gschwend, Dahm *et al*. 2002). In another study, 5-year recurrence-free survival was 76% in patients with T1 tumours, 74% for T2, 52% in T3, and 36% in T4 tumours (Madersbacher, Hochreiter *et al*. 2003).

According to a multi-institutional database of 888 consecutive patients undergoing cystectomy and lymphadenectomy for bladder cancer the outcome at 5 years was 58% for a mean recurrence-free survival and 66% for bladder cancer-specific survival (Shariat, Karakiewicz *et al*. 2006).

Owing to its anatomy, the bladder offers advantages to adjuvant therapy because it allows high local concentrations of either BCG or any chemotherapeutic drug (Bevers, Kurth *et al*. 2004). BCG and/or chemotherapeutic agent instillations are important to reduce the risk for recurrence and progression of NMIBC (Sylvester, Oosterlinck *et al*. 2008). The efficacy of BCG in preventing recurrences of TaT1 tumours has been confirmed, having a long lasting effect. The advantages of BCG were also observed in patients with intermediate-risk tumours, being more effective than other agents (Malmstrom, Sylvester *et al*. 2009, Sylvester, Brausi *et al*. 2010). Moreover, BCG therapy has been correlated with prevention of tumour progression, fewer metastases, a better overall and disease-specific survival (Sylvester, Brausi *et al*. 2010). In cases of recurrence, maintenance schedule of BCG therapy showed an optimal efficacy (Houghton, Chalasani *et al*. 2012). BCG instillations are classically given according to the empirical 6-weekly induction schedule, and different maintenance schedules have been used with up to 30 instillations given over 3 years (Lamm, Blumenstein *et al*. 2000). Regarding cases of Tis, there are no reliable prognostic factors that can be used to predict the course of the tumour (Babjuk, Oosterlinck *et al*. 2011). Tis cannot be resolved by endoscopic procedure alone, being necessary histological evaluation followed by further treatment, either intravesical instillations or radical cystectomy. Yet no consensus exists about whether conservative therapy (intravesical BCG instillations) or aggressive therapy (cystectomy) should be performed (Sylvester, van der Meijden *et al*. 2005). Recent *in vitro* studies on bladder cancer cell lines suggests that different commercial BCG trains could exert different anti-tumour activities (Secanella-Fandos, Luquin *et al*. 2013). However, data from a meta-analysis of the published data of 24 randomized clinical trials with progression information on 4863 patients suggests that at least the five most commonly used BCG strains, *i.e.* Tice, Pasteur, Connaught, RIVM and A. Frappier strains; do not differ in terms of preventing tumour progression (Sylvester, van der Meijden *et al*. 2002). A recent prospective comparative study on 129 patients with Ta/T1 and Tis that underwent intravesical BCG therapy with the Tokyo or Connaught strains, also reported no outcome differences in terms of the rates of complete response (CR), recurrence-free

survival (RFS) or adverse events (Sengiku, Ito *et al*. 2013). Nevertheless, up to 90% of all patients will experience some sort of side effect of BCG therapy among which common cystitic symptoms are by far the most frequent. Sepsis and even death following intravesical BCG have been reported (Witjes, Palou *et al*. 2008). For this, full acceptance of this therapy is still debated. In addition, despite BCG therapy success, patients either fail to respond to the intravesical pharmacotherapy or recur in 50-70% of the cases, being 10-15% of these patients prone to develop progression of the disease to muscle invasion within a 5-years period (Witjes and Hendricksen 2008). A continuous follow-up of bladder cancer patients is though required due to high recurrence rates (Babjuk, Oosterlinck *et al*. 2011).

**Bladder cancer burden**

The social and economic burden of bladder cancer is expected to increase dramatically with the increase of the life expectancy (Shariat, Milowsky *et al*. 2009). Bladder cancer has the highest lifetime treatment costs per patient of all cancers. The high recurrence rate and ongoing invasive monitoring requirement are the key contributors to the economic and human toll of this disease (Sievert, Amend *et al*. 2009). The bladder cancer burden is highest in developed communities but with the increasing age and exposure to risk factors of the population in the developing Countries, it is expected an increase in such Countries in the forthcoming years (Ploeg, Aben *et al*. 2009). Long-term cost benefits can be achieved through reduced tumour recurrence and potentially reduced progression rates (Sievert, Amend *et al*. 2009).

## *I.2 Insights into Glyco-Oncology*

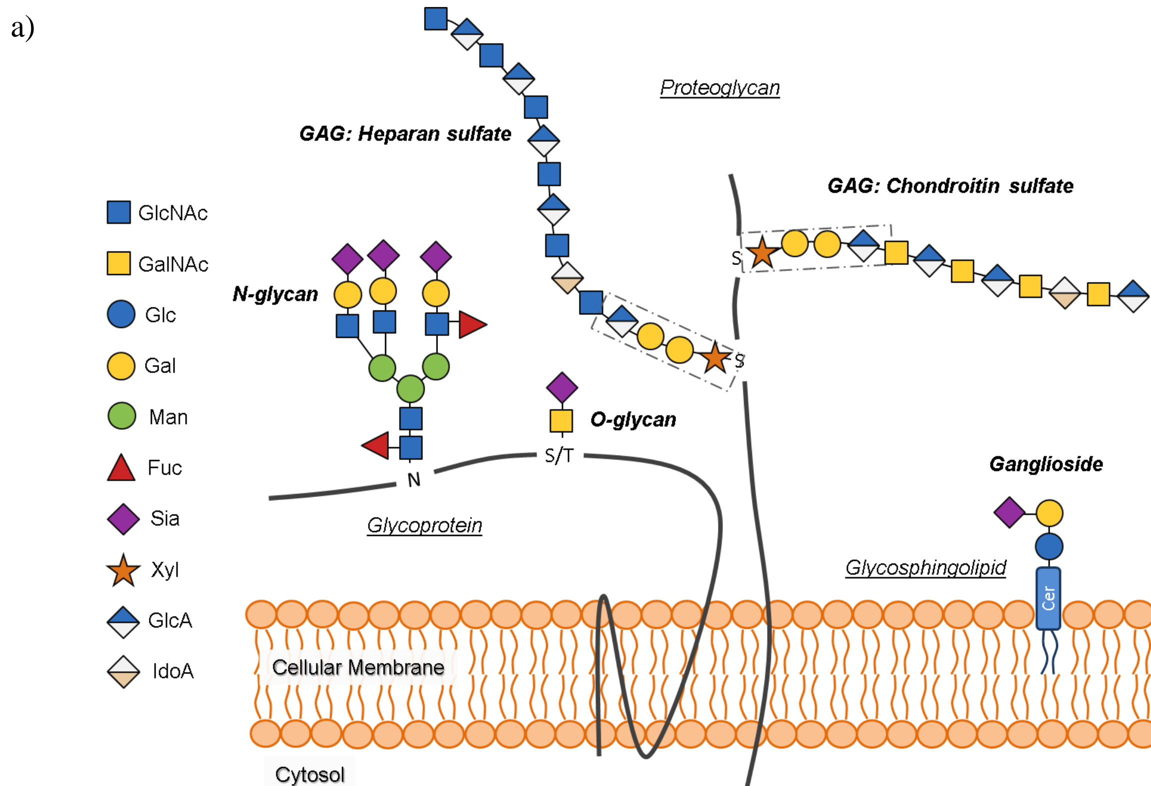
### Introduction to Glycobiology

Glycosylation is one of the most frequent modifications of proteins and lipids. This process consists in the covalent attachment of one or more glycans to a protein or a lipid, forming a glycoconjugate (Figure 1.5) (Varki, Kannagi *et al*. 2009). Unlike the biosynthesis of nucleic acids and proteins, which are deterministic, template-driven processes, glycosylation is a stochastic process, regulated mainly by the relative abundance, cellular localization and specificities of biosynthetic enzymes (glycosyltransferases) and catabolic enzymes (glycosidases). The stochastic nature of the glycosylation process is at the basis of the phenomenon known as microheterogeneity, which means that the structure of the sugar chains attached to a specific glycosylation site in a given glycoconjugate displays a certain degree of variability (Nairn, York *et al*. 2008, Lauc, Rudan *et al*. 2010). The synthesis of glycoconjugates occurs mainly in the lumen of the endoplasmic reticulum (ER) and in the Golgi apparatus (Li and Richards 2010) and is mediated by glycosyltransferases, a family of enzymes which transfer a sugar residue from a donor, which is frequently a nucleotide-sugar (*e.g.* GDP-fucose, UDP-galactose or CMP-sialic acid) to an acceptor which can be a sugar, an amino acid or a lipid. Glycosyltransferases are classified on the basis of the sugar they transfer (*e.g.* fucosyltransferases, galactosyltransferases, sialyltransferases). Moreover, members of each glycosyltransferase family are distinguished on the basis of the structure they recognize as acceptor and of the isomeric linkage they form. Glycoconjugates can be grouped in glycolipids, proteoglycans and glycoproteins. In glycolipids, the sugar portion is usually attached through a glucose (Glc) residue to the hydrophilic portion of a membrane lipid, which is often ceramide. In this case the glycolipid is referred to as glycosphingolipid. Usually, a galactose (Gal) residue is β1,4-linked to Glc. Based on their basic glycan structures, glycosphingolipids are classified into four groups, namely, globo-series, lacto-series, neolacto-series and ganglio-series, classified according to the sugar types linked to the (Gal) residue (Schnaar, Suzuki *et al*. 2009). The basic structure of proteoglycans is comprised of a core protein and one or more covalently attached glycosaminoglycan (GAG) side chains. GAGs are polysaccharides composed of repeating disaccharide units formed by an amino sugar (*N*-acetylglucosamine (GlcNAc) in

heparan sulphate or *N*-acetylgalactosamine (GalNAc) in chondroitin sulphate) linked to *D*-glucuronic acid (GlcA) or *L*-iduronic acid (IdoA). The linkage of GAGs to the protein core involves a specific trisaccharide composed of two Gal and one xylose (Xyl) sugars (GAG-GalGalXyl-*O*-$CH_2$-protein). GAGs exist also as free molecules, as is the case of hyaluronan (Esko and Lindahl 2001). In glycoproteins, the two types of glycans attached to the peptide are the *N*- and *O*-glycans, which may co-exist in the same protein. The *N*-linked chains are attached through a GlcNAc residue to the asparagine residue of the sequence N-X-S/T (X represents any amino acid, except proline), while the *O*-linked chains are usually linked through a GalNAc residue to serine or threonine.

The biosynthesis of *N*-linked chains requires the assembly of an oligosaccharide precursor comprised of two GlcNAc, nine mannose (Man) and three Glc residues associated to the membrane lipid dolichol-phosphate. This structure is then transferred *en bloc* to an asparagine residue of a the nascent polypeptide chain (Kornfeld and Kornfeld 1985). Successively, the protein-linked oligosaccharide first undergoes trimming of the glucose and of some of the mannose residues. Then GlcNAc, Gal, sialic acid (Sia) and fucose (Fuc) residues are added forming "complex type" glycans. The trisaccharide units comprised of Sia-Gal-GlcNAc are referred to as branches or *antennae*. When a GlcNAc residue is β1,4-linked to the innermost Man residue is referred to as “bisecting GlcNAc” and is not elongated further. The presence of a Fuc residue α1,6-linked to the innermost GlcNAc of the core is referred to as “core fucosylation” (Takahashi, Kuroki *et al*. 2009). *O*-linked glycans are attached to the hydroxyl group of serine or threonine in the Golgi apparatus, through the stepwise addition of single monosaccharides. After the addition of the first GalNAc residue, the addition of other sugars is a stepwise process which accompanies the maturation of the glycoprotein (Brockhausen 1999). In the ‘mucin-type’ *O*-glycans, the first GalNAc is further extended with Gal, GlcNAc, and Sia. There are also several types of non-mucin *O*-glycans, in which the peptide-bound sugar can be *O*-Fuc, or *O*-Xyl, *O*-Man or *O*-GlcNAc (Varki, Kannagi *et al*. 2009). In this thesis, the term *O*-glycan will refer to “mucin type” *O*-glycans.

a)




b)

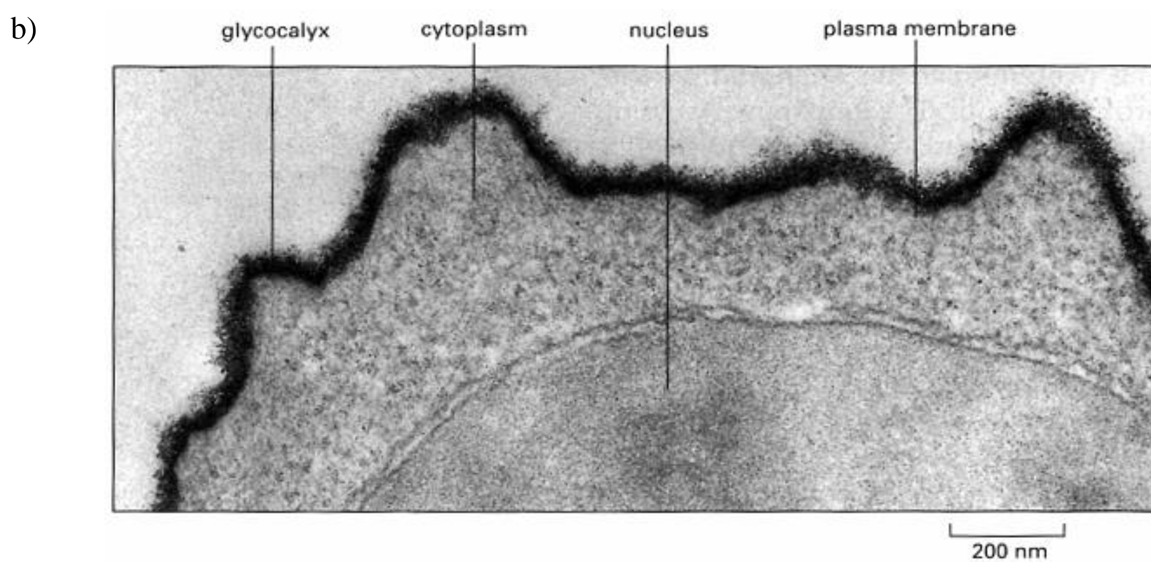



**Figure 1.5. Glycoconjugates in mammalian cells.** a) Glycans can be attached to proteins as in glycoproteins and proteoglycans. Glycoproteins are decorated with *N*- and/or *O*-glycans and proteoglycan with glycosaminoglycans (GAG). Glycosphingolipids are mainly associated with membranes and are formed by a glycan attached to the lipid moiety ceramide. A ganglioside contains one or more residues of sialic acid (Sia). The glycan structures presented for each *N*-linked, *O*-linked, glycosaminoglycan (GAG) and glycosphingolipids exemplify one of the possible structures (Severino, Silva *et al.* 2012). b) The electron micrograph of the surface of a lymphocyte stained with ruthenium red emphasizes the thick carbohydrate layer surrounding the cell, a.k.a. glycocalyx (Alberts, Johnson *et al.* 2002).

A large number of complex carbohydrates of animals and microorganisms are terminated by Sia. This sugar exists in various chemical forms linked as monomers or polymers in an outstanding position, rendering them versatile modulators in cell biology and pathology (Schauer 2009). Sialic acid structures are involved in many cellular functions, both in physiological and pathological processes (Varki and Varki 2007). The addition of sialic acid to glycoconjugates is mediated by sialyltransferases. These enzymes transfer sialic acid from the donor substrate CMP-Sia to the terminal positions of the oligosaccharide chains of glycoproteins and glycolipids in different linkages. Sia can be linked through α2,3- or α2,6- linkages to a βGal residues, α2,6-linkage to βGalNAc or to βGlcNAc residues, or even α2,8- linkage to other sialic acid residues in ganglioside or polysialic acid chains (Harduin-Lepers, Vallejo-Ruiz *et al*. 2001). Sialyltransferases constitute a family of over 20 enzymes usually associated with the membrane of the Golgi apparatus. Their expression is strongly dependent on the tissue, the cell type or the developmental stage (Harduin-Lepers, Vallejo-Ruiz *et al*. 2001).

Glycoconjugates are involved in many physiological and pathological processes, including differentiation, migration and cell signalling, host-pathogen interactions, tumour invasion and metastasis (Ghazarian, Idoni *et al*. 2011, Dall'olio, Malagolini *et al*. 2012). The expression of glycan structures, which are usually cell type-dependent, are profoundly modified in cancers (Varki, Kannagi *et al*. 2009). These modifications contribute to determine the phenotype of cancer cells, including their aberrant growth, adhesion to endothelia, angiogenesis and the ability to escape apoptotic death and immune recognition (Dall'olio, Malagolini *et al*. 2012). The alteration of glycosylation is an early step in the process of tumour invasion and metastasis and is therefore regarded as a "quasi-universal" modification in tumour cells (Reis, Osorio *et al*. 2010). Aberrant glycosylation in tumour cells include either a loss or a gain of expression of certain glycan structures, the appearance of truncated structures or even new structures. Up-regulation and/or down-regulation of specific glycosyltransferases are often responsible for these changes (Dall'Olio, Malagolini *et al*. 1989, Malagolini, Dall'Olio *et al*. 1989, Videira, Amado *et al*. 2007, Videira, Correia *et al*. 2009, Dall'olio, Malagolini *et al*. 2012). Frequently, glycosyltransferases are under the control of signal transduction pathways whose alteration in cancer cells leads to altered glycosyltransferase expression (Dall'olio, Malagolini *et al*. 2012). Examples of this mechanism are provided by GlcNAc transferase-V and sialyltransferase ST6GAL1. The first is under the control of oncogenes

*SRC* (Buckhaults, Chen *et al*. 1997), *HER2* (Chen, Zhang *et al*. 1998) and *HRAS* (Lu and Chaney 1993), through transcription factor *ETS1* (Ko, Miyoshi *et al*. 1999). The second is controlled by the Ras pathway (Easton, Bolscher *et al*. 1991, Le Marer, Laudet *et al*. 1992, Dalziel, Dall'Olio *et al*. 2004). Often, the carbohydrate structures formed by cancer cells resemble those expressed by the corresponding normal tissue during the foetal life (onco-developmental regulation) and are largely tissue-specific (Dall'olio, Malagolini *et al*. 2012). Still, some structures appear to be widely expressed by cancers of different histological origin. This is probably due to two main reasons: first, their biosynthesis is strictly controlled by the mechanism altered in cell transformation (*i.e.* activation of oncogenes, inactivation of tumour-suppressor genes, altered pattern of epigenetic regulation etc); second, their expression provides cancer cells with growth advantage, resulting in the selection of cells expressing a given antigen during tumour growth (Dall'olio, Malagolini *et al*. 2012). The relevance of epigenetic changes in cancer progression is increasingly recognized (Murr 2010). Many “glycogenes”, including glycosyltransferases, galectins, enzymes involved in the biosynthesis of sugar nucleotides, transporters, are aberrantly regulated in cancer by epigenetic mechanisms (Zoldos, Horvat *et al*. 2013). Altered glycosyltransferase expression generates a flow of information from the nucleus to the cell surface (inside-out) altering the structure of cell membrane glycoproteins. Many cell membrane receptors are crucially involved in the regulation of cell adhesion, such as integrins (Seales, Jurado *et al*. 2005) or cadherins (Zhao, Nakagawa *et al*. 2006, Pinho, Seruca *et al*. 2011), or of cell growth, such as growth factor receptors (Lau and Dennis 2008). These receptors are indeed glycoproteins and their aberrant glycosylation can result in inappropriate signaling. This altered flow of information from the cell membrane to the nucleus (outside-in) can support the growth of cancer cells, fuelling a vicious loop (Dall'olio, Malagolini *et al*. 2012). Tumour-associated carbohydrate structures allow tumour cells to invade and metastasize (Schultz, Swindall *et al*. 2012, Tsuboi, Hatakeyama *et al*. 2012). One of the best examples of relationship between expression of a cancer-associated carbohydrate structure and metastasis formation is provided by the β1,6-branching, which consists in the presence of an antenna β1,6-linked to the trimannosyl-core of *N*-linked chains. Although the mechanism(s) linking this structure with metastasis have not been completely elucidated, the conclusive evidence about the causative role played by this structure in metastasis formation came from mouse studies in which the enzyme responsible for this modification, β1,6 GlcNAc transferase V (GnT5, product of the *MGAT5* gene) was knocked down (*MGAT5*$^{-/-}$).

Mice expressing the polyomavirus middle T antigen (PyMT) from a transgene in mammary epithelium spontaneously develop mammary tumours. When these mice were crossed with *MGAT5*$^{-/-}$ mice, the tumours grew slower than in the PyMT-transgenic littermate expressing *MGAT5* and metastasis formation was almost completely inhibited (Granovsky, Fata *et al*. 2000). Another example of glycosylation-metastasis relationship is provided by ganglioside GD3 which enhances *in vivo* growth and metastasis formation through interactions with the growth factor receptor c-Met (Cazet, Lefebvre *et al*. 2010).

One of the most important cancer-associated glycosylation change is the incomplete glycosylation of *O*-glycans, leading to the formation of truncated oligosaccharides and consequently to the increased expression of the Thomsen Friedenreich (TF) related antigens (Ju, Wang *et al*. 2013). These glycosidic antigens include the T and Tn antigens, and their sialylated forms sialyl-T (sT) and sialyl-Tn (sTn) antigens (Figure 1.6). The Tn antigen (GalNAc-*O*-Ser/Thr) is the precursor of the T antigen (Galα1,3GalNAc-*O*-Ser/Thr), also known as *core 1* structure. Its biosynthesis is catalysed by core 1 β1,3*N*-galactosyltransferase (C1GALT1) or T synthase present in most tissues. This enzyme is peculiar because it requires the presence of a molecular chaperone, the product of the gene *C1GALT1C1* (coding for Cosmc) (Ju, Lanneau *et al*. 2008) which, in the endoplasmic reticulum, binds to T synthase preventing its ubiquitin-mediated proteosomal degradation.

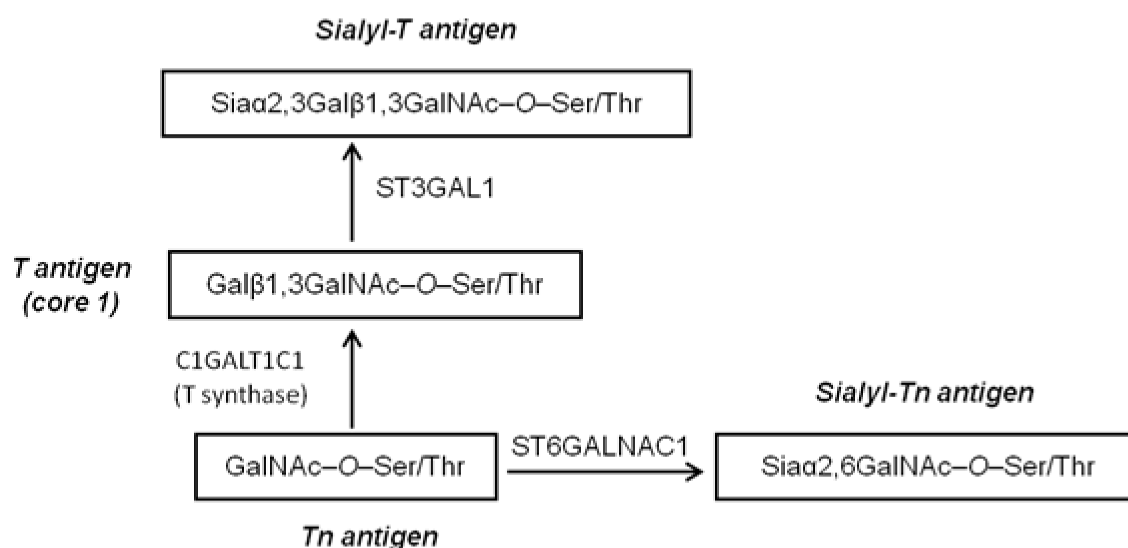



**Figure 1.6. Structures of Thomsen-Friedenreich related antigens.** Glycan structures and the main enzymes responsible for their synthesis are shown.

The T antigen (Yu 2007) accumulates in breast (Hanisch and Baldus 1997) and colon cancer (Cao, Karsten *et al*. 1995), while in normal colon its expression is masked by sialylation (Campbell, Finnie *et al*. 1995). In breast cancer, despite the overexpression of the T antigen, sialyltransferase *ST3GAL1* which synthesizes the alternative sT antigen, is usually elevated (Burchell, Poulsom *et al*. 1999). Overexpression of *ST3GAL1* in epithelial breast cells of a murine model of breast carcinogenesis, leads to the development of breast tumours with a short latency, independently on the expression of the cognate product, the sT antigen. This unexpected result has been interpreted suggesting that *ST3GAL1* acts as a tumour promoter (Picco, Julien *et al*. 2010). Interestingly, an elevation of *ST3GAL1* mRNA was reported also in bladder cancer specimens (Videira, Correia *et al*. 2009), even though the role of this modification in the biology of bladder cancer remains to be established. sTn antigen is expressed by cancers of the stomach (Conze, Carvalho *et al*. 2010), liver (Cao, Karsten *et al*. 1999), pancreas (Itzkowitz, Kjeldsen *et al*. 1991) and other tissues. In breast cancer its expression correlates with a poorly differentiated state (Cho, Sahin *et al*. 1994) and resistance to adjuvant therapy in node-positive patients (Miles, Happerfield *et al*. 1994), while in colon cancer, it correlates with a worse prognosis (Cao, Karsten *et al*. 1995). The impact of sTn overexpression has been studied by over expressing sialyltransferase *ST6GALNAC1*, which is the major sTn synthase (Marcos, Pinho *et al*. 2004), in different cellular model systems. In murine carcinoma cells overexpressing *ST6GALNAC1*, a major carrier of sTn antigen are β1-integrins. These cells displayed major morphological changes and reduced ability to migrate on extracellular matrix components (Clement, Rocher *et al*. 2004). In human breast cancer cell lines overexpressing *ST6GALNAC1*, a major carrier of sTn antigen were mucin 1 (MUC1) and other high molecular weight glycoproteins. These cells exhibited reduced cell adhesion and increased cell migration (Julien, Adriaenssens *et al*. 2006). sTn was recently reported by our group to be also overexpressed in bladder cancer carried by MUC1 and CD44. The relationship between sTn and bladder cancer is the subject of a recent study (Ferreira, Videira *et al*. 2013). Both sT and sTn and their synthesis enzymes will be discussed thoroughly in the Discussion and Conclusions section. In different epithelial tissues, the carriers of T and sTn antigens are different. In fact, they are carried mainly by a high molecular weight splice variant of CD44 (Singh, Campbell *et al*. 2001) and MUC1 (Storr, Royle *et al*. 2008) in colon cancer, by MUC2 in gastric cancer (Conze, Carvalho *et al*. 2010) and by MUC1 in breast cancer (Storr, Royle *et al*. 2008).

The somatic inactivation of the gene *C1GALT1C1* is associated with the expression of Tn and sTn antigens in colon cancer and melanoma cell lines (Ju, Lanneau *et al*. 2008), indicating a general mechanism of regulation of the biosynthesis of these structures. It was previously reported that the expression of the T antigen increased Natural Killer (NK) cells susceptibility of cancer cells (Sotiriadis, Shin *et al*. 2004), and that peptides containing the T antigen were able to elicit a specific and major histocompatibility complex (MHC) class-I-restricted anti-tumour cytotoxic T lymphocyte response (Xu, Gendler *et al*. 2004, Heimburg, Yan *et al*. 2006). These findings supported the idea of the T antigen as a possible target for cell-mediated anti cancer immunity. Initial insights into the unique repertoire of glycans expressed on tumour cells emerged from the increased ability of tumours to bind a range of plant carbohydrate-binding proteins, the lectins (Raedler and Schreiber 1988). In fact, a large number of the studies on the alterations of glycosylation in cancer has been performed using lectins as probes (Wu, Lisowska *et al*. 2009). Lectins exhibit a typical protein folding that defines the carbohydrate-binding protein families and bind defined monosaccharide or oligosaccharide structures with good specificity (Wu, Lisowska *et al*. 2009) in a 'lock-and-key' fashion (Weis and Drickamer 1996, Gabius, Andre *et al*. 2011). The carbohydrate-binding proteins are divided into several types depending on their structures and the nature of the interaction they establish (Wu, Lisowska *et al*. 2009). Long before the role of carbohydrate-protein interactions had been explored, many lectins were identified, characterized and applied as useful tools in studying glycoconjugates. Therefore, lectins have been widely used for preparative and analytical purposes in biochemistry, cell biology, immunology, oncology, pathology and related areas (Wu, Lisowska *et al*. 2009).

**Glyco-Oncology of bladder cancer**

Several reports showed drastic changes in the glycosylation of lipids in bladder tumours and correlated these modifications with tumour invasiveness and progression. For instance, while NMIBC shows an accumulation of GM3 ganglioside and a decrease of Gb3 and Gb4 glycolipids, invasive tumours show a decreased expression of GM3 and an accumulation of Gb3 and Gb4 (Kawamura, Ohyama *et al*. 2001, Guan, Handa *et al*. 2009). Also ABH and Lewis blood group systems, a group of carbohydrate structures expressed at the terminal part of both glycoproteins (*O*- and *N*-glycans) and glycolipids on red blood cells, endothelial and epithelial cells (Magnani, Nilsson *et al*. 1982, Shimodaira, Nakayama *et al*. 1997), were correlated with bladder cancer. The loss of ABH structures and the increased expression of Lewis related antigens are the most evident changes, among the blood group antigens, related with poor prognosis in bladder cancer, suggesting a pathophysiological role in bladder cancer progression (Hegele, Mecklenburg *et al*. 2010). The glycosylation of epithelial cadherin (E-cadherin), the main constituent of the adherens junctions, critically regulates its biological properties (Pinho, Seruca *et al*. 2011). It's down-regulation was reported in malignant human urothelium and found associated with tumour recurrence, progression and poor survival (Clairotte, Lascombe *et al*. 2006). High levels of the proteolytic cleavage product of the E-cadherin (sE-cadherin) can be found in serum of patients with TCC and are detectable also in urine (Matsumoto, Shariat *et al*. 2003, Shariat, Matsumoto *et al*. 2005). Integrins are also important glycoproteins that are involved in cell growth and differentiation, proliferation and migration, tissue organization, recruiting of lymphocytes and inflammation, cancer invasion and metastasis (Cox, Brennan *et al*. 2010). The integrin α3β1 was shown to take part in the bladder cancer T24 cell line migration on fibronectin being proposed that its function may be altered by glycosylation (Pochec, Litynska *et al*. 2006). MUC1, is a heavily glycosylated high-molecular-mass glycoprotein from the Mucin family that can be present both in membrane-bound and secreted forms, representing a major component of mucus (Hollingsworth and Swanson 2004). Radioactively labelled anti-MUC1 antibodies, when intravenously administrated to TCC patients, stained tumour tissues in a stage dependent manner (Hughes, Perkins *et al*. 2001, Scholfield, Simms *et al*. 2003). MUC7 is also atypically expressed in exfoliated cells expelled in the urine of patients with bladder cancer (Okegawa, Kinjo *et al*. 2003). Invasion by tumour cells involves also the alteration of cell-matrix interactions

(Fuster and Esko 2005). For example, the expression of the hyaluronic acid (HA), a non-sulfated GAG comprised of a repeating disaccharide (GlcA-GlcNAc), has been implicated in promoting tumour cell proliferation, invasion and metastasis and represents an accurate diagnostic marker for bladder cancer. The measurement of HA and of its degrading enzyme hyaluronidase (HAase) in urine has been applied as a commercial urinary test, the HA-HAase test, to the screening of bladder cancer (Lokeshwar, Schroeder *et al*. 2002).

Some lectins were found to be useful in differentiating bladder tumours from normal urothelium. Lectins like *Ricinus communis* agglutinin (RCA, recognizing Galβ1,4GlcNAcβ1-R) and wheat germ lectin (WGA, recognizing GlcNAcβ1,4GlcNAcβ1,4GlcNAc-R) can bind to all urothelial cells in the normal tissue, while some lectins like peanut agglutinin (PNA recognizing Galβ1,3GalNAc), concanavalin A (ConA, recognizing branched α-mannosidic structures and biantennary complex type *N*-Glycans) and soybean lectin (SBA, recognizing GalNAc) show little binding (Ward, Stewart *et al*. 1987). Initially, by using cell suspensions from transitional cell carcinoma, it was found that aneuploid cells bound PNA more extensively and were less reactive with WGA than diploid cell population (Langkilde, Wolf *et al*. 1992). Drugs or delivery systems modified by lectins have been thought as potential tools for more efficient, local adjuvant intravesical treatment for bladder cancer (Neutsch, Plattner *et al*. 2011). The calcium-independent galactose-specific lectins, the galectins, represent a family of carbohydrate-binding proteins widely distributed in all living organisms (Lahm, Hoeflich *et al*. 2000), that play a number of important roles in cancer by contributing to tumourigenesis, proliferation, angiogenesis, and metastasis (Liu and Rabinovich 2005). This group of lectins are the best characterized carbohydrate-binding proteins in bladder cancer (Cindolo, Benvenuto *et al*. 1999).

A glycopeptide decorated with a trisaccharide similar, if not identical, with sT was identified in the urine of patients affected by interstitial cystitis, a painful disease caused by thinning or denudation of bladder epithelium. This molecule, which is referred to as antiproliferative factor (APF) exerts a strong anti-proliferative activity on bladder cells and is involved in the pathogenesis of interstitial cystitis (Keay, Szekely *et al*. 2004).

## *I.3 BCG Immunotherapy*

### Adhesion and Internalization of BCG

The intravesical therapy with the bacillus Calmette-Guérin is commonly used in the treatment of non-muscle invasive urothelial carcinomas. BCG is a sub-attenuated strain of *Mycobacterium bovis*, developed between 1908 and 1921, by Calmette and Guérin, in order to generate a vaccine against human tuberculosis (TBC). The notion that TBC could have some anti-tumour effect born in the early twentieth century, when Raymond Pearl reported a low frequency of cancer in patients with TBC. However, the use of BCG as anti-tumour therapy began later. In 1976 Alvaro Morales carried out the first intravesical instillation of BCG to treat superficial bladder cancer, and since then BCG immunotherapy for superficial bladder cancer underwent few modifications. This therapeutic procedure begins one to three weeks after transurethral resection (TUR) of the bladder, and consists of a weekly intravesical instillation for six weeks. After six weeks, a maintenance therapy can be continued, depending on the grade and the stage of the tumour (Brandau and Suttmann 2007, Kresowik and Griffith 2009). Following instillation, BCG accumulates near the wall of the bladder, allowing the establishment of non-specific and specific contacts, with urothelial cells. The lumen of the bladder is covered with a hydrophilic layer of highly sulfated glycosaminoglycans (GAGs), which protects the bladder from pathogens and toxic compounds. The fact that both the GAGs layer and BCG cell wall are negatively charged allows BCG local accumulation, without adherence, at a close docking distance to the bladder wall. In normal conditions, BCG would only reversibly adsorb to the bladder wall, nevertheless, when GAG layer is damaged, like for instance in a case of neoplastic tissue, this will allow an increased BCG docking and adherence (Bevers, Kurth *et al*. 2004). Interestingly, it was reported that medications that inhibit fibrin clot formation diminished the side effects of BCG therapy but also adversely affected efficacy (Boorjian, Berglund *et al*. 2009). Recent *in vitro* findings support the notion that the fibronectin attachment protein (FAP) from BCG has an important role in BCG targeting to cells (Coon, Crist *et al*. 2011). After intravesical instillation, BCG is internalised and processed by tumour cells, triggering an inflammatory response (Bevers, Kurth *et al*. 2004). Glycoproteins and lipoproteins from degraded and processed BCG by tumour cells are

presented at the cell surface. This process induces phenotypic alterations in tumour cells which eventually trigger an immune reaction against the tumour (Bevers, Kurth *et al*. 2004). It has been reported that the interaction of BCG with urothelial malignant cells causes the activation of multiple signaling pathways, contributing to the transcription of early genes, including genes encoding the inhibitor of cyclin-dependent kinase p21 and interleukin 6 (IL-6), leading to cell cycle arrest in G1 phase and to the induction of necrosis of urothelial cells (Chen, Zhang *et al*. 2009). Activation of these pathways also promotes the secretion of several intracellular proteins (Chen, Zhang *et al*. 2009, See, Zhang *et al*. 2009). However it has been suggested that BCG might trigger also the formation of metastasis via the secretion of vascular endothelial growth factor (VEGF) (Ping, Wu *et al*. 2012).

**Immune response triggered by BCG**

BCG mycobacteria have a characteristic outer cell wall consisting of various complex structural biomolecules. The immunologically active components of these biomolecules are nowadays referred to as pathogen-associated molecular patterns (PAMPs). PAMPs on the mycobacterial cell wall include peptidoglycan, mycolic acids, lipomannan and lipoarabinomannan (Brennan 2003). The precise mechanisms responsible for BCG anti-tumour effect have not been fully elucidated, but a massive, complex and local activation of the immune system has been well documented (Bevers, Kurth *et al*. 2004, Videira, Calais *et al*. 2009). BCG interacts with urothelial cells which, after BCG-challenging, produce various proinflammatory cytokines, including IL-6, IL-8 and TNFα, which trigger the activation and the recruitment of inflammatory cell populations into the urothelium. Cell populations which respond to this stimulus infiltrating the wall of the urothelium, include neutrophils, monocytes and macrophages. Neutrophils and macrophages produce additional cytokines and chemokines which attract to the bladder several lymphocyte populations, including CD4 T lymphocytes and later CD8 T lymphocytes (Kresowik and Griffith 2009).

**Purpose of the work**

The present work is based on the following premises:

1) Although BCG therapy represents a very effective approach for the treatment of bladder cancer and one of the few examples of effective cancer immunotherapy, its mechanism of action remains partially obscure;

2) Thomsen-Friedenreich related antigens are aberrantly expressed in bladder cancer and some of them are related with malignancy but the role they play in bladder cancer biology remains to be established. In particular, it is not clear whether and how the expression of a given TF related antigen affects the response to BCG.

To investigate these two points, it has been taken advantage from the availability of the cellular models established in recent years by the collaboration of the two supervisors. The model is comprised of the bladder cancer cell lines HT1376 and MCR, retrovirally transduced with the cDNA of sialyltransferases *ST3GAL1* and *ST6GALNAC1*, respectively. As a consequence, the transduced HT1376 cells expressed the sialyl-T (sT) antigen and reduced expression of T antigen, while transduced MCR cells expressed the sialyl-Tn (sTn) antigen. However, the cell lines originated by transduction turned out to express heterogeneously the desired antigens. Thus, this work begins with a preliminary purification step of cell populations expressing homogeneously the desired antigen.

The objectives of this work are the following:

**1) The refinement of the cellular model of bladder cancer:**

1a. Isolation of cell populations expressing homogeneously the desired carbohydrate antigens sT and sTn

1b.Characterization of the cell population by FACS analysis and for the expression of sialyltransferase activities and mRNA

**2) The study of the response of bladder cancer cell lines to BCG:**

2a. Study of BCG internalization

2b. Effect of sialyltransferase expression and BCG interaction on the whole gene expression profile and on cytokine secretion

**3) The study of the response of macrophages to BCG-challenged BC cell lines:**

3a. Cytokine secretion by macrophages in response to the secretome of BCG-challenged bladder cancer cells

3b. Phagocytosis of apoptotic BCG-challenged bladder cancer cell lines by macrophages

# CHAPTER II

## *Materials and Methods*

## Cell lines

The HT1376 cell line was initially isolated from a 58 year old Caucasian woman with invasive transitional cell cancer (TCC) of the bladder (Rasheed, Gardner *et al*. 1977) while the MCR cell line was isolated from a subcutaneous metastatic lesion of a 51 year old male diagnosed with invasive TCC (Zoli, Ricotti *et al*. 2004). Cells were grown in 25 $cm^2$ flasks with DMEM (4.5 $g.L^{-1}$ glucose, Sigma), 10% Foetal Calf Serum (FCS – Sigma), 2 mM *L*-glutamine (Sigma) and 100 $\mu g.mL^{-1}$ penicillin/streptomycin (Sigma) in a 5% $CO_2$ humidified atmosphere. Culture media were renewed twice a week, washing the cells when necessary with sterile phosphate buffered saline (PBS) solution (1.47 mM of $KH_2PO_4$, 4.29 mM of $Na_2HPO_4.7H_2O$, 137 mM of NaCl and 2.68 mM of KCl, pH 7.3, in ultrapure water). Cells were routinely passaged when approximately 80% confluent by trypsin/EDTA (Sigma) treatment.

## Flow cytometry

Peanut (PNA), *Maackia amurensis* (MAA) and *Sambucus nigra* (SNA) lectins (Sigma) were conjugated with Fluorescein Isothiocyanate (FITC) as previously described (Hoebeke, Foriers *et al*. 1978). Briefly, 10 µg of FITC (Invitrogen) dissolved in acetone was added to 1 mg of lectin dissolved in 1 mL of $Na_2CO_3$ 1 M (pH 10). Each Lectin-FITC preparation was incubated overnight at 4 °C and dialyzed extensively against PBS. The anti-sTn antibody (TKH2 hybridoma clone) was kindly provided by Professor Celso Reis. Labelled cells were analysed in a FACSAria flow cytometer (Becton Dickinson). Fluorescence-activated cell sorting (FACS) data were processed with the Flowing Software v.2.3.3 (by Perthu Terho, Turku Centre for Biotechnology, Finland).

For lectin staining, cells were incubated with 1:20 diluted PNA-FITC, 1:40 diluted MAA-FITC or 1:25 diluted SNA-FITC at 4 ºC for 15 min in the dark. For anti-sTn staining, cells were incubated with 1:50 diluted TKH2 supernatant for 45 min at 4 ºC, and then with 1:100 diluted polyclonal anti-Ig-FITC (Dako) for 15 min at 4 ºC in the dark. After a washing by centrifugation, cells were FACS analysed.

**Preparation of cell lines constitutively expressing sialyltransferases**

HT1376 and MCR cells were previously transduced with a retroviral vector generated with the ViraPower Lentiviral Expression System (Invitrogen), according to manufacturer's instructions. The whole coding regions of human *ST3GAL1* (cDNA isolated from the 5637 bladder cancer line) or of *ST6GALNAC1* (kindly provided by Professor Celso Reis, IPATIMUP, University of Porto, Portugal) were PCR amplified and cloned in the pLenti6/V5 Directional TOPO cloning vector. The vector drives the expression of inserted genes through the cytomegalovirus promoter and contains the selectable marker for blasticidin. A negative control vector was prepared with an empty plasmid. After transduction with mock- or sialyltransferase-expressing vectors, HT1376 and MCR cells were selected with 4 $\mu g.mL^{-1}$ blasticidin. Pooled blasticidin-resistant cells were used for successive studies.

*Isolation of cell populations homogeneously expressing sialylated antigens*

To obtain cell populations homogeneously expressing sialylated antigens from the heterogeneous cell populations which resulted from the transduction steps, two different approaches were used for HT1376 and MCR cells.

HT1376 cells expressing sT antigen – $HT1376_{sT}$

HT1376 cells homogeneously negative for PNA expression ($HT1376_{sT}$) were obtained by selection of individual clones. About 100 transduced HT1376 cells were seeded in a 10 cm Petri dish. Fifty percent of the growing medium was renewed every 3 to 5 days. After approximately one month, individual colonies were observed and collected with an inoculation loop. Nine colonies were randomly selected and the presence of the T antigen (Galβ1-3GalNAcα1-Ser/Thr) was detected by PNA-FITC staining and FACS analysed.

MCR cells expressing sTn antigen – $MCR_{sTn}$

The transduced MCR cells were enriched in their sTn positive population, using a commercial system for cell separation from MACS Technology (Miltenyi Biotec) as recommended by the manufacturer. Briefly, cells were incubated with 1:50 diluted anti-sTn antibody (HB-STn1 clone, Dako) for 45 min at room temperature. After washing with Beads Buffer (0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid in PBS) by centrifugation at 1000 x g for 5 min, cells were resuspended in Beads

Buffer and incubated with anti-mouse IgG Microbeads for 30 min at 4 ºC. After washing, cells were resuspended in Beads Buffer and dispensed into a MS column attached to the MiniMAC magnetic support. Unlabelled cells were washed out with Beads Buffer. After this step, the column was removed from the magnetic support, and the positive fraction of cell suspension was collected by flushing out with Beads Buffer. Cells of the sTn-positive fraction were grown and their sTn expression was evaluated by FACS.

**Sialyltransferase activity assays**

Cell pellets, stored at -80 °C, were homogenized in water. The protein concentration of the homogenates was determined by Lowry method (Lowry, Rosebrough *et al*. 1951). Sialyltransferase activities of these homogenates were measured according to the protocols described below, in the range of linearity with regard to time and substrate concentrations. Homogenates were conserved at -80 °C.

ST3GAL1 activity

ST3GAL1 activity was assayed as previously described by Piller *et al* (Piller, Piller *et al*. 1990) with some modifications. The reaction mixture contained in a 25 µL volume: 50 mM of 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer pH 6.5, 0.5% Triton X-100, 23.5 µg of Galβ1,3GalNAcα1-*O*-benzyl (benzyl-T; Sigma) as acceptor substrate, 15 µM (640 Bq) of CMP-[$^{14}$C]Sia (Amersham) and 50 µg of lysate proteins. Endogenous controls were prepared in the absence of the acceptor substrate. The enzyme reactions were incubated at 37 ºC for 2 h and then the products were isolated by hydrophobic chromatography in SepPak C18 Classic Cartridge (Waters), eluted and measured in a liquid scintillation counter. The incorporation on endogenous substrates was subtracted.

ST6GALNAC1 activity

ST6GALNAC1 activity was assayed as previously described by Dall'Olio *et al* (Dall'Olio, Mariani *et al*. 1997) with some modifications. The reaction mixture contained in a 50 µL volume: 80 mM sodium cacodylate buffer pH 6.5, 0.5% Triton X-100 (Sigma), 0.3 mg of asialo bovine submaxillary mucin (asialo-BSM, prepared by acid desialylation of BSM) as acceptor substrate, 30 µM (1280 Bq) of CMP-[$^{14}$C]Sia (Amersham) and 0.1 mg of homogenate. Endogenous controls were prepared in the absence of acceptor

substrate. The enzyme reactions were incubated at 37 ºC for 2 h and the radioactivity of the pellets was measured in a Wallac Guardian 1414 Liquid Scintillation Counter (Perkin Elmer) as previously described (Dall'Olio, Mariani *et al*. 1997). The incorporation on endogenous substrates was subtracted.

### Neuraminidase treatment

Cells were treated with 20 mU of *Clostridium perfringens* sialidase (Roche Diagnostics) for 90 min at 37 °C. After a wash with PBS, cells were stained with lectins or antibodies and analysed as described above.

### Growth of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG)

Different available strains of BCG were studied, namely the laboratory Pasteur strain (kindly provided by Professor Yi Luo, Harvard Medical School, Boston), and the commercial strains RIVM (RIVM-BCG – Medac, Germany) and Connaught (Connaught-BCG – ImmuCyst, Sanofi Pasteur SA, France).

Pasteur-BCG was maintained as described previously by Luo *et al* (Luo, Szilvasi *et al*. 1996). In short, Pasteur-BCG was grown in 25 mL of Middlebrook 7H9 (Sigma) supplemented with 0.22% glycerol (Sigma), 0.05% Tween 80 (Sigma), 10% of Albumin Dextrose Catalase medium (ADC, Sigma) and 30 μg.mL$^{-1}$ kanamycin (Sigma) for approximately one week. The number of colony forming units (CFU) was monitored daily through the formula CFU.mL$^{-1}$ = $A_{600}$ x 3.2 x $10^7$. When the density of the culture reached 1 unit of optical density (O.D.), BCG was recultured with a starting O.D. of 0.1, or stored in growing medium at -80 ºC. Aliquots were cultured in blood agar plates for 24-48 h at 37 ºC to search for the presence of contaminating microorganisms. Commercial BCG strains were resuspended in PBS with 0.05% of Tween 80 and stored at -80 ºC. Before each assay, BCG aggregates were eliminated by centrifugation (300 x g for 5 min). When required, about 1 unit of $O.D._{600nm}$ of single cell BCG was labelled with 2 μg.mL$^{-1}$ of orange cell tracker dye (OCT – CMTMR, Invitrogen) for 2 h in growth conditions, and incubated for other 2 h in one volume of growing medium.

### BCG internalization by cell lines

BCG internalization by bladder cancer cell lines was accomplished by co-incubating cell lines with different strains of OCT-stained BCG in a cell:bacteria proportion of approximately 1:10. Cell lines, seeded in 24 wells plates, were incubated with 0.25 $O.D._{600nm}$ units of BCG, for 2, 4, 6 or 12 h. Internalization of BCG-OCT by cell lines was evaluated by FACS.

### Real time RT-PCR

Total RNA was isolated using either the GenElute Mammalian Total RNA Purification kit and DNase treatment (Sigma), according to the manufacturer's instructions, or by the guanidinium thiocyanate-method described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). The purity of RNA was determined by $A_{260}/A_{280}$ and $Abs_{260nm}/Abs_{230nm}$, absorbance ratios. Only samples with ratios between 1.9 and 2.1 were used. The absence of genomic DNA was confirmed by electrophoresis in 1% of agarose gel (Invitrogen).

Approximately 1 µg of total RNA was reverse transcribed, using the random-primers-based High Capacity cDNA Archive Kit (Applied Biosystems). Briefly, 50 µL of reaction mixture, containing 2x of random primers, 8 mM of deoxynucleotides solution, 250 U of reverse transcriptase, 2x of buffer and ultrapure water, was added to RNA samples in a 1:1 ratio. The expression levels of the set of sialyltransferases selected (Table 2.1) were evaluated with the TaqMan assay system (Applied Biosystems). Real time PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using the TaqMan Universal PCR Master Mix Fast (Applied Biosystems) as described previously by Videira *et al* (Videira, Calais *et al*. 2009). Briefly, each reaction mixture of 10 µL contained 1x of TaqMan Universal PCR Master Mix Fast, 1.8 µL of cDNA reverse transcribed previously, 1x of each probe and nuclease-free ultrapure water in a 96-well microplate (Fast Optical 96-Well Reaction). An initial denaturation step (95 °C, 20 s) was followed by 40 cycles of denaturation (95 °C, 30 s) and annealing (60 °C, 30 s). During cDNA exponential amplification the product

formation was proportional to the fluorescence emission resulting from the TaqMan probe degradation (van der Velden, Hochhaus *et al*. 2003, Wong and Medrano 2005). The mRNA levels relative to the expression of *ACTB*, which was taken as a suitable endogenous control for bladder cancer cells (Videira, Calais *et al*. 2009). Results were analysed using ΔΔCt method (Livak and Schmittgen 2001).

**Table 2.1. Set of housekeeping gene and sialyltransferases gene assays ID from Applied Biosystems.**

| Gene | Gene Assay ID |
|---|---|
| *ACTB* | Hs00355741_m1 |
| *ST3GAL1* | Hs00161688_m1 |
| *ST3GAL2* | Hs00199480_m1 |
| *ST3GAL3* | Hs00196718_m1 |
| *ST3GAL4* | Hs00272170_m1 |
| *ST3GAL5* | Hs00187405_m1 |
| *ST3GAL6* | Hs00196086_m1 |
| *ST6GAL1* | Hs00174599_m1 |
| *ST6GALNAC1* | Hs00300842_m1 |
| *ST6GALNAC2* | Hs00197670_m1 |
| *ST6GALNAC3* | Hs00541761_m1 |
| *ST6GALNAC4* | Hs00205241_m1 |
| *ST6GALNAC5* | Hs00229612_m1 |
| *ST6GALNAC6* | Hs00203739_m1 |

### Whole transcriptome analysis by expression microarray

Total RNA was prepared as described above, converted to labelled single strand DNA (ssDNA) by the commercial Whole Transcript Expression kit (Ambion) and hybridized into a GeneChip Human Gene 1.0 ST Array (Affymetrix) according to the manufacturer's instructions. In short, 250 ng of sample RNA were spiked-in with the same quantity of control poly-A RNA and converted into cDNA in two steps. The first was important for an accurate synthesis of the first DNA strand with an incorporated T7 promoter; the second was important for the synthesis of the second DNA strand and for the removal of the initial RNA template. After this, an *in vitro* transcription of complementary RNA (cRNA) was performed. cRNA samples were purified from nucleotides, salts and enzymes in a magnetic platform (Affymetrix), by taking advantage of the affinity of RNA for nucleic acid binding beads. From pure cRNA, a complementary ssDNA was obtained by reverse transcription of only the first strand. ssDNA sequences are equivalent to the

RNA sequences existing in the original samples. Samples of ssDNA were purified from nucleotides, salts and enzymes as above. ssDNA sequences with incorporated uracil nucleotides were fragmented by the activities of the uracil-DNA glycosylase (UDG) and the apurinic/apyrimidinic endonuclease 1 (APE1), and labelled at the 3' end with a proprietary biotin-labelled deoxynucleotide analogue using the Terminal Deoxynucleotidyl Transferase (TdT). Labelled ssDNA fragments virtually covering the whole transcriptome of transduced bladder cancer cell lines were then hybridized in a Human Transcriptome Array 2.0 overnight. Fluorescences were read in a GeneChip Scanner 3000 7G (Affymetrix). Raw data were background-subtracted, normalised and summarised with the robust multi-array average (RMA) algorithm implemented in the affy package of Bioconductor (http://www.bioconductor.org). Differential genes between query and control assay were selected by a modified *t*-test implemented in limma package, with $p = 0.05$ cut-off. Array data were analysed by the ArrayStar v2.0 software (DNASTAR).

## Preparation of monocyte derived macrophages

Macrophages were obtained by differentiation of peripheral human monocytes as follows. Blood from the blood collection service of the Pizzardi Hospital of Bologna was diluted one fold with PBS and centrifugated at 200 x g for 30 min at 20 ºC. The interface, containing the majority of the white blood cells, was diluted two folds with PBS and gently stratified in Ficoll-Hypaque (GE Healthcare) in a proportion of 5:3.7. Stratified preparations were centrifuged at 1200 x g for 30 min at 20 ºC and the white interface was carefully collected. This phase, containing the mononuclear cells, was washed abundantly with PBS and resuspended in RPMI 1640 (Sigma) medium supplemented with 20% FCS, 2 mM L-glutamine (Sigma) and 100 $\mu g.mL^{-1}$ penicillin/streptomycin (Sigma). Mononuclear cells were dispensed in plastic Petri dishes and incubated in growth conditions. Medium was daily renewed. After 7 days, monocyte derived macrophages were detached with a cell scraper and dispensed in 24 well plates at approximately 50% of confluence for further assays.

**Induction of cytokine secretion**

*Stimulation in bladder cancer cell lines*

Bladder cancer cell lines were incubated with BCG for 2 h at 37 °C. Then the medium was removed, cells were washed twice with PBS and incubated with fresh medium without BCG for 16 h. Conditioned media were collected and stored at -80 for further analysis. Cells were washed, harvested and used for RNA preparation. The media and cells mock treated in parallel without BCG were used as controls.

*Stimulation in macrophages*

The response of macrophages to components secreted by BCG-treated cell lines was assessed by incubating macrophages with conditioned media from BCG-challenged or unchallenged cell lines obtained as described above. After 24 h, conditioned media were removed and stored at -80 °C for cytokine expression analysis.

**Detection of secreted cytokines**

Conditioned media from cell lines challenged or unchallenged with BCG and those from macrophages incubated with media conditioned by cell lines were characterized for their cytokine content by enzyme-linked immunosorbent assay (ELISA) and multiplex immune-beads assay (MIBA). IL-6 was detected with a commercial 96 wells ELISA kit (Raybiotech) as recommended by manufacturer instructions. In short, ELISA plates coated with anti-IL-6 were incubated at room temperature for 2.5 h with 3:10 diluted supernatants and successively with biotin-conjugated anti-IL-6 (biotin-anti-IL-6) for 1 h with horseradish peroxidase conjugated streptavidin (HRP-streptavidin) for 45 min and with 3,3',5,5'-Tetramethylbenzidine (TBM) substrate solution for 30 min. At the end, an acidic stop solution was added and colorimetric results were read at 450 nm in a multi-channel reader (Multiskan EX, Labsystems). After evaluation of IL-6, ten interleukins (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IFNγ and TNFα) were evaluated in a 96-well strip plate from a commercial MIBA kit (Bio-Rad) as recommended by manufacturer's instructions. In short, a mix of colour-coded micro beads coated with each specific antibody recognizing each studied cytokine were incubated with each supernatant sample in a filtered bottom 96 wells MIBA plate for

30 min at room temperature. Further steps of incubation with a mix of antibodies for 30 min and with Phycoerythrin-streptavidin (PE-streptavidin) for 10 min at room temperature were performed. Fluorescences were read in a Luminex 100 Bio-Plex Liquid Array Multiplexing System reader (Bio-Rad) and the data were analysed with the Bio-Plex Manager v5 software (Bio-Rad).

**Phagocytosis assay**

Phagocytosis was evaluated by incubating apoptotic BCG-challenged and unchallenged cell lines with macrophages as follows. Apoptosis was induced by incubation with 100 µM (for HT1376) or 200 µM (for MCR) of the zinc chelator TPEN (*N*,*N*,*N*',*N*'-tetrakis-(2-pyridylmethyl)ethylenediamine, Sigma) for 48 h. Apoptotic cells were harvested and resuspended in prewarmed serum-free DMEM with 1 µg.mL$^{-1}$ of OCT and incubated for 1 h under growth conditions. Dye solution was then replaced by supplemented DMEM, cells were incubated for another hour under growth conditions, washed with PBS and resuspended in supplemented medium. The same procedure of labelling was carried out on adherent macrophages but with the green cell tracker dye (GCT – CMFDA, Invitrogen). Labelled apoptotic cells were incubated with macrophages in a 2:1 proportion in 24 wells plates and incubated for 2 h in growth conditions. At the end, the medium was removed and wells were washed with PBS. Macrophages were detached with a cell scraper, resuspended in 500 µL of PBS and FACS analysed. The percentage of double labelled macrophages was taken as a measure of the macrophage population which phagocytosed apoptotic cells.

# CHAPTER III

## *Results*

# *III.1 Cellular Models of Bladder Cancer (BC)*

### FACS characterization of cells highly expressing sT or sTn antigens

To investigate the role of Thomsen-Friedenreich related antigens on bladder cancer, cellular models were prepared earlier by our research group (Ferreira, Videira *et al*. 2013). As discussed before, HT1376 and MCR cell lines, derived from patients with TCC of the bladder, were chosen because they presented originally low expression of the sialyl-T (sT) or sialyl-Tn (sTn) antigens and *ST3GAL1* or *ST6GALNAC1* sialyltransferases respectively (Videira, Correia *et al*. 2009). Cell lines resulting from transduction with the cDNA of the two sialyltransferases and successive cloning or enrichment steps, were characterized for the presence of carbohydrate antigens, expression of sialyltransferase mRNA and enzymatic activities.

After the transduction step, the *ST3GAL1*-transduced $HT1376_{ST3G1}$ and the *ST6GALNAC1*-transduced $MCR_{ST6GN1}$ cell lines were composed by populations of cells expressing heterogeneously the sT or sTn antigens respectively (Figure 3.1). The induction of sT antigen expression was indirectly deduced from the decreased labelling with PNA. In fact, this lectin recognizes the T antigen (Galβ1,3GalNAc), which is masked and no longer bound by the lectin after the addition of α2,3-linked sialic acid. On the other hand, sTn expression was directly assessed by the labelling with the antibody anti-sTn.

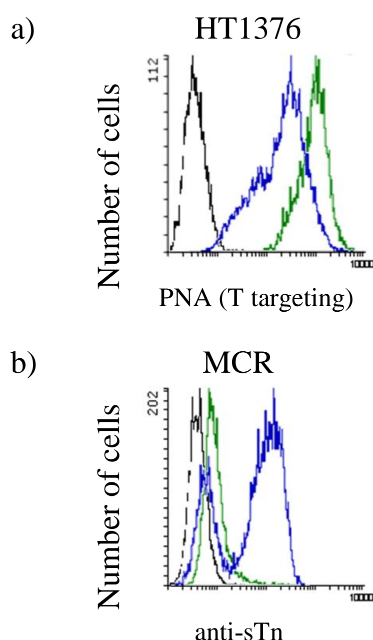



**Figure 3.1. Flow Cytometry analysis of transduced cells.** a) T antigen expression by HT1376 cells. Mock-transduced HT1376$_{NC}$ cells (green line) were strongly reactive for PNA because of the large number of Galβ1,3GalNAc termini. The *ST3GAL1*-transduced HT1376$_{ST3G1}$ cell line presented a bimodal reactivity for PNA (blue line). b) Sialyl Tn expression by MCR cells. sTn expression on mock-transduced MCR$_{NC}$ cells (green line) was negligible, while sTn expression on *ST6GALNAC1*-transduced MCR$_{ST6GN1}$ cells was bimodal (blue line) with a minority of sTn negative cells. Black line: unlabelled cell lines or isotype control antibody when labelling with lectins or antibodies was used, respectively.

To obtain cell populations homogeneously expressing sT or sTn antigens, we adopted two different strategies: clone selection for HT1376$_{ST3G1}$ cells and magnetic sorting on MCR$_{ST6GN1}$ cells. In fact, we first attempted to sort HT1376$_{ST3G1}$ clones, by negative magnetic selection, but as we still failed to obtain homogeneous populations of PNA-negative HT1376$_{ST3G1}$ cells and therefore we opted for clone selection to obtain a PNA-positive population. The new cell models (hereafter named HT1376$_{sT}$ and MCR$_{sTn}$ cells) were highly homogeneous, being comprised of more than 95% of cells either not showing PNA reactivity (Figure 3.2 – blue framed histograms) or expressing sTn (Figure 3.2 – orange framed histograms), respectively. The pattern of carbohydrate structures induced by the expression of the two sialyltransferases was also investigated by flow cytometry using two lectins specific for α2,3-linked sialic acid (*Maackia amurensis* lectin, MAA) or for α2,6-linked sialic acid (*Sambucus nigra* lectin, SNA) (Figure 3.2). *ST3GAL1* overexpression in HT1376 cells induced a slight increase of MAA reactivity while reactivity for SNA remained unchanged. *ST6GALNAC1* overexpression in MCR cells induced, beside the expected effect on sTn antigen expression, also a substantial decrease of the T antigen expression (Figure 3.2 – gray framed histograms). This effect was likely due either to the block by the addition of β1,3-linked galactose to the GalNAc (Tn antigen), since it is already linked by sialic acid (sTn antigen), or to the lack of recognition by PNA of sialyl-6-T antigen [Galβ1,3(Siaα2,6)GalNAc]. Reactivity for SNA consistently augmented, as predicted by the known specificity of SNA for sTn

antigen (Shibuya, Goldstein *et al*. 1987). Mock-transduced and *ST6GALNAC1*-transduced MCR variants showed similar reactivity with MAA. No sTn antigen was detectable in HT1376 cells (Figure 3.2 – gray framed histograms).

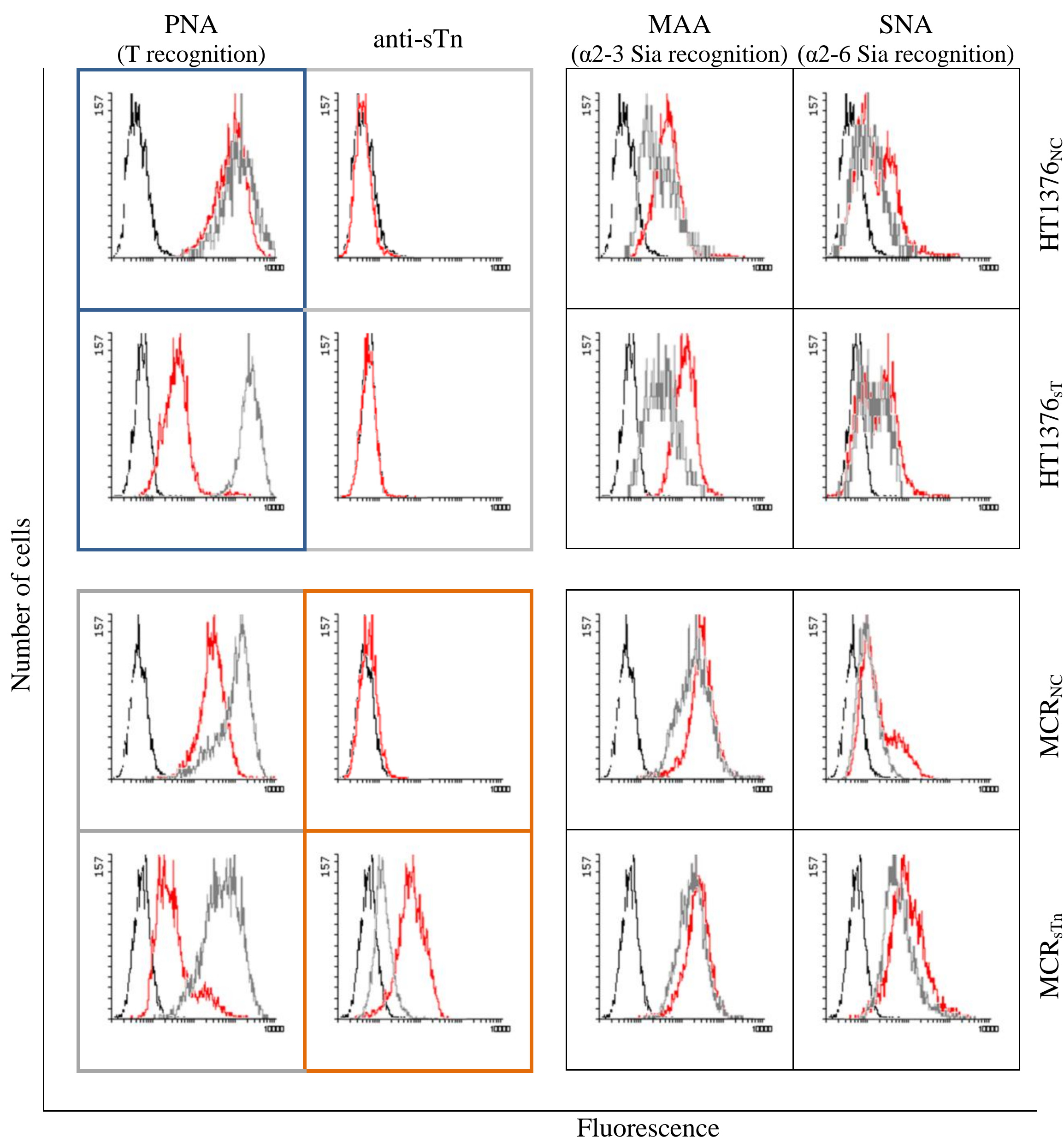



**Figure 3.2. Characterization of transduced bladder cancer cell lines by Flow Cytometry.** Bladder cancer cell lines were labelled with PNA-FITC, MAA-FITC, SNA-FITC and anti-sTn (labelled with anti-Ig-FITC) before (red line) or after (grey line) sialidase treatment. Black line: unlabelled cell lines or isotype control antibody when labelling with lectins or antibodies was used, respectively.

Sialidase treatment of the HT1376$_{sT}$ clone reverted its PNA-reactivity to the same high level of HT1376$_{NC}$ cells, confirming that α2,3-linked sialic acid was responsible for the masking of the Galβ1,3GalNAc structures. By contrast, sialidase treatment had no effect on PNA-reactivity of HT1376$_{NC}$ cells. Together, these results indicate that in HT1376 cells the T antigen is masked only by α2,3-sialylation. Sialidase treatment slightly reduced the MAA reactivity of both HT1376$_{NC}$ and HT1376$_{sT}$ cells and, although at a lesser extent, their SNA reactivity. Sialidase treatment induced a remarkable increase of PNA-reactivity in MCR$_{NC}$ cells and a much higher increase in MCR$_{sTn}$ cells. Altogether, these data suggest that in MCR$_{NC}$ cells, the T antigen is partially masked by the α2,3-sialylation of galactose (sT antigen), thus explaining the increase of PNA reactivity after sialidase treatment. mRNA expression data (see below, Figure 3.5), show that of the three α2,3 sialyltransferases which can synthesize the sT structure (*ST3GAL1*, *ST3GAL2* and *ST3GAL4*), the second and the third are expressed, albeit at a low level, in MCR cells, supporting the notion that a percentage of T antigen molecules are α2,3-sialyl substituted. In MCR$_{sTn}$ cells, in which the level of mRNA expression of transduced *ST6GALNAC1* is about 1000 fold higher than that of *ST3GAL2* and *ST3GAL4* (Figure 3.5), the level of PNA reactivity is very low, indicating that α2,6 sialylation strongly contributes to mask (or inhibit the biosynthesis) of the T antigen. The higher decrease in PNA reactivity observed in MCR$_{sTn}$, suggests that the T antigen is present but almost completely masked by α2,6-sialylation (Figure 3.3). This is consistent with the reported substrate specificity of the cloned human *ST6GALNAC1* (Ikehara, Kojima *et al*. 1999) which indicates that the enzyme can act not only on Tn, but also on T and sT structures.

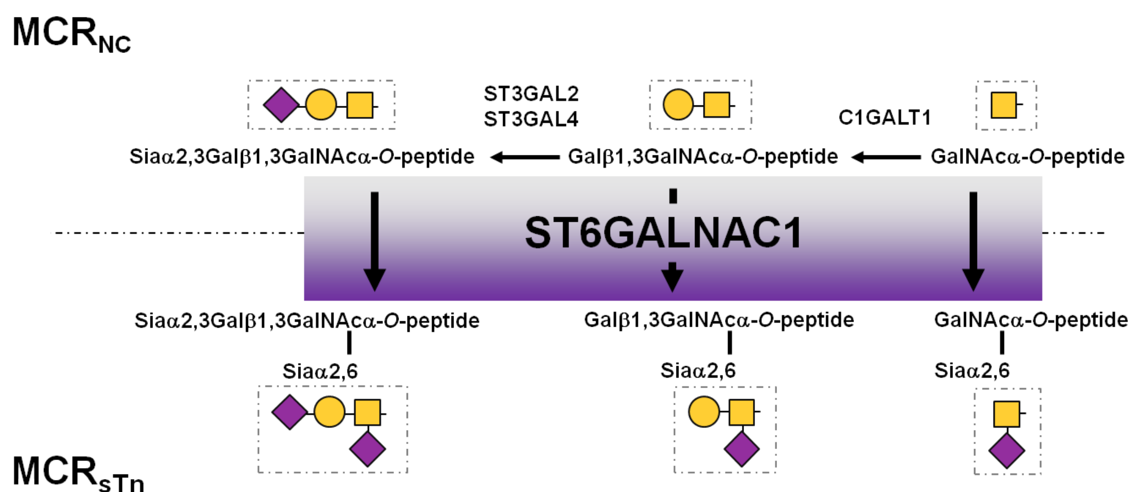



**Figure 3.3. Different PNA reactivity in mock- or *ST6GALNAC1*-transduced MCR cells.** PNA reactivity data indicate that the number of accessible T structures is much lower in $MCR_{sTn}$ cells than in $MCR_{NC}$ cells. This is due to the fact that ST6GALNAC1 acts not only on Tn antigen but also on T and sT structures, resulting in an almost complete masking of the T antigen by α2,6-linked sialic acid. GalNAc: yellow square; Gal: yellow circle; Sia: purple diamond.

## Sialyltransferase expression by selected cell populations

The activities and the mRNA levels of the two sialyltransferase of the new $HT1376_{sT}$ and $MCR_{sTn}$ cell variants were measured to confirm the success of transduction and enrichment steps. The transfer of sialic acid on exogenous acceptors catalysed by cell homogenates was measured by using benzyl-T or asialo-BSM as acceptors for ST3GAL1 or ST6GALNAC1 sialyltransferases, respectively. Enzyme activities of sialyltransferase-transduced cell lines were much higher than that of untransduced or mock-transduced cell lines (which were just above the background). $HT1376_{sT}$ cells presented a 40 fold increase of enzyme activity (Figure 3.4a) and $MCR_{sTn}$ a 20 fold increase of enzyme activity (Figure 3.4c). The specificity of the two acceptor substrates used for the two sialyltransferase assays was not absolute. Although sialyltransferases other than ST3GAL1 can act on benzyl-T, the fact that a high activity was detectable only in *ST3GAL1*-transduced cells, and not in control cells, strongly suggested that incorporation on benzyl-T was due to this enzyme. Also asialo-BSM can be sialylated by sialyltransferases other than ST6GALNAC1, but the fact that a high activity was detectable only in *ST6GALNAC1*-transduced cells strongly suggested that incorporation on asialo-BSM was due to ST6GALNAC1 activity. As expected, the mRNA level of *ST3GAL1* (Figure 3.4b) and *ST6GALNAC1* (Figure 3.4d) was also increased.

Expression of *ST3GAL1* in HT1376$_{sT}$ cells was 156‰ that of β-actin, while in HT1376$_{NC}$ cells it was undetectable. In the same way, in MCR$_{sTn}$ cells the expression of *ST6GALNAC1* was about 7000‰ that of β-actin, about 720 fold the expression of this sialyltransferase in MCR$_{NC}$ cells. These results are consistent with flow cytometry analysis.

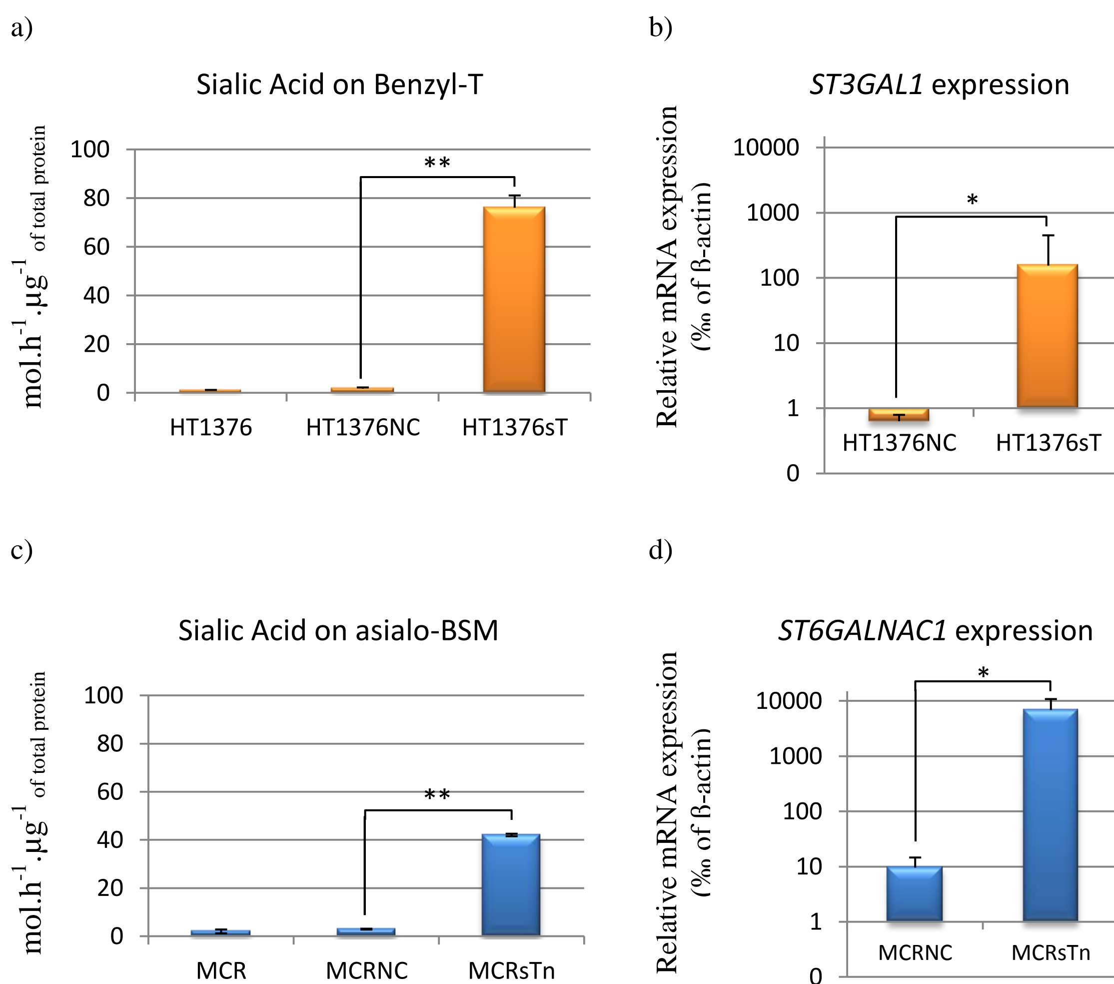
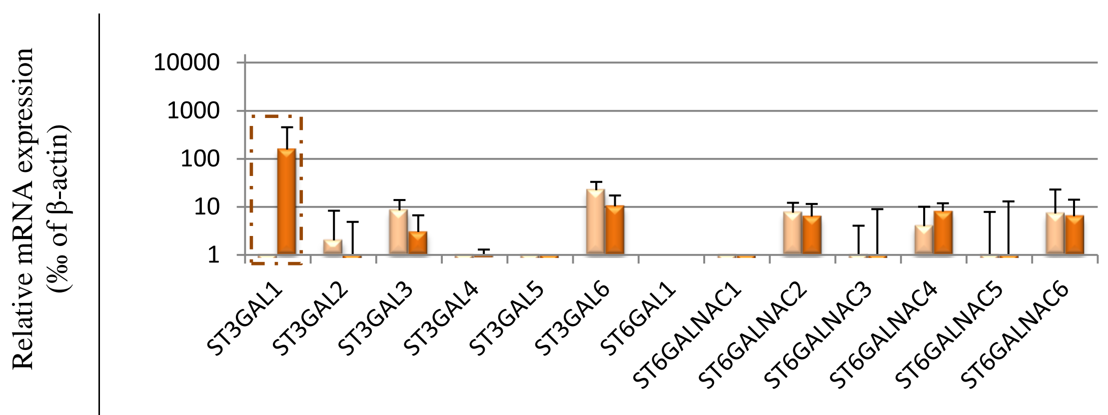



**Figure 3.4. ST3GAL1 and ST6GALNAC1 expression.** Incorporation of radioactive sialic acid ([$^3$H]-Sia) into a) benzyl-T by HT1376 cell homogenates or into c) asialo-BSM by MCR cell homogenates. The sialyltransferase activity was negligible in untransduced HT1376 or MCR, and in mock-transduced HT1376$_{NC}$ or MCR$_{NC}$ cells. Both selection procedures led to increase the respective sialyltransferase activities. Consonant expression of b) *ST3GAL1* in HT1376$_{sT}$ cells and of d) *ST6GALNAC1* in MCR$_{sTn}$ cells was found. Data are the mean ±SD of 3 experiments. ** $p < 0.001$; * $p < 0.05$.

The level of mRNA expression of several sialyltransferases in *ST3GAL1*- and *ST6GALNAC1*- transduced cell lines and in their respective negative controls was accomplished by Real-time PCR (Figure 3.5).

a)




b)

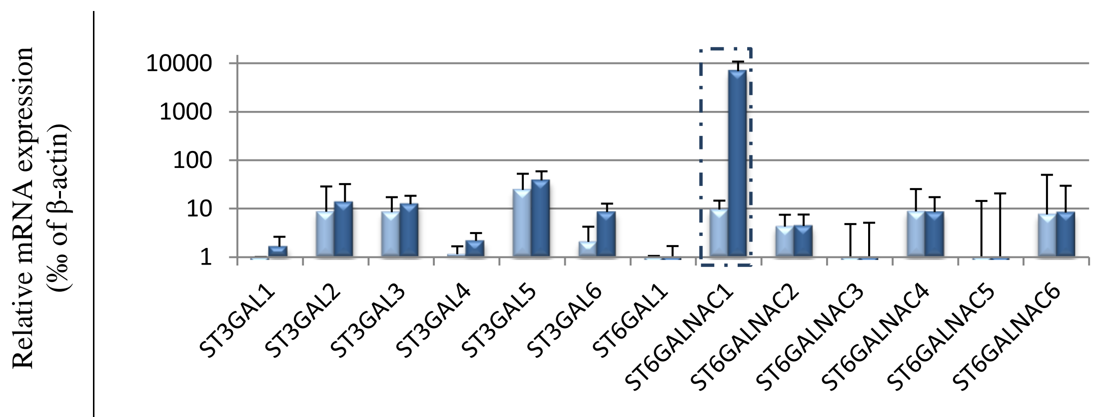



**Figure 3.5. Sialyltransferase mRNA expression in transduced cells.** Relative mRNA levels of several sialyltransferases were analysed in a) $HT1376_{NC}$ (pink bars), $HT1376_{sT}$ (orange bars) and b) $MCR_{NC}$ (light blue bars) and $MCR_{sTn}$ (dark blue bars) cells by RT-qPCR. Results were normalized for 1000 molecules of the endogenous control *β*-actin. High levels of *ST3GAL1* and *ST6GALNAC1* mRNA were detected in $MCR_{sTn}$ and in $HT1376_{sT}$ respectively, while the expression of other sialyltransferases were low or undetectable e was not interfered significantly after transduction. Data are the mean ±SD of 3 experiments. Student's t test revealed that sialyltransferases *ST3GAL1* and *ST6GALNAC1* were the only statistically modulated in $HT1376_{sT}$ and $MCR_{sTn}$, respectively. Boxed expression data are the same reported in Figure 3.4 and are repeated here for comparison.

In both HT1376$_{NC}$ and HT1376$_{sT}$ cells, low levels (usually lower than 10‰ of β-actin) of the sialyltransferases *ST3GAL3*, *ST3GAL6*, *ST6GALNAC2*, *ST6GALNAC4* and *ST6GALNAC6* mRNA were detected, while sialyltransferases *ST3GAL2*, *ST3GAL4*, *ST3GAL5, ST6GAL1*, *ST6GALNAC1*, *ST6GALNAC3* and *ST6GALNAC5* mRNA were nearly undetectable. In both MCR$_{NC}$ and MCR$_{sTn}$ cells, low levels (usually lower than 10‰ of β-actin) of the sialyltransferases *ST3GAL2, ST3GAL3, ST3GAL4 ST3GAL5*, *ST3GAL6*, *ST6GALNAC2, ST6GALNAC4* and *ST6GALNAC6* were detected, while *ST3GAL1*, *ST6GAL1*, *ST6GALNAC3* and *ST6GALNAC5* mRNA were nearly undetectable. Apparently, the expression of *ST3GAL1* and *ST6GALNAC1* did not interfere significantly on the expression of other sialyltransferases. In general, the above reported results indicate that, apart from the expression of the transduced genes and their cognate structures, the mock-transduced and the sialyltransferase-transduced cell lines showed to be similar, if not identical, allowing to establish clear cause-effect relationships between phenotype and sialyltransferases expression.

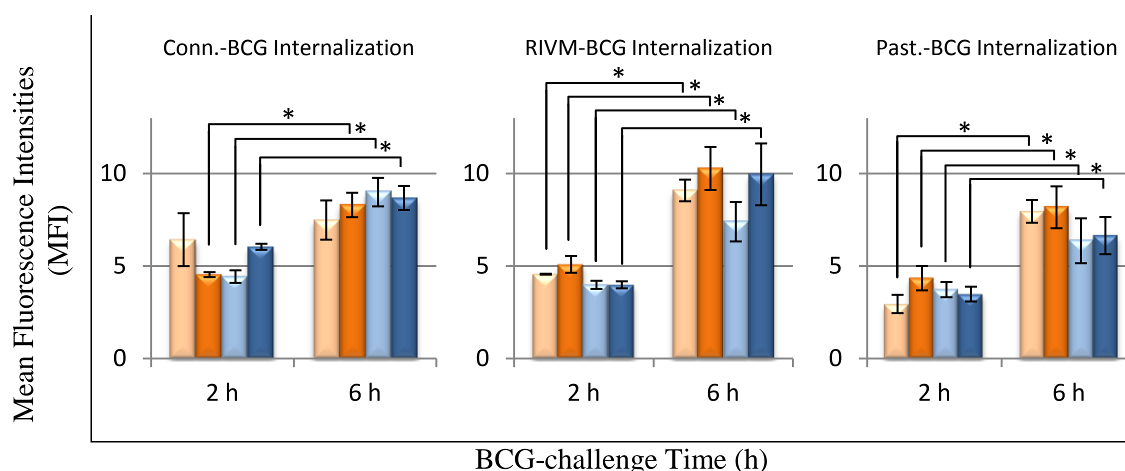
# *III.2 Response of BC cell lines to BCG*

As previously mentioned, attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is used in the therapy of TCC of the bladder. For a better understanding of the role of sialyl Thomsen-Friedenreich antigens in tumour progression, we analysed several steps of the interactions between BCG and bladder cancer cells. Each step was analysed with regard to presence/absence of the sialylated Thomsen-Friedenreich related antigens. In this section, we will describe the effects of the interactions of bladder cancer cell lines expressing sT or sTn antigens with BCG. In particular, we will analyse BCG internalization, cytokine secretion and whole transcriptome changes in bladder cancer cell lines.

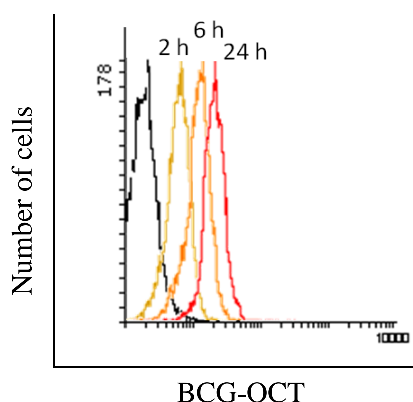
**BCG Internalization**

BCG therapy consists in the intravesical instillation of BCG for 2 hours. Internalization of BCG by bladder cancer cells was studied by using three different BCG strains (Connaught-BCG, RIVM-BCG and Pasteur-BCG). In Figure 3.6 is shown the time course of internalization of the three BCG strains by transduced $HT1376_{sT}$ and $MCR_{sTn}$ cell lines and the respective mock-transduced cell lines. Over long times, the process of BCG internalization displayed obvious time dependence (Figure 3.6b and 3.6c), while no significant differences among different BCG strains or different cell lines were observed.

a)




b)




c)

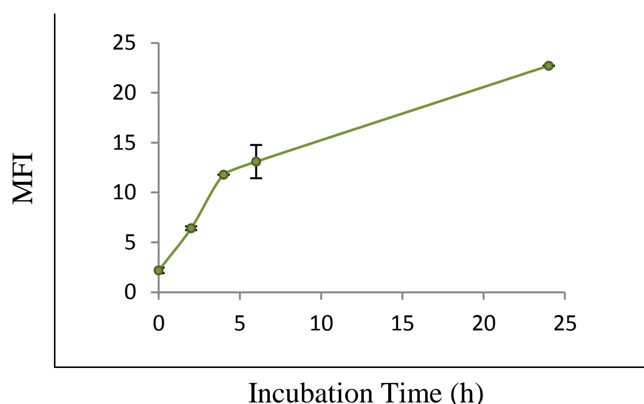



**Figure 3.6. Time course of BCG internalization by bladder cancer cell lines.** a) Analysis by FACS of the internalization of Connaught-BCG, RIVM-BCG or Pasteur-BCG by $HT1376_{NC}$ cells (light orange bars), $HT1376_{sT}$ cells (orange bars), $MCR_{NC}$ cells (light blue bars) and $MCR_{sTn}$ cells (dark blue bars), after 2 or 6 h of incubation with BCG. Internalization values were normalized with the respective controls incubated at 4 ºC. Data are the mean ± SD of 3 experiments. * $p < 0.05$. b) and c) An example of time-dependent Connaught-BCG internalization by $MCR_{sTn}$ cells $MFI_{2h} = 7$, $MFI_{6h} = 13$ and $MFI_{24h} = 23$. Black line: internalization at 4 ºC. Over longer periods of time, internalization of BCG displayed a two slope curve with tendency to saturation. OCT stands for orange cell tracker.

According to these results, BCG internalization might be independent from sT or sTn Thomsen-Friedenreich antigens since no significant differences were observed in BCG internalisation among sialyltransferase- and mock-transduced cell lines. Nonetheless the strain RIVM of BCG showed a tendency to be more internalized by $HT1376_{sT}$ or $MCR_{sTn}$. In successive experiments the Connaught-BCG strain was used with an incubation time of 2 hours, mimicking the instillation time in therapy.

## Transcriptome analysis of bladder cancer cell lines

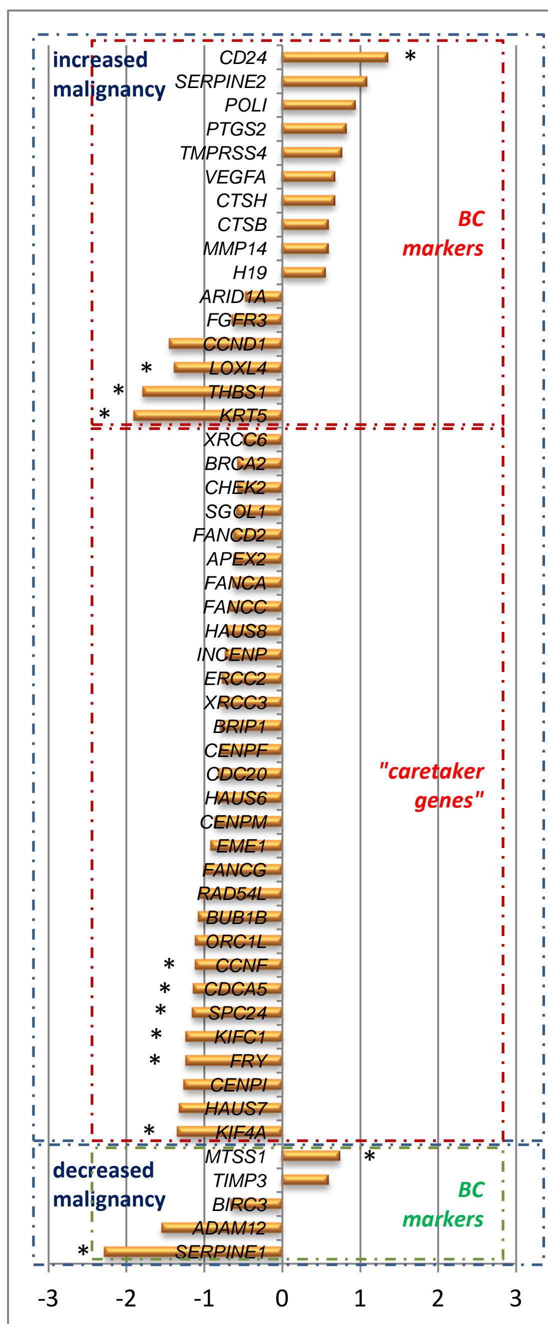
The overall impact of the expression of *ST3GAL1* or *ST6GALNAC1* sialyltransferases and of BCG-challenge on our models was evaluated by analysing by expression microarray technology the global modulation of gene expression. This technology used over than 6 million probes covering coding transcripts and non-coding transcripts. In a first step, the analysis was focused on genes displaying modulation as a response to sialyltransferase-transduction, regardless BCG-challenging. In a second step, we focused on the set of genes modulated by BCG-challenging regardless sialyltransferase expression. All significant modulations are presented as heat-maps in Supplement I section. After that, based on the literature, gene signatures of “bladder cancer markers” and “caretaker genes” were identified and are presented in Figures 3.7 and 3.9. Transcripts that showed a tendency for modulation in the context of the same gene signatures were also included.

### *Effect of ST3GAL1 expression – Gene Signatures of HT1376 cells*

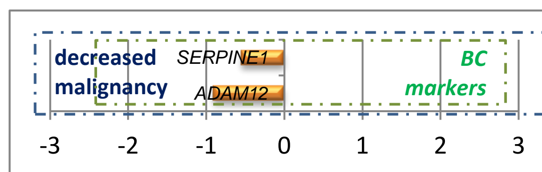
*ST3GAL1* expression in HT1376 cells impacts several genes involved in specific cell functions (Figure 3.7a). In particular, a group of genes whose products affect mitotic fidelity and chromosomal stability show a consistent down-regulation. If the analysis is restricted to genes showing modulation by at least a factor 2 (*i.e.* whose expression in $HT1376_{sT}$ cells is at least double or half that of $HT1376_{NC}$ cells), genes *ORC1L*, *SPC24*, *BUB1B*, *CENPI*, *FRY*, *KIFC1*, *HAUS7*, *CCNF* and *CDCA5*, which are involved in the accuracy of chromosomal segregation during mitosis, show down-regulation in $HT1376_{sT}$ cells. Some genes crucially involved in the mechanism of repair of double strand breaks known as homologous recombination repair (HR), such as *FANCA*, *FANCC*, *FANCD2*, *FANCG*, *RAD54L* and *KIF4A*, also show down-regulation in $HT1376_{sT}$ cells. Other genes involved in HR, such as *BRCA2*, *BRIP1*, *XRCC3*, *EME1*, as well as genes involved in other mechanisms of DNA repair, like base excision repair (BER) and nucleotide excision repair (NER) (*APEX2* and *ERCC2*, respectively) display down-regulation in $HT1376_{sT}$ cells, although at a lower extent. It can be hypothesized that, owing to the general down-regulation of the genes preserving chromosome number and integrity, which can be

collectively defined as “caretaker genes”, $HT1376_{sT}$ cells are more prone to display alterations of chromosome number and structure. $HT1376_{sT}$ cells display decreased expression of *ADAM12*, a protease involved in Notch signaling and of *SERPINE1*, but increased expression of *SERPINE2*. The two latter genes encode protease inhibitors with pleiotropic functions. Moreover, $HT1376_{sT}$ cells display decreased expression of two genes affecting cell growth, such as cyclin D1 (*CCND1*) and lysyl oxidase-like 4 (*LOXL4*), which inhibits Ras/ERK signaling. The genes encoding trombospondin 1 (*THBS1*) and keratin 5 (*KRT5*) also display down-regulation. In an attempt to predict the putative functional consequences of the gene modulation, we performed a literature search for the individual contribution of the modulated genes to the bladder neoplastic phenotype. This search revealed that the modulation of 46 genes was toward a more malignant phenotype while only 5 genes were modulated toward a less malignant phenotype. Sixteen of the genes modulated toward increased malignancy were reported in the literature as bladder cancer markers (*CD24*, *SERPINE2*, *POLI*, *PTGS2*, *TMPRSS4*, *VEGFA*, *CTSH*, *CTSB*, *H19* and *MMP14* genes were up-regulated, while *ARID1A*, *FGFR3*, *CCND1*, *LOXL4*, *THBS1* and *KRT5* genes were down-regulated), On the other hand, 5 genes reported as “bladder cancer markers” moved toward decreased malignancy (*MTSS1* and *TIMP3* genes were up-regulated, and *BIRC3*, *ADAM12* and SERPINE1 genes were down-regulated). The challenging with BCG appears to affect poorly the gene expression profile of $HT1376_{NC}$ cells whereas in $HT1376_{sT}$ cells we observed a tendency to up-regulation of the genes preserving genomic stability, although their changes is usually lower than a factor 2 (Figures 3.7b and 3.7c). A group of genes encoding proteases or their inhibitors also display a slight modulation in $HT1376_{sT}$ cells.

a) Genes modulated by *ST3GAL1* expression in HT1376 cells




b) Genes modulated by BCG-challenging in $HT1376_{NC}$ cells




c) Genes modulated by BCG-challenging in $HT1376_{sT}$ cells

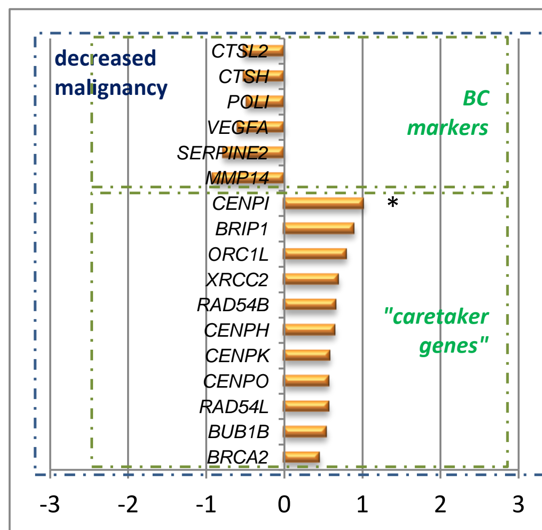



**Figure 3.7. Gene signatures in HT1376 cells.** Expression variation of relevant genes in a) $HT1376_{sT}$ cells *vs.* $HT1376_{NC}$ cells, in b) $HT1376_{NC}$ cells and in c) $HT1376_{sT}$ cells after BCG-challenging. Genes were grouped in “bladder cancer markers” and “caretaker” categories, according to putatively increased or decreased malignant phenotypes. Only variations greater then ± 0.5 were considered. Data are the mean of 3 experiments. * $p \leq 0.05$.

In Tables 3.1 to 3.3 are presented the selected signature genes presented in figure 3.7 with gene description, hosting chromosome, GenBank code and a short outline of the function.

**Table 3.1. Description of up- or down-regulated genes of HT1376$_{sT}$ cells *vs.* HT1376$_{NC}$ cells.**

| Gene Signatures modulated by *ST3GAL1* expression | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Symbol | Description | Chr | GenBank | Log2E (HT1376$_{NC}$) | Log2E (HT1376$_{sT}$) | Difference (sT-NC) | Function | Ref |
| **Changes increasing malignancy** – Bladder Cancer markers | | | | | | | | |
| CD24 | CD24 molecule | 6 | NM_013230 | 5,0 | 6,4 | 1,4 | mucin-type glycoprotein, prognostic marker (BC) | (Choi, Lee *et al.* 2007, Lee, Kim *et al.* 2009, Fenner 2012, Liu, Zheng *et al.* 2013) |
| SERPINE2 | serpin peptidase inhibitor; clade E (nexin; plasminogen activator inhibitor type 1); member 2 | 2 | NM_001136529 | 7,4 | 8,5 | 1,1 | serine peptidase inhibitor, pleiotropic functions besides haemostasis | (Bergeron, Lemieux *et al.* 2010, Nagahara, Nakayama *et al.* 2010, Xu, McKee *et al.* 2010) |
| POLI | polymerase (DNA directed) iota | 18 | NM_007195 | 9,0 | 9,9 | 0,9 | overexpressed POLI contributes to hypermutagenesis (BC) | (Yuan, Xu *et al.* 2013) |
| PTGS2 | prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) | 1 | NM_000963 | 9,8 | 10,6 | 0,8 | prostaglandins production (BC) | (Czachorowski, Amaral *et al.* 2012, Ke, Tu *et al.* 2012, Tabriz, Olfati *et al.* 2013) |
| TMPRSS4 | transmembrane protease; serine 4 | 11 | NM_019894 | 7,9 | 8,7 | 0,8 | highly expressed in pancreatic, thyroid, colon and other cancer tissues (C) | (Kim, Kuppireddy *et al.* 2012, Cheng, Kong *et al.* 2013, Cheng, Liang *et al.* 2013, Min, Lee *et al.* 2013) |
| VEGFA | vascular endothelial growth factor A | 6 | NM_00102536 | 10,3 | 11,0 | 0,7 | stimulates the formation of new blood vessels and tumour growth (BC) | (Fauconnet, Bernardini *et al.* 2009, Zaravinos, Volanis *et al.* 2012, Kopparapu, Boorjian *et al.* 2013) |
| CTSH | cathepsin H | 15 | NM_004390 | 9,9 | 10,6 | 0,7 | overexpression and release by cancerous tissues; degradation of extracellular matrix components (BC) | (Staack, Koenig *et al.* 2002, Staack, Tolic *et al.* 2004) |
| CTSB | cathepsin B | 8 | NM_147780 | 9,5 | 10,1 | 0,6 | overexpression and release by cancerous tissues; degradation of extracellular matrix components (BC) | (Eijan, Sandes *et al.* 2003, Staack, Tolic *et al.* 2004, Svatek, Karam *et al.* 2008, Kotaska, Dusek *et al.* 2012) |

**Table 3.1. Description of up- or down-regulated genes of HT1376$_{sT}$ cells *vs.* HT1376$_{NC}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| H19 | H19, imprinted maternally expressed transcript (non-protein coding) | 11 | NR_002196 | 10,4 | 11,0 | 0,6 | a long non-coding RNA induced in bladder cancer (BC) | (Matouk, DeGroot *et al*. 2007, Luo, Li *et al*. 2013, Luo, Li *et al*. 2013) |
| MMP14 | matrix metallopeptidase 14 (membrane-inserted) | 14 | NM_004995 | 9,4 | 10,0 | 0,6 | degradation of basal membranes and of the extracellular matrix (BC) | (Chaffer, Dopheide *et al*. 2005, Mohammad, Ismael *et al*. 2010, Reis, Leite *et al*. 2012) |
| ARID1A | AT rich interactive domain 1A (SWI-like) | 1 | NM_006015 | 8,8 | 8,3 | -0,5 | actin dependent regulator of chromatin structure, acting as a tumour suppressor (BC) | (Gui, Guo *et al*. 2011, Balbas-Martinez, Rodriguez-Pinilla *et al*. 2013, Guo, Zhang *et al*. 2013) |
| FGFR3 | fibroblast growth factor receptor 3 | 4 | NM_000142 | 7,6 | 7,0 | -0,6 | regulation of cell growth and division (BC) | (van Rhijn, van der Kwast *et al*. 2012, Gust, McConkey *et al*. 2013, Sjodahl, Lovgren *et al*. 2013) |
| CCND1 | cyclin D1 | 11 | NM_053056 | 9,0 | 7,6 | -1,4 | G(1)-phase cell cycle regulatory molecule (BC) | (Pantazis, Soulitzis *et al*. 2011, Sjodahl, Lovgren *et al*. 2013) |
| THBS1 | thrombospondin 1 | 15 | NM_003246 | 7,7 | 5,9 | -1,8 | antiangiogenic factor (BC) | (Goddard, Sutton *et al*. 2002, Brunner and Tzankov 2007, Donmez, Sullu *et al*. 2009, Shariat, Youssef *et al*. 2010) |
| KRT5 | keratin 5 | 12 | NM_000424 | 10,4 | 8,6 | -1,9 | KRTs are differentially expressed during epithelial tissue differentiation (BC) | (Volkmer, Sahoo *et al*. 2012, Balbas-Martinez, Rodriguez-Pinilla *et al*. 2013, Sjodahl, Lovgren *et al*. 2013) |
| LOXL4 | lysyl oxidase-like 4 | 10 | NM_032211 | 7,1 | 5,7 | -1,4 | act as tumour suppressor by inhibiting the Ras/ERK signaling pathway (BC) | (Wu, Guo *et al*. 2007) |

**Table 3.1. Description of up- or down-regulated genes of HT1376$_{sT}$ cells *vs.* HT1376$_{NC}$ cells (continued).**

| **Changes increasing malignancy** – caretaker genes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| XRCC6 | X-ray repair complementing defective repair in Chinese hamster cells 6 | 22 | NM_001469 | 10,6 | 10,1 | -0,5 | plays an essential role in the DNA double-strand break (DSB) repair pathway, *i.e.*, non homologous DNA-end-joining (NHEJ) | (Koike, Yutoku *et al*. 2011, Chang, Ke *et al*. 2012, Hsu, Yang *et al*. 2013, Xu, Zou *et al*. 2013) |
| BRCA2 | breast (C) 2, early onset | 13 | NM_000059 | 8,8 | 8,3 | -0,6 | important role in the homologous recombination pathway for double-strand DNA repair and in the G2 checkpoint following DNA-damage (BC) | (Goode, Ulrich *et al*. 2002, Wild, Catto *et al*. 2007, Catto, Abbod *et al*. 2010) |
| CHEK2 | CHK2 checkpoint homolog (S. pombe) | 22 | NM_001005735 | 6,9 | 6,3 | -0,6 | key cell cycle control gene encoding a pluripotent kinase that can cause arrest or apoptosis in response to unrepaired DNA damage (BC) | (Slojewski, Zlowocka *et al*. 2008, Zlowocka, Cybulski *et al*. 2008) |
| SGOL1 | shugoshin-like 1 (S. pombe) | 3 | NM_001012410 | 8,5 | 7,9 | -0,6 | is required for maintenance of centromeric cohesion from prophase to the metaphase-anaphase transition | (Diaz-Martinez, Gimenez-Abian *et al*. 2007, Pouwels, Kukkonen *et al*. 2007, Kang, Chaudhary *et al*. 2011) |
| FANCD2 | Fanconi anemia, complementation group D2 | 3 | NM_033084 | 8,5 | 7,9 | -0,6 | required to prevent accumulation of replication-associated DNA double-strand breaks and maintenance of normal chromosome stability (BC) | (Neveling, Kalb *et al*. 2007, Wilson, Yamamoto *et al*. 2008) |
| APEX2 | APEX nuclease (apurinic/apyrimidinic endonuclease) 2 | X | NM_014481 | 8,3 | 7,7 | -0,6 | apurinic/apyrimidinic endonuclease participating in the base excision repair (BER) system | (Nakabeppu, Tsuchimoto *et al*. 2004, Grunda, Fiveash *et al*. 2010, Al-Safi, Odde *et al*. 2012) |

**Table 3.1. Description of up- or down-regulated genes of HT1376$_{sT}$ cells *vs.* HT1376$_{NC}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FANCA | Fanconi anemia, complementation group A | 16 | NM_000135 | 8,4 | 7,7 | -0,6 | required to prevent accumulation of replication-associated DNA double-strand breaks and maintenance of normal chromosome stability (BC) | (Neveling, Kalb *et al.* 2007) |
| FANCC | Fanconi anemia, complementation group C | 9 | NM_000136 | 7,6 | 6,9 | -0,7 | required to prevent accumulation of replication-associated DNA double-strand breaks and maintenance of normal chromosome stability (BC) | (Neveling, Kalb *et al.* 2007) |
| HAUS8 | HAUS augmin-like complex, subunit 8 | 19 | NM_033417 | 6,4 | 5,7 | -0,7 | HAUS disruption results in the destabilization of kinetochore microtubules and the eventual formation of multipolar spindles | (Lawo, Bashkurov *et al.* 2009) |
| INCENP | inner centromere protein antigens 135/155kDa | 11 | NM_00104069 | 8,6 | 7,9 | -0,7 | playing a role in the regulation of sister chromatid separation in mitosis | (Pouwels, Kukkonen *et al.* 2007, Kang, Chaudhary *et al.* 2011) |
| ERCC2 | excision repair cross-complementing rodent repair deficiency, complementation group 2 | 19 | NM_000400 | 7,5 | 6,7 | -0,8 | plays a critical role in nucleotide excision repair (NER) and basal transcription (BC) | (Fontana, Bosviel *et al.* 2008, Stern, Lin *et al.* 2009, Gangawar, Ahirwar *et al.* 2010) |
| XRCC3 | X-ray repair complementing defective repair in Chinese hamster cells 3 | 14 | NM_001100119 | 6,9 | 6,2 | -0,8 | plays an important role in the repair of DNA double-strand breaks by homologous recombination (BC) | (Fontana, Bosviel *et al.* 2008, Sun, Qiao *et al.* 2010, Li, Li *et al.* 2011, Mittal, Gangwar *et al.* 2012) |
| BRIP1 | BRCA1 interacting protein C-terminal helicase 1 | 17 | NM_032043 | 8,7 | 7,9 | -0,8 | BRCA1-associated DNA helicase that contributes to homologous recombination and cross-link repair (BC) | (Figueroa, Malats *et al.* 2007) |

**Table 3.1. Description of up- or down-regulated genes of HT1376$_{sT}$ cells *vs.* HT1376$_{NC}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CENPF | centromere protein F, 350/400ka (mitosin) | 1 | NM_016343 | 10,0 | 9,2 | -0,8 | centromeric protein, important role in the fidelity of chromosome segregation | (Pouwels, Kukkonen *et al.* 2007, Cheeseman, Hori *et al.* 2008) |
| CDC20 | cell division cycle 20 homolog (S. cerevisiae) | 1 | NM_001255 | 11,3 | 10,4 | -0,8 | mediating the association of the mitotic spindle checkpoint protein MAD1L1 with the cyclosome/anaphase-promoting complex | (Kops, Kim *et al.* 2005, Famulski, Vos *et al.* 2008) |
| HAUS6 | HAUS augmin-like complex, subunit 6 | 9 | NM_017645 | 7,9 | 7,1 | -0,8 | HAUS disruption results in the destabilization of kinetochore microtubules and the eventual formation of multipolar spindles | (Lawo, Bashkurov *et al.* 2009) |
| CENPM | centromere protein M | 22 | NM_024053 | 8,4 | 7,5 | -0,9 | centromeric protein, important role in the fidelity of chromosome segregation | (Foltz, Jansen *et al.* 2006) |
| EME1 | essential meiotic endonuclease 1 homolog 1 (*S. pombe*) | 17 | NM_152463 | 7,3 | 6,4 | -0,9 | catalyses with MUS81 coordinate bilateral cleavage of model Holliday-junction structures | (Forment, Blasius *et al.* 2011, Zhao, Liu *et al.* 2013) |
| FANCG | Fanconi anemia; complementation group G | 9 | NM_004629 | 8,2 | 7,2 | -1,0 | required to prevent accumulation of replication-associated DNA double-strand breaks and maintenance of normal chromosome stability (BC) | (Neveling, Kalb *et al.* 2007, Wilson, Yamamoto *et al.* 2008) |
| RAD54L | RAD54-like (S. cerevisiae) | 1 | NM_003579 | 8,5 | 7,5 | -1,0 | recombination protein associated with RAD51, involved in NER of double-strand break and in meiotic recombination | (Li, Frazier *et al.* 2006, Nowacka-Zawisza, Brys *et al.* 2006, Romanowicz-Makowska and Smolarz 2006) |
| BUB1B | budding uninhibited by benzimidazoles 1 homolog beta (yeast) | 15 | NM_001211 | 9,8 | 8,7 | -1,1 | important role in the spindle checkpoint, reduction of BUB1B results in premature chromatid separation and aneuploidy | (Hernando, Orlow *et al.* 2001, Bohers, Sarafan-Vasseur *et al.* 2008, Izumi, Matsumoto *et al.* 2009) |

**Table 3.1. Description of up- or down-regulated genes of $HT1376_{ST}$ cells *vs.* $HT1376_{NC}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ORC1L | origin recognition complex, subunit 1-like (yeast) | 1 | NM_004153 | 8,2 | 7,1 | -1,1 | localized in the centrosomes preventing Cyclin E-dependent reduplication of centrioles and centrosomes during the cell division | (Hemerly, Prasanth *et al*. 2009, Ferguson, Pascreau *et al*. 2010, Hossain and Stillman 2012) |
| CCNF | cyclin F | 16 | NM_001761 | 9,0 | 7,9 | -1,1 | fine-tunes centrosome duplication and DNA synthesis, by promoting the elimination of RRM2 and by inducing CP110 degradation, it prevents chromosome instability | (D'Angiolella, Donato *et al*. 2012, D'Angiolella, Esencay *et al*. 2013, Li, D'Angiolella *et al*. 2013) |
| CDCA5 | cell division cycle associated 5 | 11 | NM_080668 | 8,5 | 7,4 | -1,1 | is involved in maintenance of cohesion in response to the spindle checkpoint | (Diaz-Martinez, Gimenez-Abian *et al*. 2007, Dreier, Bekier *et al*. 2011, Wu, Nguyen *et al*. 2011) |
| SPC24 | SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae) | 19 | NM_182513 | 8,4 | 7,3 | -1,1 | required to establish and maintain kinetochore-microtubule attachment | (McCleland, Kallio *et al*. 2004, Wei, Schnell *et al*. 2006) |
| KIFC1 | kinesin family member C1 | 6 | NM_002263 | 9,4 | 8,1 | -1,2 | plays an essential role for bipolar microtubule organizing centres (MTOC) formation and maintaining chromosomal stability during mitosis | (Kim and Song 2013) |
| FRY | furry homolog (Drosophila) | 13 | NM_023037 | 9,1 | 7,9 | -1,2 | FRY mediated activation of STK38 is crucial for the fidelity of mitotic chromosome alignment | (Chiba, Ikeda *et al*. 2009, Kohler, Schmitz *et al*. 2010) |
| CENPI | centromere protein I | X | NM_006733 | 8,6 | 7,4 | -1,2 | centromeric protein, important role in the fidelity of chromosome segregation | (Liu, Hittle *et al*. 2003, Izuta, Ikeno *et al*. 2006, Cheeseman, Hori *et al*. 2008) |
| HAUS7 | HAUS augmin-like complex, subunit 7 | X | NM_207107 | 7,9 | 6,5 | -1,3 | HAUS disruption results in the destabilization of kinetochore microtubules and the eventual formation of multipolar spindles | (Lawo, Bashkurov *et al*. 2009) |

**Table 3.1. Description of up- or down-regulated genes of HT1376$_{sT}$ cells *vs.* HT1376$_{NC}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| KIF4A | kinesin family member 4A | X | NM_012310 | 9,3 | 8,0 | -1,3 | associates with BRCA2, upon nucleus-specific laser micro-irradiation, Kif4A was rapidly recruited to sites of DNA damage | (Wu and Chen 2008, Wu, Zhou *et al.* 2008, Gao, Sai *et al.* 2011) |
| **Changes decreasing malignancy** – Bladder Cancer markers | | | | | | | | |
| MTSS1 | metastasis suppressor 1 | 8 | NM_014751 | 6,2 | 7,0 | 0,7 | cytoskeletal scaffold protein (BC) | (Wang, Liu *et al.* 2007, Du, Ye *et al.* 2011, Xie, Ye *et al.* 2011) |
| TIMP3 | TIMP metallopeptidase inhibitor 3 | 22 | NM_000362 | 9,0 | 9,7 | 0,6 | tissue inhibitor of metalloproteinase (BC) | (Hoque, Begum *et al.* 2008, Kandimalla, van Tilborg *et al.* 2013, Wieczorek, Reszka *et al.* 2013) |
| BIRC3 | baculoviral IAP repeat-containing 3 | 11 | NM_001165 | 9,8 | 9,2 | -0,6 | regulator of apoptosis; its overexpression may make tumours resistant to apoptosis (BC) | (Che, Yang *et al.* 2012, He, Wang *et al.* 2012) |
| ADAM12 | ADAM metallopeptidase domain 12 | 10 | NM_003474 | 8,6 | 7,0 | -1,5 | a disintegrin and metalloprotease, multifunctional zinc-dependent metzincin protease (BC) | (Frohlich, Albrechtsen *et al.* 2006, Kogure, Takawa *et al.* 2013) |
| SERPINE1 | serpin peptidase inhibitor; clade E (nexin; plasminogen activator inhibitor type 1); member 1 | 7 | NM_000602 | 9,6 | 7,3 | -2,3 | serine peptidase inhibitor, pleiotropic functions besides haemostasis (BC) | (Chen, Henry *et al.* 2009, Litlekalsoy, Hostmark *et al.* 2012, Rosser, Ross *et al.* 2013) |

*Log2E* stands for the base 2 logarithm of a given expression value.

**Table 3.2. Description of up- or down-regulated genes by BCG-challenging in HT1376$_{NC}$ cells.**

| Symbol | Description | Chr | GenBank | Log2E (HT1376NC) | Log2E (after BCG) | Difference (BCG-NC) | Function | Ref |
|---|---|---|---|---|---|---|---|---|
| **Genes modulated by BCG-challenging in HT1376NC cells** | | | | | | | | |
| **Changes decreasing malignancy** – Bladder Cancer markers | | | | | | | | |
| SERPINE1 | serpin peptidase inhibitor; clade E (nexin; plasminogen activator inhibitor type 1); member 1 | 7 | NM_000602 | 9,6 | 9,0 | -0,6 | serine peptidase inhibitor, pleiotropic functions besides haemostasis (BC) | (Chen, Henry *et al.* 2009, Litlekalsoy, Hostmark *et al.* 2012, Rosser, Ross *et al.* 2013) |
| ADAM12 | ADAM metallopeptidase domain 12 | 10 | NM_003474 | 8,6 | 7,7 | -0,9 | a disintegrin and metalloprotease, multifunctional zinc-dependent metzincin protease (BC) | (Frohlich, Albrechtsen *et al.* 2006, Kogure, Takawa *et al.* 2013) |

*Log2E* stands for the base 2 logarithm of a given expression value.

**Table 3.3. Description of up- or down-regulated genes by BCG-challenging in HT1376$_{sT}$ cells.**

| Symbol | Description | Chr | GenBank | Log2E (HT1376sT) | Log2E (after BCG) | Difference (BCG-sT) | Function | Ref |
|---|---|---|---|---|---|---|---|---|
| **Genes modulated by BCG-challenging in HT1376sT cells** | | | | | | | | |
| **Changes decreasing malignancy** – Bladder Cancer markers | | | | | | | | |
| CTSL2 | cathepsin L2 | 9 | NM_001333 | 7,2 | 6,7 | -0,5 | overexpression and release by cancerous tissues; degradation of extracellular matrix components (BC) | (Staack, Koenig *et al.* 2002, Staack, Tolic *et al.* 2004, Svatek, Karam *et al.* 2008) |
| CTSH | cathepsin H | 15 | NM_004390 | 10,6 | 10,0 | -0,5 | overexpression and release by cancerous tissues; degradation of extracellular matrix components (BC) | (Staack, Koenig *et al.* 2002, Staack, Tolic *et al.* 2004) |
| POLI | polymerase (DNA directed) iota | 18 | NM_007195 | 9,9 | 9,4 | -0,5 | overexpressed POLI contributes to hypermutagenesis (BC) | (Yuan, Xu *et al.* 2013) |

**Table 3.3. Description of up- or down-regulated genes by BCG-challenging in HT1376$_{sT}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| VEGFA | vascular endothelial growth factor A | 6 | NM_001025366 | 11,0 | 10,4 | -0,6 | stimulates the formation of new blood vessels and tumour growth (BC) | (Fauconnet, Bernardini *et al.* 2009, Zaravinos, Volanis *et al.* 2012, Kopparapu, Boorjian *et al.* 2013) |
| SERPINE2 | serpin peptidase inhibitor; clade E (nexin; plasminogen activator inhibitor type 1); member 2 | 2 | NM_001136529 | 8,5 | 7,7 | -0,8 | serine peptidase inhibitor, pleiotropic functions besides haemostasis | (Bergeron, Lemieux *et al.* 2010, Nagahara, Nakayama *et al.* 2010, Xu, McKee *et al.* 2010, Iwaki, Urano *et al.* 2012) |
| MMP14 | matrix metallopeptidase 14 (membrane-inserted) | 14 | NM_004995 | 10,0 | 9,1 | -0,9 | degradation of basal membranes and of the extracellular matrix (BC) | (Chaffer, Dopheide *et al.* 2005, Mohammad, Ismael *et al.* 2010, Reis, Leite *et al.* 2012) |
| **Changes decreasing malignancy** – caretaker genes | | | | | | | | |
| CENPI | centromere protein I | X | NM_006733 | 7,4 | 8,4 | 1,0 | centromeric protein, important role in the fidelity of chromosome segregation | (Liu, Hittle *et al.* 2003, Izuta, Ikeno *et al.* 2006, Okada, Cheeseman *et al.* 2006, Cheeseman, Hori *et al.* 2008) |
| BRIP1 | BRCA1 interacting protein C-terminal helicase 1 | 17 | NM_032043 | 7,9 | 8,8 | 0,9 | BRCA1-associated DNA helicase that contributes to homologous recombination and cross-link repair (BC) | (Figueroa, Malats *et al.* 2007) |
| ORC1L | origin recognition complex, subunit 1-like (yeast) | 1 | NM_004153 | 7,1 | 7,9 | 0,8 | localized in the centrosomes preventing Cyclin E-dependent reduplication of centrioles and centrosomes during the cell division | (Hemerly, Prasanth *et al.* 2009, Ferguson, Pascreau *et al.* 2010, Hossain and Stillman 2012) |
| XRCC2 | X-ray repair complementing defective repair in Chinese hamster cells 2 | 7 | NM_005431 | 8,1 | 8,8 | 0,7 | a member of the RecA/Rad51-related protein family that participates in homologous recombination (BC) | (Matullo, Guarrera *et al.* 2005, Figueroa, Malats *et al.* 2007) |

**Table 3.3. Description of up- or down-regulated genes by BCG-challenging in $HT1376_{sT}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| RAD54B | RAD54 homolog B (S. cerevisiae) | 8 | NM_012415 | 6,6 | 7,2 | 0,7 | involved in recombination repair of DNA damage, including DNA double-strand breaks during homologous recombination | (Romanowicz-Makowska and Smolarz 2006, Murzik, Hemmerich *et al.* 2008, Sarai, Kagawa *et al.* 2008) |
| CENPH | centromere protein H | 5 | NM_022909 | 6,2 | 6,8 | 0,7 | centromeric protein, important role in the fidelity of chromosome segregation | (Izuta, Ikeno *et al.* 2006, Okada, Cheeseman *et al.* 2006, Cheeseman, Hori *et al.* 2008) |
| CENPK | centromere protein K | 5 | NM_022145 | 8,8 | 9,4 | 0,6 | centromeric protein, important role in the fidelity of chromosome segregation | (Foltz, Jansen *et al.* 2006, Okada, Cheeseman *et al.* 2006, Cheeseman, Hori *et al.* 2008) |
| CENPO | centromere protein O | 2 | NM_024322 | 7,9 | 8,5 | 0,6 | centromeric protein, important role in the fidelity of chromosome segregation | (Foltz, Jansen *et al.* 2006) |
| RAD54L | RAD54-like (S. cerevisiae) | 1 | NM_003579 | 7,5 | 8,0 | 0,6 | recombination protein associated with RAD51, involved in NER of double-strand break | (Li, Frazier *et al.* 2006, Nowacka-Zawisza, Brys *et al.* 2006, Romanowicz-Makowska and Smolarz 2006) |
| BUB1B | budding uninhibited by benzimidazoles 1 homolog beta (yeast) | 15 | NM_001211 | 8,7 | 9,3 | 0,5 | important role in the spindle checkpoint, reduction of BUB1B results in premature chromatid separation and aneuploidy | (Hernando, Orlow *et al.* 2001, Bohers, Sarafan-Vasseur *et al.* 2008, Izumi, Matsumoto *et al.* 2009) |
| BRCA2 | breast (C) 2, early onset | 13 | NM_000059 | 8,3 | 8,7 | 0,5 | important role in the homologous recombination pathway for double-strand DNA repair and in the G2 checkpoint following DNA-damage (BC) | (Goode, Ulrich *et al.* 2002, Wild, Catto *et al.* 2007, Catto, Abbod *et al.* 2010) |

*Log2E* stands for the base 2 logarithm of a given expression value.

Owing to the particular relevance of MHC genes in BCG response, the behaviour of MHC genes which exhibit modulation by BCG in HT1376 cells is shown in Figure 3.8. In $HT1376_{NC}$ cells BCG induced up-regulation of one class I and two class II MHC genes.

a) MHC genes modulated by BCG in $HT1376_{NC}$ cells  b) MHC genes modulated by BCG in $HT1376_{sT}$ cells

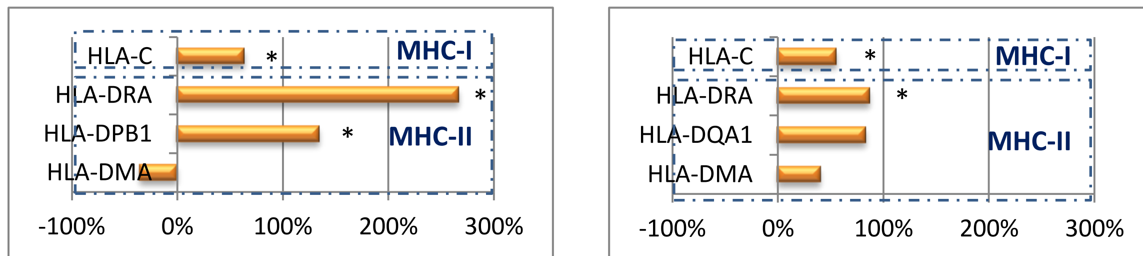



**Figure 3.8. Modulation of MHC genes by BCG internalization in HT1376 cells.** Percentage of expression variation of MHC related genes in a) $HT1376_{NC}$, b) $HT1376_{sT}$, after BCG-challenging. Only variations greater then ± 0.5 were considered. Data are the mean of 3 experiments. * $p \leq 0.05$.

*Effect of ST6GALNAC1 expression – Gene Signatures of MCR cells*

Like *ST3GAL1* expression in HT1376 cells, *ST6GALNAC1* expression in MCR cells resulted in down-regulation of a set of genes, which can be defined as “caretaker genes”, involved in maintaining genetic stability (Figure 3.9a). Genes *HAUS6* and *ERCC6L*, both involved in proper chromosomal segregation, and *XRCC4* involved in repair of double strand breaks, display the most prominent down-regulation (at least a factor 2) in $MCR_{sTn}$ cells. Other genes involved in chromosomal segregation (*CDCA5*, *KNTC1*, *CSPP1*, *HAUS3*, *SGOL1*) also show down-regulation, although at a lower extent. Other down-regulated genes are involved in different mechanisms of DNA repair. In particular, *XRCC2* and *BRCA1* are both involved in HR, *NBN* in both HR and non homologous end joining (NHEJ), while *ERCC8* and *ERCC6* are involved in NER. It is worth mentioning that homologues of many of these down-regulated genes are also down-regulated in $HT1376_{sT}$ (Table 3.4).

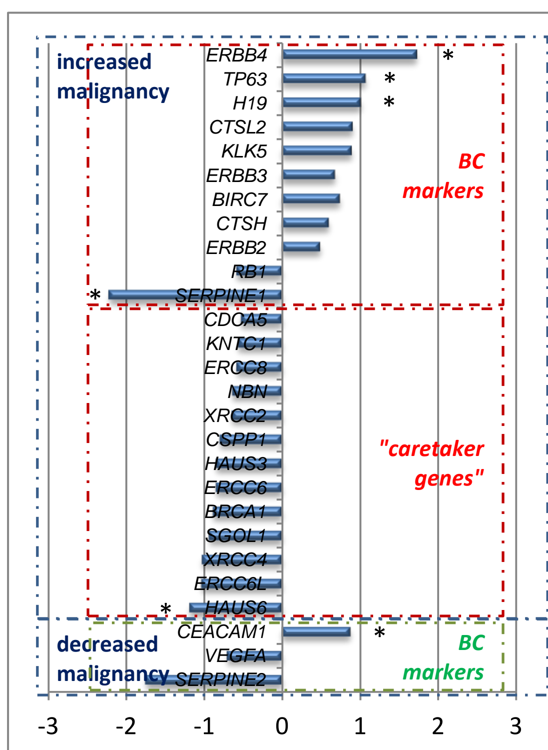
**Table 3.4. Genes with analogous or identical functions showing parallel down-regulation in sialyltransferase-transduced bladder cancer cell lines.**

| **Down-regulated in $HT1376_{sT}$** | **Down-regulated in $MCR_{sTn}$** |
|---|---|
| *HAUS7* | *HAUS3*<br>*HAUS6* |
| *CDCA5* | *CDCA5* |
| *BRCA2* | *BRCA1* |
| *XRCC3* | *XRCC2*<br>*XRCC4* |
| *ERCC2* | *ERCC8*<br>*ERCC6* |

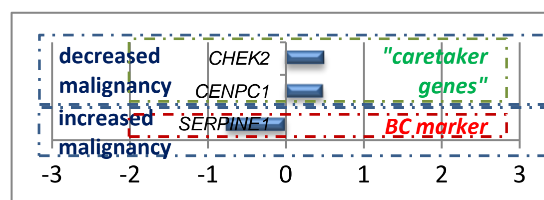
In $MCR_{sTn}$ cells, we observed a parallel and concomitant up-regulation of three of the four members of the ERBB family of membrane receptors (*ERBB4*, *ERBB3* and *ERBB2*). Although *ERBB2* showed a good level of expression also in $MCR_{NC}$ cells and little increased in $MCR_{sTn}$ cells, the expression of both *ERBB3* and of *ERBB4* in $MCR_{NC}$ cells were very low and the *ST6GALNAC1*-dependent up-regulation had the effect of a switch-on. The overexpression of *ERBB3* and *ERBB4* in the presence of high levels of

*ERBB2* can result in a strong activation of ERBB receptor signaling. In addition, the up-regulation of the tumour protein p63 and a slight decrease of Rb were also observed in $MCR_{sTn}$ cells. Although the latter remains highly expressed also in $MCR_{sTn}$ cells, its limited down-regulation could corroborate the increased cell growth fuelled by the up-regulation of receptors of the ERBB family. The overall protease activity of MCR appears to be up-regulated in $MCR_{sTn}$ cells. In fact, cathepsin L2 and H (*CTSL2* and *CTSH*) as well as kallikrein-related peptidase 5 (*KLK5*) display up-regulation which is accompanied by a strongly decreased expression of the serine protease inhibitors *SERPINE1* and *SERPINE2*. Interestingly, the latter two genes show modulation also in $HT1376_{sT}$ cells. A literature search for the global contribution of the modulated genes to the neoplastic phenotype has revealed that the modulation of 24 genes is toward a more malignant phenotype while only 3 genes were modulated toward a less malignant phenotype. Eleven of the genes modulated toward increased malignancy are reported in the literature as "bladder cancer markers" (*ERBB4*, *TP63*, *H19*, *CTSL2*, *KLK5*, *ERBB3*, *BIRC7*, *CTSH* and *ERBB2* were up-regulated while and *RB1* and *SERPINE1* were down-regulated), while 13 "caretaker genes" (*CDCA5*, *KNTC1*, *CSPP1*, *HAUS3*, *SGOL*, *ERCC6L, HAUS6, XRCC2*, *NBN*, *ERCC8*, *ERCC6*, *BRCA1* and *XRCC4)* genes were down-regulated). On the other hand, 3 genes reported as "bladder cancer markers" were modulated toward decreased malignancy (*CEACAM1* gene was up-regulated, while *VEGFA* and *SERPINE2* genes were down-regulated). When $MCR_{NC}$ cells (Figure 3.9b) were challenged with BCG, only 3 genes were specifically modulated, all toward a less malignant phenotype; two of the genes were caretakers. However, when $MCR_{sTn}$ cells were challenged with BCG (Figure 3.9c), 11 caretaker genes were modulated in the sense of decreased malignancy, while gene *BIRC3* which, according to the literature, is associated with bladder cancer, moved toward increased malignancy.

a) Genes modulated by *ST6GALNAC1* expression in MCR cells




b) Genes modulated by BCG-challenging in $MCR_{NC}$ cells




c) Genes modulated by BCG-challenging in $MCR_{sTn}$ cells

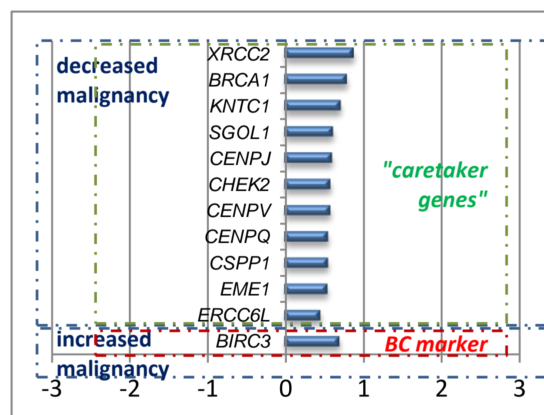



**Figure 3.9. Gene signatures in MCR cells.** Expression variation of relevant genes in a) $MCR_{sTn}$ cells *vs*. $MCR_{NC}$ cells in b) $MCR_{NC}$ cells and in c) $MCR_{sTn}$ cells after BCG-challenging. Genes were grouped in "BC markers" and "caretaker" according to their putative effect on malignancy. Only differential variations greater then ± 0.5 were considered. Data are the mean of 3 experiments. * $p \leq 0.05$.

In Tables 3.5 to 3.7 are presented the selected signature genes presented in figure 3.9 with gene description, hosting chromosome, GenBank code and a short outline of the function.

**Table 3.5. Description of up- or down-regulated genes in $MCR_{sTn}$ cells *vs*. $MCR_{NC}$ cells.**

| **Gene Signatures modulated by *ST6GALNAC1* expression** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Symbol | Description | Chr | GenBank | Log2E ($MCR_{NC}$) | Log2E ($MCR_{sTn}$) | Difference (sTn-NC) | Function | Ref |
| **Changes increasing malignancy** – Bladder Cancer markers | | | | | | | | |
| ERBB4 | v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian) | 2 | NM_005235 | 5,8 | 7,6 | 1,7 | important in diverse cellular functions, including cell proliferation, differentiation, and survival of normal cells, increased expression has been reported in cancer (BC) | (Junttila, Laato *et al*. 2003, Gunes, Sullu *et al*. 2013) |
| TP63 | tumour protein p63 | 3 | NM_003722 | 4,7 | 5,8 | 1,1 | plays a role in the regulation of epithelial morphogenesis (BC) | (Choi, Shah *et al*. 2012, Sjodahl, Lovgren *et al*. 2013) |
| H19 | H19, imprinted maternally expressed transcript (non-protein coding) | 11 | NR_002196 | 9,1 | 10,1 | 1,0 | an imprinted oncofoetal gene abundantly expressed in embryogenesis and shut off in most tissues after birth (BC) | (Matouk, DeGroot *et al*. 2007, Luo, Li *et al*. 2013, Luo, Li *et al*. 2013) |
| CTSL2 | cathepsin L2 | 9 | NM_001333 | 6,3 | 7,2 | 0,9 | overexpression and release by cancerous tissues; degradation of extracellular matrix components (BC) | (Staack, Koenig *et al*. 2002, Staack, Tolic *et al*. 2004, Svatek, Karam *et al*. 2008) |
| KLK5 | kallikrein-related peptidase 5 | 19 | NM_012427 | 6,7 | 7,6 | 0,9 | secreted enzyme belonging to the serine protease family (BC) | (Shinoda, Kozaki *et al*. 2007) |
| ERBB3 | v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) | 12 | NM_001982 | 5,7 | 6,4 | 0,7 | important in diverse cellular functions, including cell proliferation, differentiation, and survival of normal cells, increased expression have been identified in cancer (BC) | (Junttila, Laato *et al*. 2003, Gunes, Sullu *et al*. 2013) |
| BIRC7 | baculoviral IAP repeat-containing 7 | 20 | NM_139317 | 5,7 | 6,4 | 0,7 | involved in the regulation of apoptosis (BC) | (Gazzaniga, Gradilone *et al*. 2003, Song, Hong *et al*. 2007, Xi, Sheng *et al*. 2013) |
| CTSH | cathepsin H | 15 | NM_004390 | 4,9 | 5,5 | 0,6 | secreted enzyme belonging to the serine protease family (BC) | (Staack, Koenig *et al*. 2002, Staack, Tolic *et al*. 2004) |

**Table 3.5. Description of up- or down-regulated genes in MCR$_{sTn}$ cells *vs*. MCR$_{NC}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ERBB2 | v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) | 17 | NM_001005862 | 7,5 | 8,0 | 0,5 | important in diverse cellular functions, including cell proliferation, differentiation, and survival of normal cells, increased expression has been reported in cancer (BC) | (Chekaluk, Wu *et al*. 2013, Chen, Yu *et al*. 2013, Gunes, Sullu *et al*. 2013, Sjodahl, Lovgren *et al*. 2013) |
| RB1 | retinoblastoma 1 | 13 | NM_000321 | 10,0 | 9,4 | -0,6 | tumour suppressor protein (BC) | (Hurst, Tomlinson *et al*. 2008, Lindgren, Sjodahl *et al*. 2012, Sjodahl, Lovgren *et al*. 2013) |
| SERPINE1 | serpin peptidase inhibitor; clade E (nexin; plasminogen activator inhibitor type 1); member 1 | 7 | NM_000602 | 8,5 | 6,3 | -2,2 | serine peptidase inhibitor, pleiotropic functions besides haemostasis (BC) | (Chen, Henry *et al*. 2009, Litlekalsoy, Hostmark *et al*. 2012, Rosser, Ross *et al*. 2013) |
| **Changes increasing malignancy** – caretaker genes | | | | | | | | |
| CDCA5 | cell division cycle associated 5 | 11 | NM_080668 | 8,7 | 8,1 | -0,5 | is involved in maintenance of cohesion in response to the spindle checkpoint | (Diaz-Martinez, Gimenez-Abian *et al*. 2007, Dreier, Bekier *et al*. 2011, Wu, Nguyen *et al*. 2011) |
| KNTC1 | kinetochore associated 1 | 12 | NM_014708 | 7,7 | 7,2 | -0,6 | involved in mechanisms to ensure proper chromosome segregation, participate in the generation and amplification of the " wait anaphase" signal | (Wang, Hu *et al*. 2004, Kops, Kim *et al*. 2005, Famulski, Vos *et al*. 2008) |
| ERCC8 | excision repair cross-complementing rodent repair deficiency, complementation group 8 | 5 | NM_000082 | 6,7 | 6,1 | -0,6 | has a role in NER | (Latini, Frontini *et al*. 2011, Scrima, Fischer *et al*. 2011) |
| NBN | nibrin | 8 | NM_002485 | 7,1 | 6,5 | -0,6 | plays a critical role in the DSB repair pathway, functioning both in the NHEJ pathway as a sensor for DNA damage and in the HR pathway (BC) | (Sanyal, Festa *et al*. 2004, Stern, Lin *et al*. 2009, Park, Bastani *et al*. 2010) |
| XRCC2 | X-ray repair complementing defective repair in Chinese hamster cells 2 | 7 | NM_005431 | 9,5 | 8,9 | -0,6 | a member of the RecA/Rad51-related protein family that participates in homologous recombination (BC) | (Matullo, Guarrera *et al*. 2005, Figueroa, Malats *et al*. 2007) |

**Table 3.5. Description of up- or down-regulated genes in $MCR_{sTn}$ cells *vs*. $MCR_{NC}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CSPP1 | centrosome and spindle pole associated protein 1 | 8 | NM_001077204 | 7,2 | 6,4 | -0,8 | is associated with centrosomes and microtubules and may play a role in the regulation of G1/S-phase progression and spindle assembly | (Patzke, Hauge *et al*. 2005, Asiedu, Wu *et al*. 2009) |
| HAUS3 | HAUS augmin-like complex, subunit 3 | 4 | NM_024511 | 6,8 | 5,9 | -0,8 | HAUS disruption results in the destabilization of kinetochore microtubules and the eventual formation of multipolar spindles | (Lawo, Bashkurov *et al*. 2009) |
| ERCC6 | excision repair cross-complementing rodent repair deficiency, complementation group 6 | 10 | NM_000124 | 8,9 | 8,1 | -0,8 | recruits nucleotide excision repair factors to the DNA damage site and plays an important role in the repair process (BC) | (Garcia-Closas, Malats *et al*. 2006, Chen, Kamat *et al*. 2007, Chang, Chiu *et al*. 2009) |
| BRCA1 | breast (C) 1; early onset | 17 | NM_007296 | 7,6 | 6,7 | -0,9 | activating DNA repair through HR, in cooperation with BRCA2, RAD51 and RAD52 (BC) | (Agundez, Grau *et al*. 2011, Font, Taron *et al*. 2011, Golka, Selinski *et al*. 2011) |
| SGOL1 | shugoshin-like 1 (S. pombe) | 3 | NM_001012410 | 8,7 | 7,8 | -0,9 | is required for maintenance of centromeric cohesion from prophase to the metaphase‑anaphase transition | (Diaz-Martinez, Gimenez-Abian *et al*. 2007, Pouwels, Kukkonen *et al*. 2007, Kang, Chaudhary *et al*. 2011) |
| XRCC4 | X-ray repair complementing defective repair in Chinese hamster cells 4 | 5 | NM_022550 | 8,4 | 7,4 | -1,0 | involved in DNA DSB repair by NHEJ recombination (BC) | (Figueroa, Malats *et al*. 2007, Chang, Chang *et al*. 2009, Mittal, Gangwar *et al*. 2012) |
| ERCC6L | excision repair cross-complementing rodent repair deficiency; complementation group 6-like | X | NM_017669 | 7,7 | 6,7 | -1,0 | plays an important role in maintaining prometaphase chromosome architecture and in the segregating sister centromeres during anaphase | (Kurasawa and Yu-Lee 2010, Ke, Huh *et al*. 2011, Rouzeau, Cordelieres *et al*. 2012) |
| HAUS6 | HAUS augmin-like complex, subunit 6 | 9 | NM_017645 | 8,2 | 7,0 | -1,2 | HAUS disruption results in the destabilization of kinetochore microtubules and the eventual formation of multipolar spindles | (Lawo, Bashkurov *et al*. 2009) |

**Table 3.5. Description of up- or down-regulated genes in $MCR_{sTn}$ cells *vs*. $MCR_{NC}$ cells (continued).**

| **Changes decreasing malignancy** – Bladder Cancer markers | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CEACAM1 | carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein) | 19 | NM_001712 | 6,2 | 7,1 | 0,9 | is a member of the carcinoembryonic antigen family, as a tumour-suppressive role by inhibiting tumour angiogenesis (BC) | (Oliveira-Ferrer, Tilki *et al*. 2004, Tilki, Oliveira-Ferrer *et al*. 2007, Tilki, Singer *et al*. 2010) |
| VEGFA | vascular endothelial growth factor A | 6 | NM_001025366 | 9,8 | 9,2 | -0,7 | stimulates the formation of new blood vessels and tumour growth (BC) | (Fauconnet, Bernardini *et al*. 2009, Zaravinos, Volanis *et al*. 2012, Kopparapu, Boorjian *et al*. 2013) |
| SERPINE2 | serpin peptidase inhibitor; clade E (nexin; plasminogen activator inhibitor type 1); member 2 | 2 | NM_001136529 | 8,8 | 7,1 | -1,7 | serine peptidase inhibitor, pleiotropic functions besides haemostasis | (Bergeron, Lemieux *et al*. 2010, Nagahara, Nakayama *et al*. 2010, Xu, McKee *et al*. 2010, Iwaki, Urano *et al*. 2012) |

*Log2E* stands for the base 2 logarithm of a given expression value.

**Table 3.6. Description of up- or down-regulated genes by BCG-challenging in $MCR_{NC}$ cells.**

**Gene Signatures modulated by BCG-challenging in $MCR_{NC}$ cells**

| Symbol | Description | Chr | GenBank | Log2E ($MCR_{NC}$) | Log2E (after BCG) | Difference (BCG-NC) | Function | Ref |
|---|---|---|---|---|---|---|---|---|
| **Changes decreasing malignancy** – caretaker genes | | | | | | | | |
| CHEK2 | CHK2 checkpoint homolog (S. pombe) | 22 | NM_001005735 | 7,0 | 7,5 | 0,5 | cell cycle control gene encoding a pluripotent kinase, can cause arrest or apoptosis in response to unrepaired DNA (BC) | (Slojewski, Zlowocka *et al*. 2008, Zlowocka, Cybulski *et al*. 2008) |
| CENPC1 | centromere protein C 1 | 4 | NM_001812 | 7,4 | 7,9 | 0,5 | centromeric protein, important role in the fidelity of chromosome segregation | (Obuse, Yang *et al*. 2004, Foltz, Jansen *et al*. 2006, Izuta, Ikeno *et al*. 2006) |
| **Changes increasing malignancy** – Bladder Cancer markers | | | | | | | | |
| SERPINE1 | serpin peptidase inhibitor; clade E (nexin; plasminogen activator inhibitor type 1); member1 | 7 | NM_000602 | 8,5 | 7,8 | -0,7 | serine peptidase inhibitor, pleiotropic functions besides haemostasis (BC) | (Chen, Henry *et al*. 2009, Litlekalsoy, Hostmark *et al*. 2012, Rosser, Ross *et al*. 2013) |

*Log2E* stands for the base 2 logarithm of a given expression value.

**Table 3.7. Description of up- or down-regulated genes by BCG-challenging in $MCR_{sTn}$ cells.**

| Gene Signatures modulated by BCG-challenging in $MCR_{sTn}$ cells | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Symbol | Description | Chr | GenBank | Log2E ($MCR_{sTn}$) | Log2E (after BCG) | Difference (BCG-sTn) | Function | Ref |
| **Changes decreasing malignancy** – caretaker genes | | | | | | | | |
| XRCC2 | X-ray repair complementing defective repair in Chinese hamster cells 2 | 7 | NM_005431 | 8,9 | 9,8 | 0,9 | a member of the RecA/Rad51-related protein family that participates in homologous recombination (BC) | (Matullo, Guarrera *et al.* 2005, Figueroa, Malats *et al.* 2007) |
| BRCA1 | breast (C) 1; early onset | 17 | NM_007296 | 6,7 | 7,5 | 0,8 | activating DNA repair through HR, in cooperation with BRCA2, RAD51 and RAD52 (BC) | (Romanowicz-Makowska and Smolarz 2006, Ma, Cai *et al.* 2013) |
| KNTC1 | kinetochore associated 1 | 12 | NM_014708 | 7,2 | 7,9 | 0,7 | involved in mechanisms to ensure proper chromosome segregation, participate in the generation and amplification of the “ wait anaphase” signal | (Wang, Hu *et al.* 2004, Kops, Kim *et al.* 2005, Famulski, Vos *et al.* 2008) |
| SGOL1 | shugoshin-like 1 (S. pombe) | 3 | NM_001012410 | 7,8 | 8,4 | 0,6 | is required for maintenance of centromeric cohesion from prophase to the metaphase‑anaphase transition | (Diaz-Martinez, Gimenez-Abian *et al.* 2007, Pouwels, Kukkonen *et al.* 2007, Kang, Chaudhary *et al.* 2011) |
| CENPJ | centromere protein J | 13 | NM_018451 | 6,8 | 7,4 | 0,6 | centromeric protein, important role in the fidelity of chromosome segregation | (Foltz, Jansen *et al.* 2006) |
| CHEK2 | CHK2 checkpoint homolog (S. pombe) | 22 | NM_001005735 | 7,0 | 7,6 | 0,6 | key cell cycle control gene encoding a pluripotent kinase that can cause arrest or apoptosis in response to unrepaired DNA damage (BC) | (Slojewski, Zlowocka *et al.* 2008, Zlowocka, Cybulski *et al.* 2008) |
| CENPV | centromere protein V | 17 | NM_181716 | 8,5 | 9,1 | 0,6 | centromeric protein, important role in the fidelity of chromosome segregation | (Foltz, Jansen *et al.* 2006) |
| CENPQ | centromere protein Q | 6 | NM_018132 | 5,6 | 6,2 | 0,6 | centromeric protein, important role in the fidelity of chromosome segregation | (Foltz, Jansen *et al.* 2006) |

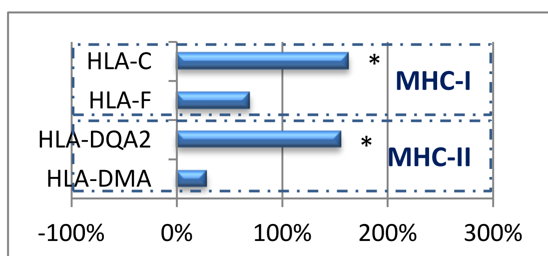
**Table 3.7. Description of up- or down-regulated genes by BCG-challenging in $MCR_{sTn}$ cells (continued).**

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CSPP1 | centrosome and spindle pole associated protein 1 | 8 | NM_001077204 | 6,4 | 6,9 | 0,6 | is associated with centrosomes and microtubules and may play a role in the regulation of G1/S-phase progression and spindle assembly | (Patzke, Hauge *et al.* 2005, Asiedu, Wu *et al.* 2009) |
| EME1 | essential meiotic endonuclease 1 homolog 1 (S. pombe) | 17 | NM_152463 | 6,4 | 7,0 | 0,5 | catalyses with MUS81 coordinate bilateral cleavage of model Holliday-junction structures | (Forment, Blasius *et al.* 2011, Zhao, Liu *et al.* 2013) |
| ERCC6L | excision repair cross-complementing rodent repair deficiency; complementation group 6-like | X | NM_017669 | 6,7 | 7,2 | 0,5 | plays an important role in maintaining prometaphase chromosome architecture and in the segregating sister centromeres during anaphase | (Kurasawa and Yu-Lee 2010, Ke, Huh *et al.* 2011, Rouzeau, Cordelieres *et al.* 2012) |
| **Changes increasing malignancy** – Bladder Cancer markers | | | | | | | | |
| BIRC3 | baculoviral IAP repeat-containing 3 | 11 | NM_001165 | 8,4 | 9,1 | 0,7 | regulator of apoptosis; its overexpression may make tumours resistant to apoptosis (BC) | (Che, Yang *et al.* 2012, He, Wang *et al.* 2012) |

*Log2E* stands for the base 2 logarithm of a given expression value.

Like for HT1376 cells, the behaviour of MHC genes in response to BCG-challenging in MCR cells was accessed and is shown in Figure 3.10. In $MCR_{NC}$ cells, BCG induced up-regulation of two class I and two class II MHC genes, while in $MCR_{sTn}$ only the two MHC class II genes displayed up-regulation.

a) MHC genes modulated by BCG in $MCR_{NC}$ cells




b) MHC genes modulated by BCG in $MCR_{sTn}$ cells

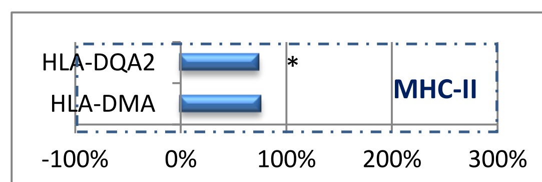



**Figure 3.10. Modulation of MHC genes by BCG internalization.** Percentage of expression variation of MHC genes in a) $MCR_{NC}$, b) $MCR_{sTn}$ cells after BCG-challenging. Only variations greater then ± 0.5 were considered. Data are the mean of 3 experiments. * $p \leq 0.05$.

Altogether, microarray results indicate that groups of genes involved in specific functions are regulated in parallel in the two cell lines, regardless the type of sialyltransferase expressed.

## Cytokine secretion by BCG-challenged bladder cancer cells

The impact of *ST3GAL1* or *ST6GALNAC1* sialyltransferase expression and BCG-challenge on cytokine secretion was evaluated in the conditioned medium of bladder cancer cell lines. The following cytokines: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IFNγ and TNFα were measured in the conditioned media of BCG-challenged or unchallenged bladder cancer cell lines 16 hours after Connaught-BCG-challenge and compared with unchallenged cells.

Only IL-6 and IL-8 were secreted by HT1376 cells (Figure 3.11). In both, unchallenged $HT1376_{NC}$ or $HT1376_{sT}$ cells, IL-6 secretion was negligible, while a slight induction was observed after BCG-challenge. IL-8 was produced by both HT1376 cell variants; and slightly more pronounced in $HT1376_{sT}$. BCG-challenge increased IL-8 secretion by about 50%.

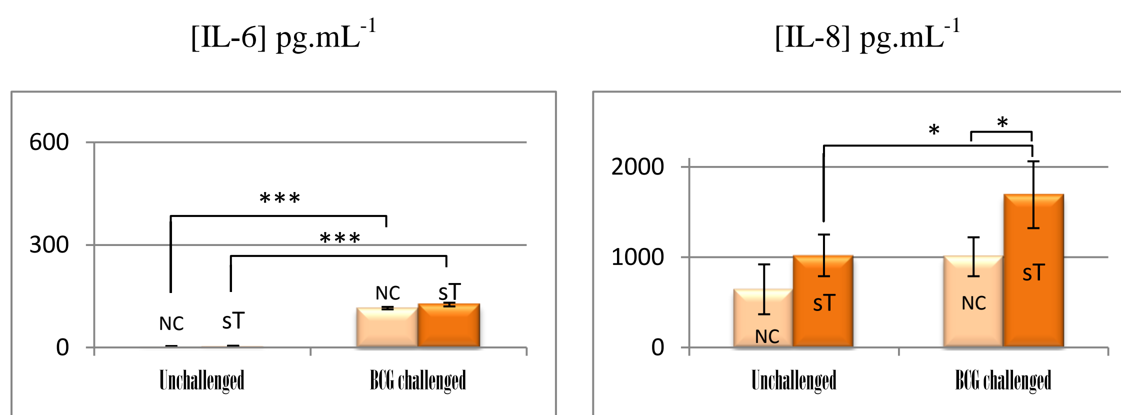



**Figure 3.11. Cytokine secretion by HT1376 cells.** An induction of both IL-6 and IL-8 was found in both $HT1376_{NC}$ (pink bars) and $HT1376_{sT}$ (orange bars) 12 h after BCG-challenging. IL-8 induction in $HT1376_{sT}$ cells was stronger than in $HT1376_{NC}$ cells. Data are the mean ± SD of 3 experiments. *** $p < 0.0001$; * $p < 0.05$.

Also in MCR cells, only IL-6 and IL-8 were detected (Figure 3.12). Similar levels of IL-6 were produced by both unchallenged MCR variants. BCG-challenge left unaltered IL-6 production by $MCR_{NC}$, while it induced a statistically significant increase in $MCR_{sTn}$. The secretion of IL-8 was negligible by unchallenged cells but increased dramatically, especially in the sTn variant, after BCG-challenging.

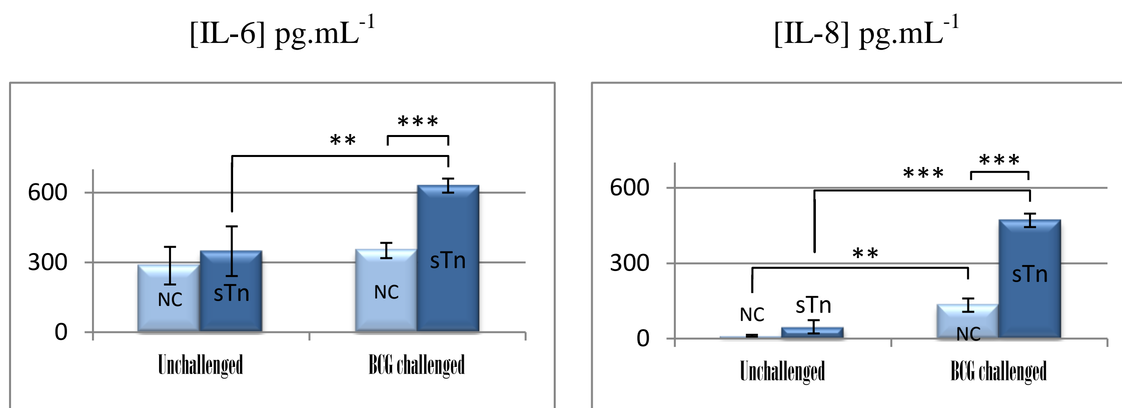



**Figure 3.12. Cytokine secretion by MCR cells.** Twelve hours after BCG-challenging, a significant induction of both IL-6 and IL-8 secretion was observed in $MCR_{sTn}$ (dark blue bars), but not in $MCR_{NC}$ (light blue bars). Data are the mean ± SD of 3 experiments. *** $p < 0.0001$; ** $p < 0.001$.

No significant differences in cytokine secretion were found just after BCG-challenging (data not shown).

## *III.3 Response of Macrophages to BCG-challenged BC cell Lines*

### Cytokine secretion by macrophages in response to bladder cancer secretome

After the characterization of the cytokine content of the microenvironment of our cell models, the effect of the conditioned medium from bladder cancer cell lines on first line defence immune cells, such as macrophages, was studied. To assess the macrophage response to the tumour microenvironment, the same set of cytokines was measured in the culture medium of macrophages stimulated for 24 hours with the secretome of BCG-challenged or unchallenged cell lines. Unstimulated macrophages secreted at high levels only IL-8. (Figures 3.13 and 3.14). Unstimulated HT1376 cells failed to induce cytokine secretion by macrophages. However, the secretome from BCG-challenged cells induced a significant secretion of IL-6, IL-8, IL-1β, TNFα and IL-10. With the exception of IL-8, whose secretion was stimulated at similar levels by both $HT1376_{NC}$ and $HT1376_{sT}$, the induction of cytokine secretion was markedly stronger when BCG-challenged cells expressed the sT antigen ($HT1376_{sT}$) (Figure 3.13). Cytokines IL-2, IL-4, IL-12, and IL-17 were never expressed by macrophages.

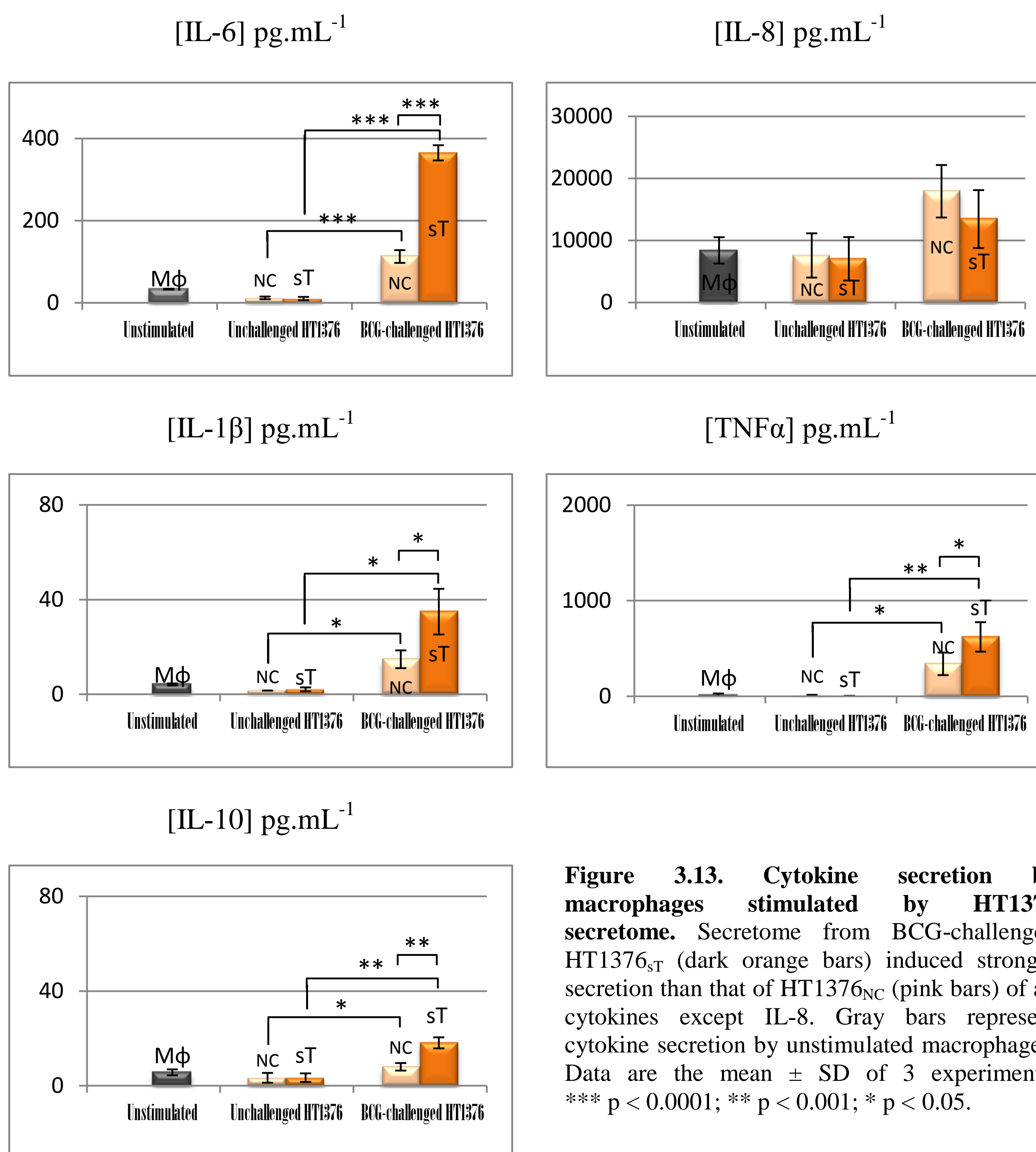



**Figure 3.13. Cytokine secretion by macrophages stimulated by HT1376 secretome.** Secretome from BCG-challenged HT1376$_{sT}$ (dark orange bars) induced stronger secretion than that of HT1376$_{NC}$ (pink bars) of all cytokines except IL-8. Gray bars represent cytokine secretion by unstimulated macrophages. Data are the mean ± SD of 3 experiments. *** $p < 0.0001$; ** $p < 0.001$; * $p < 0.05$.

When macrophages were stimulated with the secretome of unchallenged MCR cells, TNFα, IL-6 and IL-8 cytokines were slightly induced (Figure 3.14). The stimulation with the secretome of both BCG-challenged cell variants induced a strong secretion of IL-6, IL-1β, TNFα and IL-10. Interestingly the induction was much higher with the secretome from MCR$_{sTn}$ cells. Secretion of IL-8 was not affected either by BCG-challenging or *ST6GALNAC1* expression of MCR cells.

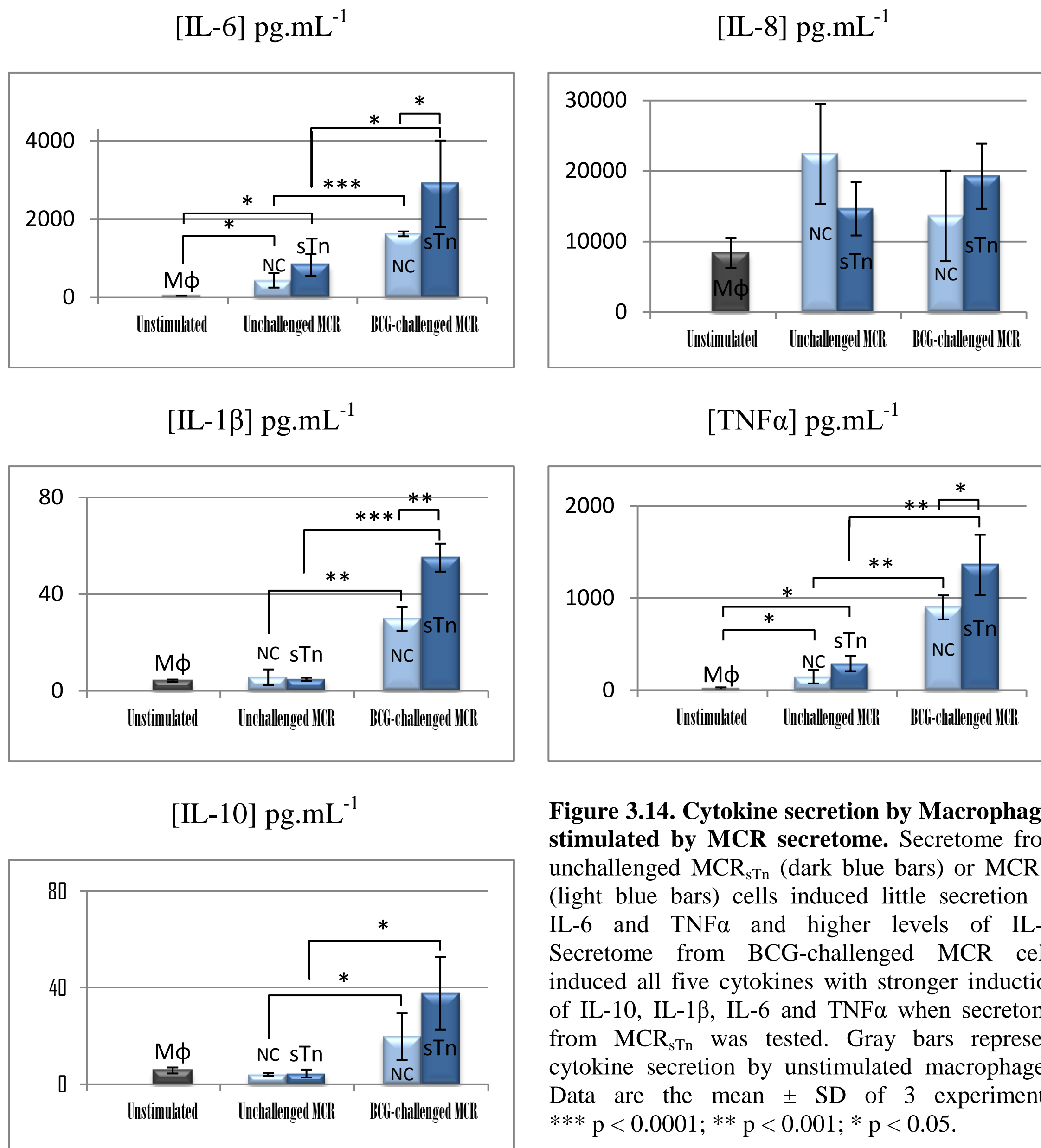



**Figure 3.14. Cytokine secretion by Macrophages stimulated by MCR secretome.** Secretome from unchallenged $MCR_{sTn}$ (dark blue bars) or $MCR_{NC}$ (light blue bars) cells induced little secretion of IL-6 and TNFα and higher levels of IL-8. Secretome from BCG-challenged MCR cells induced all five cytokines with stronger induction of IL-10, IL-1β, IL-6 and TNFα when secretome from $MCR_{sTn}$ was tested. Gray bars represent cytokine secretion by unstimulated macrophages. Data are the mean ± SD of 3 experiments. *** $p < 0.0001$; ** $p < 0.001$; * $p < 0.05$.

In general, the secretome of BCG-challenged cells, in particular those expressing either the sT or the sTn antigen, potentiated markedly the secretion of cytokines such as IL-6 and TNFα, and to a lower extent IL-1β and IL-10.

**Phagocytosis of apoptotic bladder cancer cell lines by macrophages**

A crucial step in the process of bladder cancer antigen presentation by APC to T lymphocytes is represented by the phagocytosis of apoptotic cancer cells by mononuclear phagocytes. To assess whether BCG-challenge and/or the expression of sT/sTn antigens by bladder cancer cells affected their uptake by macrophages, we induced apoptosis in BCG-challenged or unchallenged bladder cancer cell lines and measured their phagocytosis by monocyte-derived macrophages (Figure 3.15). No significant differences were seen in the propensity to undergo apoptosis by the different cell lines (data not shown). The phagocytosis of apoptotic HT1376 cells was not affected by BCG-challenging while it was higher for $HT1376_{sT}$ cells (Figure 3.15a). On the contrary, phagocytosis of MCR cells was not affected either by BCG-challenging or sTn expression (Figure 3.15b).

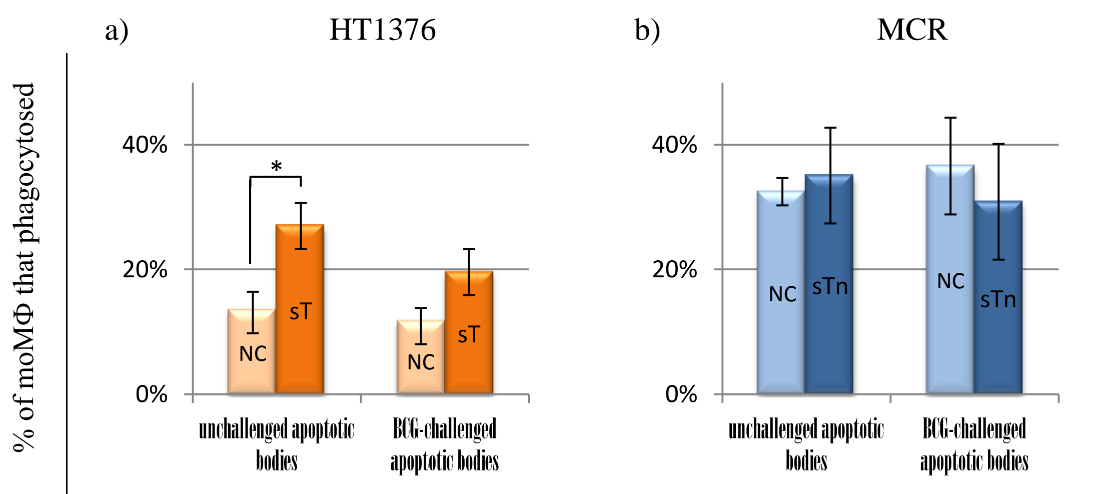



**Figure 3.15. Phagocytosis of apoptotic bladder cancer cell lines by macrophages.** a) Phagocytosis of apoptotic BCG-challenged or unchallenged $HT1376_{NC}$ (pink bars) or $HT1376_{sT}$ (orange bars) cells. Phagocytosis was not affected by BCG-challenging but was higher with $HT1376_{sT}$ cells. b) Phagocytosis of apoptotic BCG-challenged or -unchallenged $MCR_{NC}$ (light blue) or $MCR_{sTn}$ cells (dark blue). Phagocytosis was not affected either by BCG-challenging or sTn expression. Data are the mean ± SD of 3 experiments. * $p < 0.05$.

These results suggest that the sT could facilitate the phagocytosis of apoptotic bladder cancer cells by macrophages, while the previous contact with BCG does not appear to play a role. The presence of sTn antigen didn't show significant differences in the capacity of induction of phagocytosis of apoptotic bladder cancer cells by macrophages.

# CHAPTER IV

## *Discussion and Conclusions*

The aim of this thesis was the investigation of the response of bladder cancer cells to BCG in the context of a differential expression of Thomsen-Friedenreich related antigens sialyl-T (sT) and sialyl-Tn (sTn) and to investigate the response of macrophages to the secretome of BCG-challenged bladder cancer cells. To this aim, a bladder cancer model of cell lines permanently expressing the cDNA of *ST3GAL1* or of *ST6GALNAC1* was refined to obtain cell populations homogenously expressing the cognate carbohydrate antigens sT and sTn. Besides these studies, the expression of sT and sTn and their relationship in the clinic was studied in collaboration with groups of the University of Porto.

## *IV.1 Cellular Models of Bladder Cancer*

The isolation of cell populations expressing homogeneously the sTn antigen from the bladder cancer cell line MCR was successfully achieved through magnetic sorting using an anti-sTn antibody. Owing to the lack of an anti-sT antibody, the same strategy was not applicable to the sorting of sT expressing cells from the cell line HT1376. Any attempt to pursue a negative selection, through the removal of cell populations reactive with the T-specific lectin PNA turned out to be unsuccessful because the population of cells unbound by the lectin was still containing a large percentage of PNA-positive cells (*i.e.* still expressing the T antigen). For this reason it was decided to adopt a cloning strategy based on single cell cloning through which homogeneous populations of PNA-negative cells were obtained. Lectin analysis of neuraminidase-treated cells $HT1376_{sT}$ cells indicated that the masking of T antigen by α2,3-linked sialic acid is reverted by neuraminidase treatment. Expression of *ST6GALNAC1* in MCR cells induced, beside the expected appearance of sTn antigen, also profound changes in other Thomsen-Friedenreich related antigens. In particular, beside the Tn also the T antigen appeared to be profoundly inhibited by α2,6-sialylation, as revealed by the strong inhibition of PNA reactivity observed in $MCR_{sTn}$. The notion that the T antigen is present

but almost completely masked by α2,6-sialylation is confirmed by our data in collaboration with investigators of the University of Porto (Lima *et al*. 2013), showing that treatment with β1,3-galactosidase of bladder cancer tissue sections unmasked the presence of sTn antigen. The reliability of our model of bladder cancer was confirmed by the expression of the transduced sialyltransferases at both the mRNA and enzyme activity. The expression microarray technique was used to study the transcriptome of the two bladder cancer cell lines used and the impact of sialyltransferase expression on whole gene expression. According to a recent study on molecular classification of bladder cancer (Sjodahl, Lovgren *et al*. 2013), three types can be identified on the basis of the expression of a set of genes: the urobasal (Uro, which is further divided in subtypes A and B), the squamous cell cancer-like (SCCL) and the genomically unstable (GU). This classification has prognostic value in that UroA tumours are associated with a good prognosis, while UroB and SCCL tumours are associated with the worst outcome (Sjodahl, Lovgren *et al*. 2013). Unlike UroA, SCCL tumours show high expression of CDH3 (coding for P-cadherin), EGFR, KRT5, KRT14, but not FGFR2, ERBB2 and KRT20 (Chan, Espinosa *et al*. 2009, Volkmer, Sahoo *et al*. 2012, Sjodahl, Lovgren *et al*. 2013). UroB tumours demonstrate features shared by both UroA and SCCL subtypes. In contrast to SCCL tumours, GU tumours showed high CDH1 (coding for E-cadherin), ERRB2, CCNB1, MKI67, low CDH3 expression, while genes FGFR3, EGFR, KRT5, KRT6 and KRT14 were not expressed. Moreover, it was found that the transition from UroA to SCCL and GU is accompanied by a change from high expression of early cell cycle genes, in particular CCND1, to high expression of late cell cycle genes, *e.g.*, CCNE1 (Sjodahl, Lauss *et al*. 2012). In Table 4.1 are presented the expression levels detected by microarray analysis in both cell models of genes marking the described molecular subtypes. A group of genes showed high expression in HT1376 cells and very low expression in MCR cells: gene *CDH1* and five genes belonging to the cytokeratin group (*KRT5*, *KRT6A*, *KRT6B*, *KRT6C* and *KRT14*). In HT1376, the high *CDH1* expression is consistent with a GU phenotype, while high expression of KRT genes is consistent with a SCCL phenotype. Cyclin genes were expressed at a good level by both cell lines, although in MCR is prevalent the expression of an early cell cycle cyclin, like *CCND1*, while in HT1376 a late cell cycle cyclin, like CCNE2 prevails. This feature is consistent with literature data indicating an invasive SCCL or GU phenotype for both cell lines (Rasheed, Gardner *et al*. 1977, Zoli, Ricotti *et al*. 2004), rather than an UroA or UroB phenotype. Even though transduction with either sialyltransferase causes

profound changes in gene expression, no significant changes were observed for the genes listed in Table 4.1.

**Table 4.1. Molecular classification of HT1376 and MCR bladder cancer cells according to Sjodahl *et al.***

<table>
<tr><th>molecular subtype</th><th>HT1376 (Log2E)</th><th>Gene symbol</th><th>Gene product</th><th>MCR (Log2E)</th><th>molecular subtype</th></tr>
<tr><td rowspan="6">SCCL or GU</td><td>9.0</td><td>CCND1</td><td>cyclin D1</td><td>11.2</td><td rowspan="6">SCCL</td></tr>
<tr><td>9.8</td><td>CCND3</td><td>cyclin D3</td><td>9.7</td></tr>
<tr><td>9.8</td><td>CCNB1</td><td>cyclin B1</td><td>9.5</td></tr>
<tr><td>9.6</td><td>CCNB2</td><td>cyclin B2</td><td>10.0</td></tr>
<tr><td>8.0</td><td>CCNE1</td><td>cyclin E1</td><td>8.4</td></tr>
<tr><td>9.4</td><td>CCNE2</td><td>cyclin E2</td><td>7.9</td></tr>
<tr><td>GU</td><td>11.1</td><td>CDH1</td><td>cadherin 1, or E-cadherin (epithelial)</td><td>5.0</td><td>SCCL</td></tr>
<tr><td>SCCL or GU</td><td>8.8</td><td>CDH3</td><td>cadherin 3, or P-cadherin (placental)</td><td>8.2</td><td>SCCL or GU</td></tr>
<tr><td>SCCL or GU</td><td>7.6</td><td>FGFR3</td><td>fibroblast growth factor receptor 3</td><td>8.4</td><td>SCCL or GU</td></tr>
<tr><td>SCCL</td><td>11.3</td><td>EGFR</td><td>epidermal growth factor receptor</td><td>10.7</td><td>SCCL</td></tr>
<tr><td>GU</td><td>8.4</td><td>ERBB2</td><td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 2</td><td>7.5</td><td>SCCL</td></tr>
<tr><td>SCCL</td><td>10.4</td><td>KRT5</td><td>keratin 5</td><td>5.6</td><td>GU</td></tr>
<tr><td>SCCL</td><td>9.9<br>7.1<br>10.9</td><td>KRT6A<br>KRT6B<br>KRT6C</td><td>keratin 6</td><td>5.8<br>6.3<br>6.5</td><td>GU</td></tr>
<tr><td>SCCL</td><td>11.7</td><td>KRT14</td><td>keratin 14</td><td>6.5</td><td>GU</td></tr>
<tr><td>GU</td><td>10.6</td><td>MKI67</td><td>MKI67 (FHA domain) interacting nucleolar phosphoprotein</td><td>10.4</td><td>GU</td></tr>
</table>

The maintenance of life requires that DNA replication is exceptionally accurate. Errors in these processes result in mutations that might cause cancer. The fact that the DNA in cancer cells is different from the DNA in normal cells indicates that carcinogenesis involves substantial errors in DNA replication, deficits in DNA repair, and alterations in chromosomal segregation (Loeb 2001). Once the rarity of mutations in normal cells cannot explain the large numbers of mutations observed in human cancers, it has been suggested that cancer cells are frequently characterized by an intrinsic tendency to accumulate mutations (Schmitt, Prindle *et al.* 2012, Datta, Gutteridge *et al.* 2013). Microsatellite instability and chromosomal instability are two types of genetic instability (Lengauer, Kinzler *et al.* 1997, Orr-Weaver and Weinberg 1998). Aneuploidy and DNA errors represent a general feature of bladder cancer malignancy (Phillips and Richardson

2006, Puntoni, Zanardi *et al*. 2007). Cancers that exhibit extensive microsatellite instability are those with mutations or inactivation of mismatch repair genes; those that exhibit predominantly chromosomal instability are those with mutations that affect the segregation of chromosomes during mitosis (Loeb 2001). Many genes, including those involved in maintaining genome stability, chromosomal segregation, DNA repair genes, cell cycle checkpoints, if changed could induce a mutator phenotype (Loeb 2001).

Constitutive expression of *ST3GAL1* in HT1376 cells led to a decreased expression of several genes whose common feature is the preservation of genomic stability. In particular, we found down-regulated genes involved in different mechanisms of DNA repair, including homologous recombination (HR), nucleotide excision repair (NER), base excision repair (BER). Moreover, we found down-regulated several genes involved in the accuracy of chromosomal segregation during mitosis. Interestingly, constitutive expression of *ST6GALNAC1* in MCR cells also results in a down-regulation of several genes involved in different mechanisms of DNA repair and in chromosomal segregation. Strikingly, the two sialyltransferases affect in a parallel manner groups of genes with similar functions. These data suggest that the expression of either sialyltransferase in the two cell lines leads to a decreased ability to fix mutations and to an inherent tendency to aneuploidy, resulting in genetic instability and tumour progression. In MCR cells, *ST6GALNAC1* turns on genes *ERBB3* and *ERBB4*, in the presence of good levels of expression of *ERBB2*. It should be remembered that the human gene *ERBB2* (or *HER-2*) encodes a 185 kDa cell surface transmembrane glycoprotein ($p185^{erbB2}$) with intrinsic tyrosine kinase activity that is frequently amplified or over-expressed in breast cancer and other epithelial cancers. Most studies have linked *ERBB2* over-expression with poor clinical prognosis in breast cancer patients (Slamon, Clark *et al*. 1987, Eccles 2001). Members of the ERBB family undergo ligand-induced heterodimerization. Although ERBB2 is unique within this family as having no known ligand, it is nevertheless the favoured heterodimerization partner of the other three family members. It has been shown that ERBB2 is normally expressed in an activated conformation, *i.e.* ready for heterodimer formation (Cho, Mason *et al*. 2003). In contrast, activation of the other ERBB family members is dependent on ligand binding. Therefore, rather than being a growth factor receptor *per se*, the role of *ERBB2* may be that of an essential common subunit of ERBB receptor signal transduction. Thus, in the presence of *ERBB2*, the higher expression of other ERBB family members would have the effect of strengthen

the ligand-induced ERBB signalling. These changes, in addition with an increase of the tumour protein p63 coding gene (*TP63*) and a decrease of the tumour suppressor *RB* in $MCR_{sTn}$ cells are consistent with a moderate positive effect on cell growth. Another functional group of genes affected by *ST6GALNAC1* expression is represented by those encoding proteases. The up-regulation of cathepsin L2 and H (*CTSL2* and *CTSH*) as well as kallikrein-related peptidase 5 (*KLK5*) in association with the down-regulation of the serine protease inhibitors *SERPINE1* and *SERPINE2* is consistent with an increase of the overall protease activity in $MCR_{sTn}$ cells.

In a recent study in collaboration with investigators of the University of Porto (Ferreira *et al*. 2013), we have shown that $MCR_{sTn}$ cells display slightly increased proliferation, increased cell motility and increased Matrigel invasion, data which are fully consistent with the changes in gene expression profile. Also consistent with current data are those of clinical specimens (Ferreira, Videira *et al*. 2013), showing that the sTn antigen is preferentially expressed by high-grade and muscle invasive cancers.

## *IV.2 Response of Bladder Cancer to BCG*

The interaction of BCG with urothelial endothelium is a key step in the development of anti-tumour response. Thus, after instillation of BCG, it accumulates near the wall of the bladder, allowing the establishment of specific and unspecific contacts to urothelial cells. The lumen of the bladder allows to accumulate BCG, specifically without adhering to the cell wall of the bladder (Bevers, Kurth *et al*. 2004). In clinical studies, intravesical BCG treatment has reduced bladder cancer recurrence rates by inducing a series of complex systemic humoral and cellular immune responses (Agarwal, Agrawal *et al*. 2010). It has been reported that the internalization process is dependent on the dose of BCG and time of incubation, as well as the state of differentiation of tumour cells, being that cells poorly differentiated (high grade) have a higher rate of internalization (de Boer, Bevers *et al*. 1996). Data from a large clinical study suggest that the five most commonly used strains Connaught, RIVM, Pasteur, Tice and A. Frappier do not differ in terms of preventing tumour progression (Sylvester, van der Meijden *et al*. 2002) even though recent *in vitro* studies on bladder cancer cell lines suggest that different commercial BCG strains could exert different anti-tumour activities (Secanella-Fandos, Luquin *et al*. 2013).

In this study is shown that internalization of BCG occurs for both cell lines in a time-dependent manner, independently on sTn or sT expression. Nevertheless, it should be noticed that the strain RIVM of BCG showed a tendency to be more internalized by $HT1376_{sT}$ or $MCR_{sTn}$ when confronting with non expressing sT or sTn antigen cells. A higher internalization of BCG might be correlated with the higher internalization of BCG, by sTn expressing bladder cancer cells, was concurrent with a higher loss of viability of these cells after BCG internalization. The observations that sTn cancer cells are more susceptible to BCG may be one of the mechanisms associated with the fact that bladder cancer patients expressing sTn respond better BCG (Lima, Severino *et al*. 2013). Lima, Severino *et al* paper is presented in Supplement II section. After internalization by tumour cells, BCG is processed and degraded and its antigens are presented at the cell surface in the context of MHC complex. This response of bladder cancer cells to BCG-challenging is complex and only partially understood. Among the functional consequences of BCG interaction is the modulation of the expression of MHC molecules (Ikeda, Toida *et al*. 2002) and the secretion of cytokines (Bevers, de Boer *et al*. 1998,

Bevers, Kurth *et al*. 2004). Consistently, gene expression analysis revealed increased expression of some transcripts encoding for class I or II MHC antigens upon BCG-challenging in both cell lines.

The analysis of transcriptome changes in BCG-challenged HT1376 and MCR cells reveals that the number of genes modulated by BCG-challenging is much higher in the cell lines expressing *ST3GAL1* or *ST6GALNAC1*. Among the genes which are consistently up-regulated only in sialyltransferase expressing cells are "caretaker" genes, suggesting a BCG-induced turn toward increased genomic stability, but only in sialyltransferase expressing cells. The mechanism(s) leading a cell surface change, such as the expression of Thomsen-Friedenreich related antigens on one or more glycoproteins, to a change in gene expression can be at the moment only hypothesized. However, the "outside-in" flow of information going from the cell membrane to the nucleus, triggered by the stimulation of membrane receptor and eventually leading to gene transcription can be conceivably modulated by postranslational modifications (including glycosylation) of these receptors.

Among the ten cytokines whose presence we investigated in the secretome of our bladder cancer cell lines, only IL-6 and IL-8 were expressed and modulated by BCG-challenging. IL-6 was produced by HT1376 cells only after BCG-challenging, regardless of *ST3GAL1* expression. By contrast, unchallenged MCR cells produced good levels of IL-6, which were doubled by BCG-challenging only in *ST6GALNAC1* expressing cell population. IL-6 is a multifunctional proinflammatory cytokine, playing multiple functions in angiogenesis and vascular modelling that may contribute to tumour cell proliferation and differentiation (Wei, Kuo *et al*. 2003, Culig 2011). Several studies have suggested that IL-6 is associated with a number of biological functions in bladder cancer, including cell proliferation, cell transformation, inflammation, and detrusor smooth muscle contractility (Han, Lee *et al*. 2009, Weng, Wu *et al*. 2009, Escudero-Lourdes, Medeiros *et al*. 2010, Li, Li *et al*. 2010). IL-6 levels in serum and urine have been also associated with progression in bladder cancer (Andrews, Shariat *et al*. 2002, Chen, Lin *et al*. 2013). Previous studies have indicated that BCG also increased IL-6 production in human and murine bladder cancer cell lines; however the mechanisms by which IL-6 affects human bladder carcinoma cells remain unclear (Chen, Crist *et al*. 2002, Chen, Zhang *et al*. 2009). Recently Tsui *et al* reported that IL-6-knockdown experiments in T24 cells and IL-6 overexpression experiments in HT1376 cells resulted in IL-6 reduced cell proliferation,

migration, and invasion *in vitro*. Xenograft studies indicated that the overexpression of IL-6 down-regulated tumourigenesis of bladder cells and that IL-6 knockdown reversed this effect (Tsui, Wang *et al*. 2013). IL-8 was expressed at a good level by HT1376 cells but not by MCR cells. BCG-challenging turned on IL-8 production only in cells expressing either sialyltransferase. IL-8 is a neutrophil chemoattractant and an inducer of angiogenesis (Koch, Polverini *et al*. 1992, Mukaida 2000). Some authors have found that high levels of IL-8 production early during the treatment of bladder cancer are associated with better clinical responses to BCG and are responsible for immune cells recruitment (Rabinowitz, Smith *et al*. 1997, Kumar, Dubey *et al*. 2002). However, others reported high urinary IL-8 levels during intravesical instillation in patients who developed recurrence compared with the levels in those in remission (Sheryka, Wheeler *et al*. 2003, Sagnak, Ersoy *et al*. 2009). Recently, Secanella-Fandos *et al* reported that IL-6 and IL-8 inhibited tumour growth in a dose dependent manner. When both interleukins were present at low concentrations, a synergistic effect on growth inhibition was observed. However, at higher concentrations inhibition was increased only with respect to the IL-8 dose alone (Secanella-Fandos, Luquin *et al*. 2013). The apparently contradictory relationship between IL-6 and IL-8 production and anti-tumoural response should be considered in the context of the relationship between inflammation and cancer which is double edge. In fact, on one hand the inflammatory reaction is useful to eradicate the tumour while on the other hand the inflammatory environment is the necessary soil for cancer growth and progression. The finding that both cytokines are preferentially released by *ST3GAL1*- or *ST6GALNAC1*- expressing cells strongly indicates that the expression of these sialyltransferases might profoundly influence the clinical response to BCG. This could happen through different and not mutually exclusive mechanisms. First, at the level of the BCG interaction with bladder cells; second, at the level of a differential activity of receptors decorated by Thomsen-Friedenreich related antigens; and third, indirectly through a differential pattern of gene expression.

## IV.3 Response of Macrophages to BCG-challenged BC cell lines

Although the mechanism of action of BCG immunotherapy has not been fully elucidated, it is clear that it induces a local immune response characterized by massive inflow and infiltration of inflammatory cells to the lining and bladder lumen, which results in the production of various cytokines and in the development of a robust cellular response (Lockyer and Gillatt 2001, Kresowik and Griffith 2009). A key role in these processes is played by macrophages, a cell population present in almost all tissues which provides a link between innate and adaptive immunity (Gordon and Taylor 2005, Hao, Lu *et al*. 2012). Macrophages can be activated by a variety of stimuli and polarized to functionally different phenotypes. Two distinct subsets of macrophages have been described: classically activated M1 and alternatively activated M2 macrophages (Hao, Lu *et al*. 2012). M1 macrophages are classically activated by microbial products, secrete cytokines such as IL-12, which through the cooperation of other cell types, polarize the differentiation of T helper cells toward the Th1 phenotype whose hallmark is the cytokine IFNγ. This response leads to antimicrobial responses. On the other hand, M2 macrophages are involved in type 2 responses, during which T helper 2 lymphocytes secrete IL-4, IL-5 and IL-10. M2 macrophages are mainly involved in angiogenesis and tissue repair and are thought to provide help to tumour growth (Balkwill, Charles *et al*. 2005, Mantovani, Allavena *et al*. 2008). However, M1 and M2 phenotypes are extremes in a continuum spectrum of functional conditions. The hallmark of M1 or M2 phenotypes can be considered the IL-12/IL-10 ratio, which is high for M1 and low for M2 macrophages. The expression of other cytokines such as IL-1β, IL-6 or TNFα can be shared by macrophages of both phenotypes (Mantovani, Allavena *et al*. 2004). In our unstimulated monocyte-derived macrophages, the level of IL-10 is low while IL-12 is undetectable. Moreover, cytokines IFNγ and IL-4, which are the hallmarks of Th1 and Th2 lymphocyte differentiation, respectively, are barely or not detectable. On the contrary, inflammatory cytokines, such as IL-1β, IL-6 and TNFα and the anti-inflammatory IL-10 are induced only by the secretome of BCG-challenged bladder cancer cells, particularly when expressing either sialyltransferase. IL-8 was produced at a high level even by unstimulated macrophages and exhibited little variations upon BCG-challenging. Altogether, these results indicate that the macrophages used in our model system, after secretome from BCG-challenged and sT or sTn expressing bladder

cancer cells, display a phenotype oriented toward a M1 type accordingly to ability to produce inflammatory cytokines like M1 type. Nevertheless, the expression of IL-10 suggests an intermediate phenotype between M1 and M2. The stimulation of inflammatory cytokine production by macrophages is crucially dependent on the secretome of BCG-challenged bladder cancer cells expressing sialylated Thomsen-Friedenreich related antigens, and in the case of sTn antigens, this may also explain the better response to BCG observed in sTn positive bladder cancer patients. Particularly the expression of sT in our bladder cancer model showed also a tendency for higher phagocytosis level of apoptotic bladder cancer cells by macrophages. In fact, in the latest publication of Lima *et al.*, clinical data correlated a worst response to BCG when M2 type macrophages were present (Lima, Oliveira *et al*. 2013), and accordingly to our most recent results, it was found that the expression of sTn and of s6T antigens (the biosynthesis of both is dependent on *ST6GALNAC1*) is predictive of BCG response and is associated with a better recurrence free survival (Lima, Severino *et al*. 2013). Lima, Severino *et al* paper is presented in Supplement II section. Thus, BCG treatment turns the expression of sTn/s6T from an unfavourable condition associated with increased progression to a favourable condition associated with a better prognosis. Current results are fully consistent with these clinical data because they show how the whole gene expression profile is modulated by the expression of either sialyltransferase and that the secretion of inflammatory cytokines, a crucial step in the establishment of an effective anti-tumour response, is dramatically dependent on sT or sTn expression.

## *IV.4 Conclusions*

In conclusion, it appears that the expression of either sialyltransferase in the two cell lines induces the down-regulation of several genes involved in proper chromosomal segregation and repair of DNA alterations. Although the level of modulation is rarely high, it is reasonable to expect that the concomitant and parallel down-regulation of many genes involved in the same cellular function results in a phenotype more prone to chromosomal instability. The changes of expression of genes encoding members of the ERBB family we observed in *ST6GALNAC1*-transduced cells are consistent with a more sustained cell growth. Moreover, these cells display also a modulation of the protease genes and of their inhibitors which is consistent with the recently observed increased proteolytic activity of these cells (Ferreira, Videira *et al*. 2013). The effect of BCG on gene expression appears to be remarkably stronger on cells expressing either sialyltransferase rather than on mock-transduced cells, while the cytokine production and the ability to induce cytokine secretion by macrophages are also crucially dependent on sTn or sT antigens. Together with the clinical studies showing that carbohydrate antigens are major determinants in predicting successful BCG therapy, Thomsen-Friedenreich related antigens emerge as key determinants in the biology of bladder cancer and in the clinical response to BCG immunotherapy.

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# SUPPLEMENT I

## *Array Heat Maps for HT1376 or MCR cells after ST3GAL1 or ST6GALNAC1 transduction and/or after BCG challenging*

*Effect of ST3GAL1 expression*

In Figure I.1 is presented, as a heat-map, the *ST3GAL1*-dependent modulation of genes in HT1376 cells. Up- or down-regulation refer to the change in $HT1376_{ST}$ compared with $HT1376_{NC}$ cells.

**Figure I.1. Genes showing up- or down-regulation in $HT1376_{ST}$ cells compared with $HT1376_{NC}$ cells.** Heat-maps are presented for the 2log expression ratio ($\leq$ -0.9 in green or $\geq$ 0.9 in red) between $HT1376_{ST}$ cells and $HT1376_{NC}$ cells. Constitutive expression of *ST3GAL1* induced significant ($p \leq 0.05$) overexpression of 197 genes and down-regulation of 107 genes. Data are the mean of 3 experiments.

In HT1376$_{sT}$ cells, constitutive *ST3GAL1* overexpression induced the down-regulation of 197 genes and the up-regulation of 107 genes.

*Effect of BCG challenging on HT1376 cells transcriptome*

The impact of BCG challenging on gene expression of HT1376 cells (HT1376$_{NC}$ and HT1376$_{sT}$ cells) is shown in Figure I.2. The analysis was restricted to genes modulated by BCG but not modulated by the *ST3GAL1* overexpression (*i.e.* the genes which showed modulation by both *ST3GAL1* overexpression and BCG treatment were excluded from this analysis). BCG induced up-regulation of 68 genes and down-regulation of 18 genes.

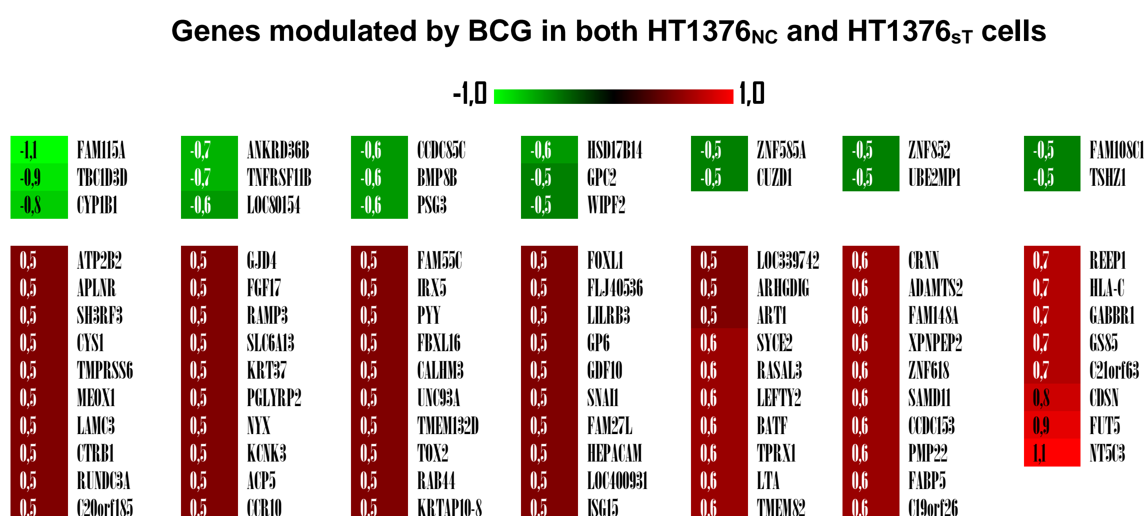
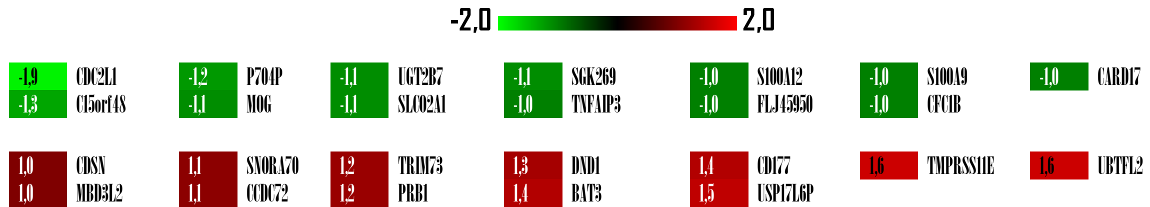



**Figure I.2. Genes showing up- or down-regulation in both HT1376$_{NC}$ and HT1376$_{sT}$ after BCG challenging.** Heat-maps are presented for the 2log expression ratio (≤ -0.5 in green or ≥ 0.5 in red) between BCG-challenged and unchallenged HT1376 (NC and sT) cell lines. BCG challenging induced down-regulation of 18 genes and up-regulation of 68 genes. Data are the mean of 3 experiments. $p \leq 0.05$.

These results showed an overall up-regulation of the transcriptome of HT1376 cells after BCG challenging. When HT1376$_{NC}$ or HT1376$_{sT}$ were analysed separately for their response to BCG, we found that 25 genes were modulated only in HT1376$_{NC}$ (Figure I.3a) (13 genes down-regulated and 12 genes up-regulated), whereas 107 genes were modulated (80 genes down-regulated and 27 up-regulated) only in BCG-challenged HT1376$_{sT}$ cells (Figure I.3b).

a)

**Genes modulated by BCG only in $HT1376_{NC}$ cells**




b)

**Genes modulated by BCG only in $HT1376_{sT}$ cells**

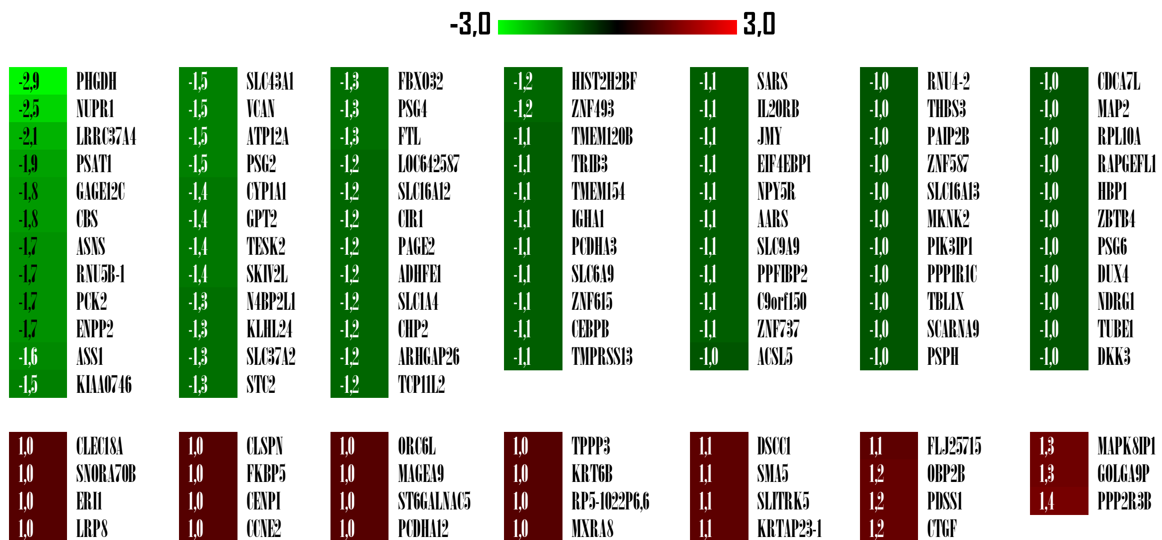



**Figure I.3. Genes showing differential modulation after BCG-challenging in $HT1376_{NC}$ or $HT1376_{sT}$ cells.** Heat-maps are presented for the 2log expression ratio ($\leq$ -1.0 in green or $\geq$ 1.0 in red) between BCG-challenged $HT1376_{NC}$ or $HT1376_{sT}$ cells and unchallenged cells. a) Group of genes modulated by BCG challenging in $HT1376_{NC}$, but not in $HT1376_{sT}$ cells. b) Group of genes modulated by BCG challenging in $HT1376_{sT}$, but not in $HT1376_{NC}$ cells. Twenty five genes showed modulation (12 up-regulation, 13 down-regulation) in $HT1376_{NC}$, cells, but not in $HT1376_{sT}$. On the contrary, 107 genes displayed modulation in $HT1376_{sT}$ cells but not in $HT1376_{NC}$. Of these, 27 displayed up-regulation and 80 down-regulation. Data are the mean of 3 experiments. $p \leq 0.05$.

This finding is consistent with the notion that *ST3GAL1* expression makes the genome of bladder cancer cells more prone to BCG-induced modulation.

*Effect of ST6GALNAC1 expression*

In Figure I.4 is presented as a heat-map the modulation of genes whose expression was modulated by *ST6GALNAC1*. In $MCR_{sTn}$ cells, the constitutive *ST6GALNAC1* overexpression induced the down-regulation of 114 genes and the up-regulation of 83 genes.

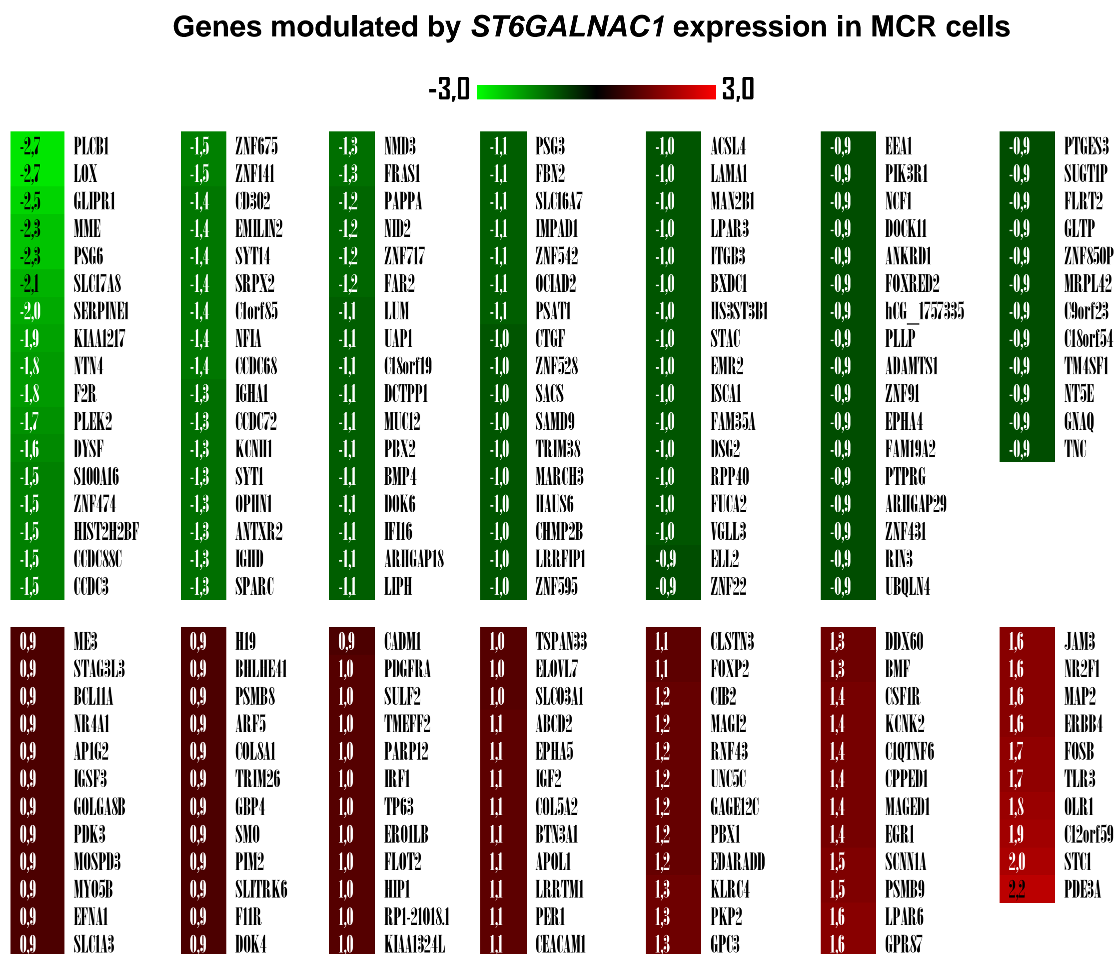



**Figure I.4. Genes showing up- or down-regulation in $MCR_{sTn}$ cells compared with $MCR_{NC}$.** Heat-maps are presented for the 2log expression difference ($\leq$ -0.9 in green or $\geq$ 0.9 in red) between $MCR_{sTn}$ cells and $MCR_{NC}$ cells. In $MCR_{sTn}$ cells, constitutive *ST6GALNAC1* overexpression induced the down-regulation of 114 genes and the up-regulation of 83 genes. Data are the mean of 3 experiments. $p \leq 0.05$.

*Effect of BCG challenging on MCR cells transcriptome*

The gene expression profile of BCG-challenged MCR ($MCR_{NC}$ and $MCR_{sTn}$ cells) cells confronted with that of unchallenged cells is presented in Figure I.5. The analysis was restricted to genes modulated by BCG but not modulated by the *ST6GALNAC1* overexpression (*i.e.* the genes which showed modulation by both ST6GALNAC1 expression and BCG treatment were excluded from this analysis). BCG induced up-regulation of 25 genes and down-regulation of 24 genes.

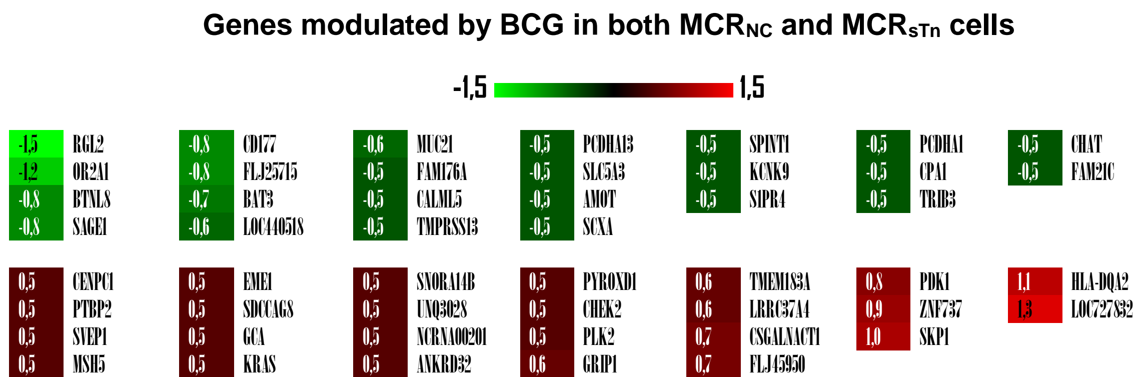



**Figure I.5. Genes showing up- or down-regulation in both $MCR_{NC}$ and $MCR_{sTn}$ after BCG challenging.** Heat-maps are presented for the 2log expression ratio ($\leq$ -0.5 in green or $\geq$ 0.5 in red) between BCG-challenged and unchallenged MCR (NC and sTn) cell lines. BCG challenging, 24 genes are down-regulated and 25 genes are significantly up-regulated. Data are the mean of 3 experiments. $p \leq 0.05$.

These results showed an overall equivalent up- and down-regulation of MCR cell transcriptome after BCG challenging. When the BCG response of $MCR_{NC}$ or $MCR_{sTn}$ cells was analysed separately (Figure I.6) we found that 17 genes were modulated only in $MCR_{NC}$ cells (11 genes down-regulated and 6 up-regulated), whereas 38 genes were modulated only in $MCR_{sTn}$ cells (29 were up-regulated and 9 down-regulated).

a)

**Genes modulated by BCG only in $MCR_{NC}$ cells**

b)

**Genes modulated by BCG only in $MCR_{sTn}$ cells**

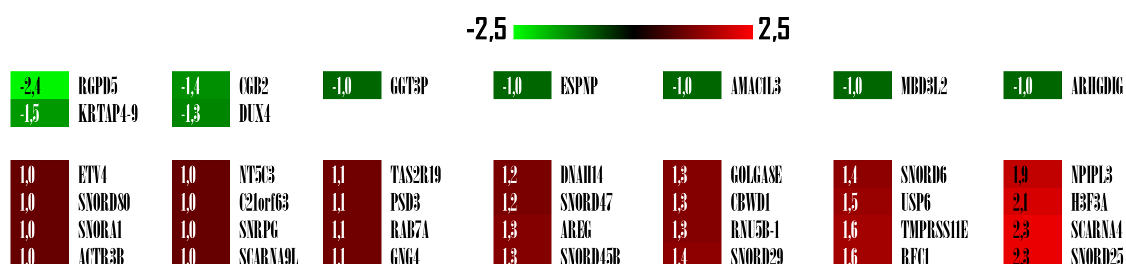



**Figure I.6. Genes showing differential modulation after BCG challenging in $MCR_{NC}$ or $MCR_{sTn}$ cells.** Heat-maps are presented for the 2log expression difference (≤ -1.0 in green or ≥ 1.0 in red) between BCG-challenged $MCR_{NC}$ or $MCR_{sTn}$ cells and their respective unchallenged cells. a) Group of genes modulated by BCG challenging in $MCR_{NC}$, but not in $MCR_{sTn}$ cells. b) Group of genes modulated by BCG challenging in $MCR_{sTn}$, but not in $MCR_{NC}$ cells. In $MCR_{sTn}$ bladder cancer cells, after BCG challenging, a) 17 genes failed to respond to BCG whether in $MCR_{NC}$ these responded negatively (11 genes) or positively (6 genes). On the other hand b) 38 new genes differentially respond to BCG challenging (29 genes were up-regulated and 9 were down-regulated). Data are the mean of 3 experiments. $p \leq 0.05$.

This finding is consistent with the notion that *ST6GALNAC1* expression makes the genome of bladder cancer cells more prone to BCG-induced modulation.

# SUPPLEMENT II

## *Response of high-risk of recurrence/progression bladder tumours expressing sTn and s6T to BCG immunotherapy – paper*

BJC

FULL PAPER

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Keywords: Bacillus Calmette-Guérin; BCG immunotherapy; bladder cancer; sialyl-Tn; sialyl-6-T; tumour glycosylation

# Response of high-risk of recurrence/progression bladder tumours expressing sialyl-Tn and sialyl-6-T to BCG immunotherapy

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**Background:** High risk of recurrence/progression bladder tumours is treated with Bacillus Calmette-Guérin (BCG) immunotherapy after complete resection of the tumour. Approximately 75% of these tumours express the uncommon carbohydrate antigen sialyl-Tn (Tn), a surrogate biomarker of tumour aggressiveness. Such changes in the glycosylation of cell-surface proteins influence tumour microenvironment and immune responses that may modulate treatment outcome and the course of disease. The aim of this work is to determine the efficiency of BCG immunotherapy against tumours expressing sTn and sTn-related antigen sialyl-6-T (s6T).

**Methods:** In a retrospective design, 94 tumours from patients treated with BCG were screened for sTn and s6T expression. *In vitro* studies were conducted to determine the interaction of BCG with high-grade bladder cancer cell line overexpressing sTn.

**Results:** From the 94 cases evaluated, 36 had recurrence after BCG treatment (38.3%). Treatment outcome was influenced by age over 65 years (HR = 2.668; (1.344–5.254); $P = 0.005$), maintenance schedule (HR = 0.480; (0.246–0.936); $P = 0.031$) and multifocallity (HR = 2.065; (1.033–4.126); $P = 0.040$). sTn or s6T expression was associated with BCG response ($P = 0.024$; $P < 0.0001$) and with increased recurrence-free survival ($P = 0.001$). Multivariate analyses showed that sTn and/or s6T were independent predictive markers of recurrence after BCG immunotherapy (HR = 0.296; (0.148–0.594); $P = 0.001$). *In vitro* studies demonstrated higher adhesion and internalisation of the bacillus to cells expressing sTn, promoting cell death.

**Conclusion:** s6T is described for the first time in bladder tumours. Our data strongly suggest that BCG immunotherapy is efficient against sTn- and s6T-positive tumours. Furthermore, sTn and s6T expression are independent predictive markers of BCG treatment response and may be useful in the identification of patients who could benefit more from this immunotherapy.

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Bladder cancer is the fifth most common cancer in Western society (van Rhijn *et al*, 2009), with the highest recurrence rate among solid tumours and poor prognosis when the tumour invades the muscularis propia (Babjuk *et al*, 2011). To reduce the risk of recurrence and progression to muscle invasion, non-muscle invasive high-grade tumours, multifocal and recurrent lesions are submitted to intravesical instillations with live attenuated Bacillus Calmette-Guérin (BCG) after complete transurethral resection of bladder tumour (TURBT; Babjuk *et al*, 2011). Although the management of the disease has significantly improved with this therapeutics, 30–40% of the patients relapse and approximately 15% progress to muscle invasive tumours (Palou Redorta, 2006). Predicting patients who could be best served by an alternative treatment or early cystectomy, would avoid progression, reduce disease charge and decrease health expenses. It is consensual that the integration of multiple biomarkers may hold predictive value; still such biomarker panel remains to be established (Lima *et al*, 2012; Zuiverloon *et al*, 2012).

The exact mechanism by which BCG mediates anti-bladder cancer immunity remains elusive (Alexandroff *et al*, 2010). However, the capability of the bacillus to recognise and efficiently bind to tumour cells has a determinant role in the therapeutics outcome (Alexandroff *et al*, 2010). The bacillus is then internalised triggering tumour cell apoptosis or host adaptive immune responses (Becich *et al*, 1991; Ratliff, 1992). The bacterial adhesion, fibronectin attachment protein (FAP), was recognised as the main factor mediating BCG attachment and internalisation by bladder tumour cells (Sinn *et al*, 2008; Alexandroff *et al*, 2010). FAP binds to $\alpha 5\beta 1$ integrins expressed by tumour cells via a fibronectin bridge and to be responsible for the uptake of BCG–fibronectin–integrin complexes (Sinn *et al*, 2008; Alexandroff *et al*, 2010).

Malignant transformations may be accompanied by a premature stop in the *O*-glycosylation of proteins by sialylation, originating the sialyl-Tn (sTn, Neu5Ac$\alpha$2-6GalNAc$\alpha$-*O*-Ser/Thr) and sialyl-6-T antigens (s6T, Gal$\beta$1-3(Neu5Ac$\alpha$2-6)GalNAc$\alpha$-*O*-Ser/Thr; Dall'Olio *et al*, 2012). We recently reported that approximately 75% of high-grade bladder tumours, presenting elevated proliferation indexes and high risk of recurrence/progression expressed sTn (Ferreira *et al*, 2013). sTn expression enhanced the invasive capability of bladder cancer cells and was considered a surrogate biomarker of tumour aggressiveness (Ferreira *et al*, 2013). Hence, efficient therapies to manage these tumours are needed to avoid disease progression and poor outcomes.

sTn expression is known to interfere with cell–cell adhesion, cell–matrix interaction, including integrin-fibronectin binding, modulate cell morphology (Clement *et al*, 2004; Julien *et al*, 2006; Pinho *et al*, 2007) and immune responses (Gilewski *et al*, 2007; Julien *et al*, 2009; Takamiya *et al*, 2013). Thus, we hypothesise it may modulate BCG attachment to tumour cells and/or immune response and consequently influence BCG immunotherapy outcome. sTn is also a biomarker of concomitant molecular alterations that may further determine the tumour behaviour (Ohno *et al*, 2006). As such, this work is devoted to evaluating the response of sTn-positive bladder tumours to BCG immunotherapy. The sTn structurally related antigen was also evaluated for the first time in the context of bladder cancer.

## MATERIALS AND METHODS

**Patient cohort.** This study was performed in a retrospective series of 94 cases with high-risk non-muscle invasive bladder cancer. Patients were treated with TURBT and then submitted to BCG immunotherapy in the Portuguese Oncology Institute of Porto, between 1998 and 2006. No second-look TURBT was performed, although the majority of the samples had muscularis propria tumour free. All received intravesical instillation of BCG for 6 consecutive weeks (induction BCG scheme, iBCG) and 56.4% were submitted to maintenance BCG schedule (iBCG + maintenance protocol with two weekly instillations every 3 months during 2 years, mBCG). The iBCG group includes patients treated before the European Association of Urology guidelines recommending the mBCG (Oosterlinck *et al*, 2006) scheme and patients showing significant intolerance to long BCG treatment.

The male/female sex ratio was of 78:16. The patients were followed every 3 months for the first year, every 6 months for the second year and every 12 months thereafter by cystoscopy and urine cytology. Recurrence was defined as the appearance of a tumour once the treatment has begun, with at least one tumour-free cystoscopy and cytology in-between. These recurrences were also available for study. The non-responders were defined as patients submitted to BCG treatment with tumour recurrence. Finally, recurrence-free survival (RFS) was defined as the period of time between the beginning of treatment and recurrence or the most recent tumour-free cystoscopy and cytology. All procedures were performed after patient's informed consent and approved by the Ethics Committee of IPO-Porto. All clinicopathological information was obtained from patients' clinical records. All tumour samples were revised by a pathologist, regarding 2004 WHO grading criteria.

**Expression of STn in bladder tumours.** Formalin-fixed paraffin-embedded tissue sections were screened for sTn by immunohistochemistry using the avidin/biotin peroxidase method, as described by Ferreira *et al* (2013). sTn expression was evaluated with anti-sTn mouse monoclonal antibody clone TKH2 (Ferreira *et al*, 2013). The s6T antigen was evaluated in sTn-negative tumours using the same antibody, after treatment with a recombinant $\beta$-(1-3)-galactosidase from *Xanthomonas campestris* (R&D systems, Minneapollis, MN, USA) for 1 h at 37°C. This enzyme removes the *O*-3-linked Gal residues exposing the sTn antigen (Figure 1A).

Both antigens were assessed double-blindly by three independent observers. Upon disagreement, the slides were reviewed, until a consensus was reached. Tumours were classified as positive when immunoreactivity of anti-sTn TKH2 antibody was observed.

Structural assignments were validated by a combination of enzymatic treatments. For sTn, positive tissues were first treated with a $\alpha$-neuraminidase from *Clostridium perfringens* (Sigma-Aldrich, St Louis, MO, USA) for 2 h at 37 °C to remove the sialic acid and then screened for sTn expression. For s6T, positive tissues were primarily incubated with the $\beta$-(1–3)-galactosidase, followed by incubation with the neuraminidase. The absence or decrease in immunoreactivity of TKH2 monoclonal antibody confirmed the presence of these structures.

**Adhesion and internalisation of BCG to bladder cancer cell line**

*Cell lines culture.* The human bladder cancer cell line MCR and the transduced variants of MCR (MCRnc and MCRsTn$^+$) were grown as described by Videira *et al* (2009). The MCRsTn$^+$ cell line results from the stable transduction of MCR cells with a lentivirus expressing the coding region of the human *ST6GalNAc.I* gene, the enzyme responsible by the biosynthesis of sTn (Ferreira *et al*, 2013). The MCRnc cell line, not expressing sTn, was used as control (Ferreira *et al*, 2013).

*Bacterial strain and labelling.* BCG strain RIVM (Medac, Hamburg, Germany) was used in this study. Briefly, $10^8$ viable BCG cells were labelled with 10 $\mu$g fluorescein isothiocyanate (FITC; Invitrogen, Carlsbad, CA, USA) in 1 ml of 50 mM sodium carbonate buffer (pH 9.2) for 30 min at 20 °C. The labelled bacteria (BCG-FITC) were washed three times with PBS containing 0.05% of Tween-80 (Sigma-Aldrich) and centrifuged for 10 min at 13.000 g to remove excess FITC.

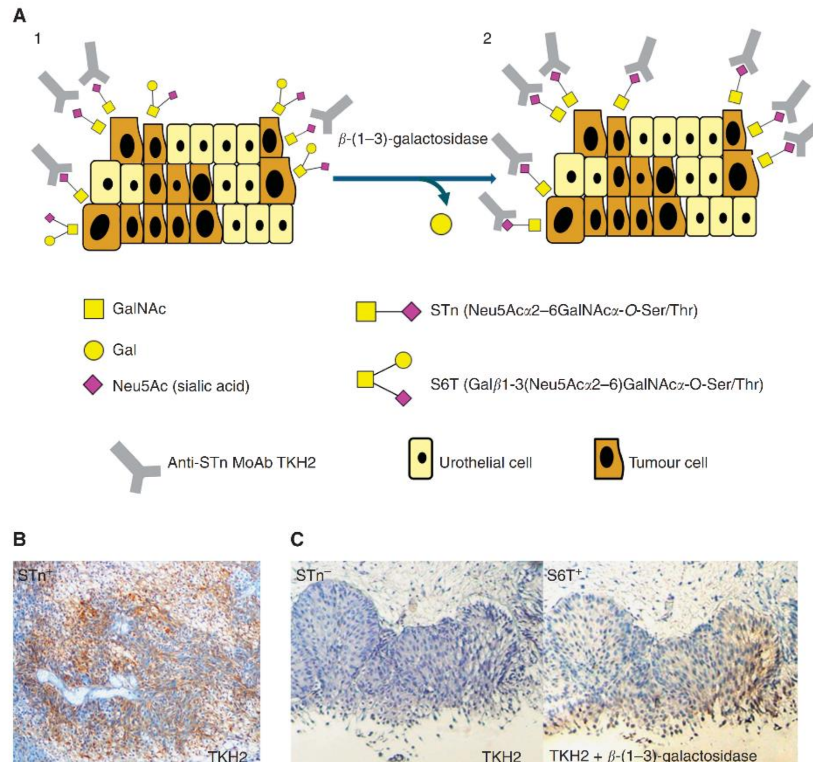


**Figure 1. Expression of sTn and s6T in bladder tumours.** Schematic representation of a bladder tumour expressing sTn and s6T antigens (**A**). Urothelial cell-surface glycoproteins present more or less elongated *O*-glycan chains often terminated with ABO and/or Lewis blood group determinants (not represented). Conversely, some malignant cells express the sTn and s6T (STn + Gal residue) antigens that result from a premature stop in the biosynthesis of *O*-glycans (**A**). The sTn, but not the s6T antigen, can be recognised by mouse monoclonal antibody TKH2 (**A**1). The digestion of the FFPE sections with a β-(1-3)-galactosidase removes the Gal residue from s6T allowing detection by TKH2 (**A**2). Bladder tumour expressing the sTn antigen (**B**). Bladder tumour expressing the s6T antigen but not sTn (**C**). The FFPE tissue was positive for sTn only after digestion of the FFPE tissue with a β-(1-3)-galactosidase. This is the first report on s6T antigen expression in bladder tumours.

*BCG adhesion and internalisation assay.* MCR cells were plated into 24-well plates at $0.2 \times 10^6$ cell per well. At confluence, cells were washed and BCG-FTIC was added to the cultures at a ratio of 10:1 (BCG/cells). After 2, 6 or 24 h incubation time, the cells were gently washed to remove the excess BCG, harvested and centrifuged at 350 **g** for 5 min. In order to differentiate between internalised and surface-bound BCG, trypan blue was added to quench surface-attached fluorescence bacteria. Flow cytometry was performed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and data were analysed using the Flowing *v*2.4 software (Turku Center for Biotechnology, Turku, Finland).

*Estimation of cell viability after exposure to BCG.* The influence of BCG treatment on MCR cell viability after the 24 h of exposure was assessed through the visualisation of morphologic changes by flow cytometry. Dot plots of forward-angle light scatter (FSc) *vs* side-angle light scatter (SSc) of MCR cells before and after exposure to BCG were analysed using the Flowing *v*2.4 software (Turku Center for Biotechnology).

For a shorter period of time of BCG exposure (6 h), apoptotic status of cells was evaluated by labelling with Annexin V (Sigma-Aldrich) as indicated by manufacture instructions.

**Statistical analysis.** Statistical data analysis was performed with IBM Statistical Package for Social Sciences—SPSS for Windows (version 20.0; IBM, Armonk, NY, USA). Chi-square analysis was used to compare categorical variables. Kaplan–Meier survival curves were used to evaluate correlation between glycans expression and RFS, log-rank statistical test was used for curves comparison. Multiple Cox regression analysis was used to assess the effect of both antigens on the time to recurrence in BCG-treated patients and to adjust for potential confounders. Non-parametric Mann–Whitney test was used to compare the differences in the BCG attachment and internalisation to MCRnc and MCRsTn+ cells.

## RESULTS

**Clinicopathological features and BCG treatment outcome.** From the 94 cases evaluated, 36 had recurrence after BCG

**Table 1.** Relation between patients clinical and tumour characteristics and response to BCG treatment and time to recurrence

| Variables | Total *n* (%) | Responders *n* (%) | Non-responders *n* (%) | $P^a$ | HR (95% CI) | $P^b$ |
|---|---|---|---|---|---|---|
| **Age (years)** | | | | | | |
| <65 | 51 (54.3) | 37 (63.8) | 14 (38.9) | **0.018** | 1.0 | |
| ⩾65 | 43 (45.7) | 21 (36.2) | 22 (61.1) | | 2.668 (1.355–5.254) | **0.005** |
| **Sex** | | | | | | |
| Male | 78 (83.0) | 47 (81.0) | 31 (86.1) | 0.524 | 1.0 | |
| Female | 16 (17.0) | 11 (19.0) | 5 (13.9) | | 0.883 (0.342–2.283) | 0.798 |
| **Stage** | | | | | | |
| Ta | 40 (42.6) | 23 (39.7) | 17 (47.2) | 0.471 | 1.0 | |
| T1 | 54 (57.4) | 35 (60.3) | 19 (52.8) | | 0.838 (0.435–1.613) | 0.596 |
| **Grade** | | | | | | |
| Low | 38 (40.4) | 24 (41.4) | 14 (38.9) | 0.843 | 1.0 | |
| High | 56 (59.6) | 34 (58.6) | 22 (61.1) | | 1.295 (0.661–2.537) | 0.450 |
| **Size (cm)** | | | | | | |
| <3 | 62 (66.7) | 38 (65.5) | 24 (68.6) | 0.762 | 1.0 | |
| ⩾3 | 31(33.3) | 20 (34.5) | 11 (31.4) | | 0.787 (0.384–1.613) | 0.513 |
| **Tumour number** | | | | | | |
| Unifocal | 51 (54.3) | 30 (51.7) | 13 (36.1) | 0.140 | 1.0 | |
| Multifocal | 43 (45.7) | 28 (48.3) | 23 (63.9) | | 2.065 (1.033–4.126) | **0.040** |
| **CIS** | | | | | | |
| No | 88 (93.6) | 54 (93.1) | 34 (94.4) | 1.000 | 1.0 | |
| Yes | 6 (6.4) | 4 (6.9) | 2 (5.6) | | 0.783 (0.188–3.267) | 0.737 |
| **Recurrence status** | | | | | | |
| Primary | 48 (51.0) | 31 (53.4) | 17 (47.2) | 0.557 | 1.0 | |
| Recurrent | 46 (49) | 27 (46.6) | 19 (52.8) | | 1.327 (0.686–2.564) | 0.401 |
| **BCG schedule** | | | | | | |
| iBCG | 41 (43.6) | 20 (34.5) | 21 (58.3) | **0.023** | 1.0 | |
| mBCG | 53 (56.4) | 38 (65.5) | 15 (41.7) | | 0480 (0.246–0.936) | **0.031** |

Abbreviations: BCG = Bacillus Calmette Guérin; CI = confidence interval; CIS = Carcinoma *in situ*; HR = hazard ratio; iBCG = induction BCG; mBCG = maintenance BCG.
Bold values indicate $P<0.05$.
[a]Chi-square test.
[b]Wald test.

treatment (38.3%). The median follow-up time of the patients free of recurrence was 68.5 months (range: 6.0–135.0) and the median time of recurrence was 38.5 months (range: 10.0–122.0). The median follow-up time considering all the cases under analysis was 61.0 months (range: 6.0–135.0). Table 1 summarises patients and tumour clinicopathological features and its association with BCG response and RFS after treatment. We found that 61.1% of the non-responders were over 65 years old at the time of tumour resection, whereas only 36.2% of responders were over 65 years old ($P = 0.018$). Furthermore, patients over 65 years have approximately three-fold increased risk of recurrence (HR = 2.668; (1.344–5.254); $P = 0.005$). Moreover, it was observed a higher percentage of patients treated only with iBCG in the non-responder group when compared with the responder group (58.3% *vs* 34.5%, $P = 0.018$). Therefore, patients treated with mBCG scheme showed a 52% reduced risk of recurrence (HR = 0.480; (0.246–0.936); $P = 0.031$). It was also found that patients with multifocal tumours had an increased risk of recurrence after BCG treatment (HR = 2.065; (1.033–4.126); $P = 0.040$). No association was found for other characteristics, such as gender, tumour stage, grade or size, CIS presence and prior recurrence.

**Expression of sTn and s6T and association with clinicopathological features.** Approximately 66% of the studied bladder tumours were sTn positive (Figure 1A and B) and in all cases the antigen was observed in more than 5% of the tumour area. Additionally, 10 out of 32 sTn negative cases were positive for s6T (Figure 1C), which is structurally related to sTn. However, s6T assumed a more diffuse expression that did not exceed 5% of the tumour area in all cases.

The expression of sTn alone or in combination with s6T (sTn/s6T) was associated with high-grade tumours ($P = 0.007$; $P = 0.037$ Table 2) and also with primary tumours ($P = 0.001$; $P = 0.039$).

**STn and sTn/s6T as predictors of BCG treatment outcome.** sTn antigen was expressed by 74.1% of BCG responders and only by 47.2% of non-responders ($P = 0.034$; Table 3). When sTn and s6T were evaluated together, a similar relationship was observed ($P = 0.0001$; Table 3).

From the 94 patients included in this study, 36 had recurrences after treatment and 75% of these tumour specimens were available for sTn and s6T screening. All non-responders who presented sTn-

Table 2. Association between sTn and s6T antigens and clinicopathological characteristics

| | sTn | | | sTn – s6T | | |
|---|---|---|---|---|---|---|
| Variables | Negative *n* (%) | Positive *n* (%) | P[a] | Negative *n* (%) | Positive *n* (%) | P[a] |
| **Age (years)** | | | | | | |
| <65<br>⩾65 | 21 (65.6)<br>11 (34.4) | 30 (48.4)<br>32 (51.6) | 0.112 | 13 (59.1)<br>9 (40.9) | 38 (52.8)<br>34 (47.2) | 0.603 |
| **Sex** | | | | | | |
| Male<br>Female | 27 (84.4)<br>5 (15.6) | 51 (86.1)<br>11 (17.7) | 0.796 | 17 (77.3)<br>5 (22.7) | 61 (84.7)<br>11 (15.3) | 0.517 |
| **Stage** | | | | | | |
| Ta<br>T1 | 16 (50.0)<br>16 (50.0) | 24 (38.7)<br>38 (61.3) | 0.294 | 10 (45.5)<br>12 (54.5) | 30 (41.7)<br>42 (58.3) | 0.753 |
| **Grade** | | | | | | |
| Low<br>High | 19 (59.4)<br>13 (40.6) | 19 (30.6)<br>43 (69.4) | **0.007** | 13 (59.1)<br>9 (40.9) | 25 (34.7)<br>47 (65.3) | **0.042** |
| **Size (cm)** | | | | | | |
| <3<br>⩾3 | 23 (74.2)<br>8 (25.8) | 39 (62.9)<br>23 (37.1) | 0.276 | 16 (76.2)<br>5 (23.8) | 46 (63.9)<br>26 (36.1) | 0.431 |
| **Tumour number** | | | | | | |
| Unifocal<br>Multifocal | 12 (37.5)<br>20 (62.5) | 13 (50.0)<br>31 (50.0) | 0.249 | 7 (31.8)<br>15 (68.2) | 36 (50.0)<br>36 (50.0) | 0.134 |
| **CIS** | | | | | | |
| No<br>Yes | 29 (90.6)<br>3 (9.4) | 59 (95.2)<br>3 (4.8) | 0.406 | 19 (86.4)<br>3 (13.6) | 69 (95.4)<br>3 (4.2) | 0.112 |
| **Recurrence status** | | | | | | |
| Primary<br>Recurrent | 9 (28.1)<br>23 (71.9) | 39 (62.9)<br>19 (37.1) | **0.001** | 7 (31.8)<br>15 (68.2) | 41 (56.9)<br>31 (43.1) | **0.039** |
| **BCG schedule** | | | | | | |
| iBCG<br>mBCG | 14 (43.8)<br>18 (56.2) | 27 (43.5)<br>35 (56.5) | 0.985 | 11 (50.0)<br>11 (50.0) | 30 (41.7)<br>42 (58.3) | 0.490 |

Abbreviations: BCG = Bacillus Calmette-Guérin; iBCG = induction BCG; CIS = Carcinoma *in situ*; mBCG = maintenance BCG.
Bold values indicate $P<0.05$.
[a]Chi-square test.

Table 3. STn and sTn/s6T frequencies and risk of recurrence after BCG therapy

| | Responders *n* (%) | Non-responders *n* (%) | P value[a] |
|---|---|---|---|
| **sTn** | | | |
| Negative<br>Positive | 15 (25.9)<br>43 (74.1) | 17 (47.2)<br>19 (52.8) | 0.034 |
| **sTn and/or s6T** | | | |
| Negative<br>Positive | 6 (10.5)<br>51 (89.5) | 16 (43.2)<br>21 (56.8) | 0.0001 |

Abbreviation: BCG = Bacillus Calmette-Guérin.
[a]Chi-square test.

negative tumours prior treatment had sTn-negative recurrences. From the 15 non-responder patients who had sTn-positive tumours, 40% presented sTn-negative recurrences.

Kaplan–Meier analysis was used to evaluate if sTn with or without s6T influenced the RFS after BCG treatment. No differences were found regarding sTn expression alone (Figure 2A). However, when sTn and s6T were considered together, significant differences were found ($P=0.001$; Figure 2B). Patients with sTn/s6T-positive tumours had higher RFS than negative tumours (100.1 *vs* 63.2 months).

A Cox regression analysis, adjusted to age, tumour number and treatment scheme, was performed to assess the individual effect of these antigens in recurrence after BCG. Patients with sTn-positive tumours presented a trend to a lower-risk recurrence after BCG (HR = 0.544; 95% CI: (0.275–1.076); $P=0.080$; Table 4). Likewise, cases positive for sTn/S6T showed a significant lower risk of recurrence (HR = 0.296; 95% CI: (0.148–0.594); $P=0.001$).

**BCG interaction with MCRsTn+ cell line.** To evaluate the affinity of BCG for cells expressing sTn, we set up *in vitro* assays with MCRnc and MCRsTn+ cell lines, two genetically modified variants of the original MCR bladder cancer cell line. MCRnc cells

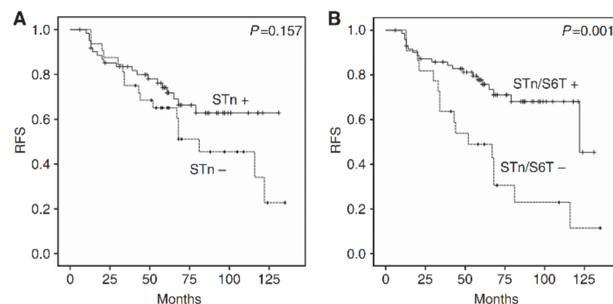


**Figure 2. Effect of sTn and s6T expression in recurrence-free survival (RFS).** Kaplan–Meier analysis to evaluate the association between RFS in the studied patients and: (**A**) sTn expression; (**B**) STn plus s6T presence (sTn/S6T). Comparison performed by log-rank test (**A**: $P=0.157$; **B**: $P=0.001$); ± censored sTn or sTn/s6T-negative tumours; ♦ censored sTn or sTn/s6T-negative tumours.

**Table 4.** Multivariate analysis and risk estimation of sTn and s6T influence on BCG therapy outcome

| | HR[a] | 95% CI | *P* value |
|---|---|---|---|
| **sTn** | | | |
| Negative | 1.0 | Referent | |
| Positive | 0544 | 0275–1.076 | 0.080 |
| **sTn + s6T** | | | |
| Negative | 1.0 | Referent | |
| Positive | 0296 | 0148–0.594 | 0.001 |

Abbreviations: BCG = Bacillus Calmette-Guérin; CI = confidence interval; HR = hazard ratio.
[a]Adjusted for age, tumour number and BCG schedule.

were transduced with an empty vector and MCRsTn$^+$ with the cDNA coding for the ST6GalNAc.I sialyltransferase. The phenotype of these transduced variants was previously described by Ferreira *et al* (2013). MCRsTn$^+$ presents a continuous and stable expression of the antigen sTn, whereas the MCRnc does not as shown in Supplementary Figure 1 (in the supplementary section).

MCRsTn$^+$ and its negative control MCRnc where then treated with BCG. Fluorescent-labelled BCG was internalised significantly over time, with a significant higher internalisation after 6 h incubation, when comparing with 2 h incubation (Figure 3A). Interestingly, after 6 h, MCRsTn$^+$ cells showed a tendency to internalise higher amounts of BCG than MCRnc cells (Figure 4A and B). Small amounts of BCG are internalised, resulting in small shifts of FITC fluorescence of the cells after internalisation (Figure 4B).

We then evaluated apoptosis status in MCR cells after 6 h of BCG challenging, using Annexin V an earlier labelling marker for cells undergoing apoptosis. It was observed a consistent tendency for a higher cell death in MCRsTn$^+$ after BCG challenging (higher Annexin V labelling—$MFI_{MCRnc} = 2560$) compared with MCRnc cells ($MFI_{MCRsTn+} = 2640$). In addition, a population of cells presenting stronger Annexin V labelling was also observed after 6 h BCG, which was higher (7%) in MCRsTn$^-$ cell than MCRnc (4%; Figure 4A). After a longer period of BCG challenging (24 h), MCRsTn$^+$ cells significantly decreased their size and granularity (80% of FSC$^{low}$SSC$^{low}$), which is usually typical of a rupture of plasma membrane and leakage of the cell's contents (Figure 4B). Conversely, MCRnc cells underwent little physical changes, presenting only 10% of FSC$^{low}$SSC$^{low}$ (Figure 4B). Furthermore, the internalisation of BCG by viable MCRsTn$^+$ cells at 24 h was markedly increased in relation to the controls (MCRnc; Supplementary Figure 2, supplementary section), therefore, in accordance with the observations made for 6 h.

These results present evidence that both BCG internalisation and loss of cell viability are correlated and both features are enhanced in cells expressing sTn antigen. Altogether, these findings suggest that the bacillus acts more efficiently in cells expressing sTn probably due to its higher internalisation.

## DISCUSSION

A significant percentage of high risk of recurrence/progression bladder tumours, conservatively treated with BCG immunotherapy after surgery, express cell-proteins yielding the sTn antigen (Ferreira *et al*, 2013). Despite the malignant potential of these tumours (Ferreira *et al*, 2013) and evidences that sTn expression may modulate the cell–BCG interaction (Clement *et al*, 2004; Julien *et al*, 2006; Pinho *et al*, 2007) as well as immune responses (Gilewski *et al*, 2007; Julien *et al*, 2009; Takamiya *et al*, 2013), nothing is known about the way patients exhibiting sTn-positive tumours respond to treatment.

We first observed that treatment outcome was influenced by age, treatment scheme and tumour multifocallity, as showed in other reports (Bohle and Bock, 2004; Joudi *et al*, 2006; Fernandez-Gomez *et al*, 2008; Malmstrom *et al*, 2009; Kohjimoto *et al*, 2010; Ajili *et al*, 2012). To overcome the samples heterogeneity, these variables were taken into account in multivariate analysis models to assess the influence of tumour-associated glycans in BCG response.

We also found that sTn expression was associated with high-grade tumours, which is in agreement with our previous observations (Ferreira *et al*, 2013). STn expression was also associated with primary tumours. However, this may result from fact that the percentage of high-grade tumours was much higher among primary cases (90%) than in recurrences (67%). Altogether, these data reinforce the notion that sTn is a surrogate marker of high-risk bladder cancer.

Furthermore, we report for the first time that bladder tumours express the sTn-related carbohydrate antigen s6T. From the structural point of view, s6T may be considered a form of the sTn antigen masked by a Gal residue *O*-3 linked to the GalNAc moiety. To our knowledge, s6T has only been observed in human cancer cell lines (Marcos *et al*, 2004; Julien *et al*, 2006; Pinho *et al*, 2007). We also describe that the incubation of tissue

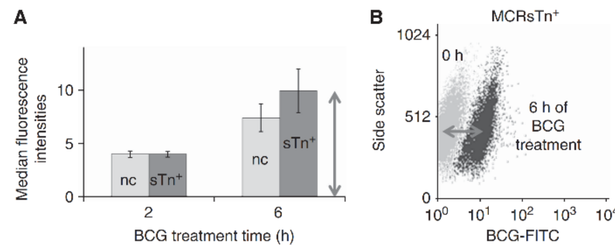


**Figure 3. BCG internalisation by bladder cancer cell lines over time.** (**A**) Internalisation of fluorescent-labelled BCG by mock-transduced MCRnc cells (light grey bars) and by *ST6GalNAc.I*-transduced MCRsTn$^+$ cells (dark grey bars), after 2 or 6 h of exposure with BCG. An obvious time-dependent internalisation was observed, and a tendency for higher fluorescent-labelled BCG internalisation rates was observed by MCRsTn$^+$ cells. Data are the average of three independent experiments (mean fluorescence intensity). (**B**) A representative flow cytometry dot plots of MCRsTn$^-$ cells before (light grey dots) and after 6 h (dark grey dots) of fluorescent-labelled BCG exposure. Horizontal and transversal double sense arrows represent the MFI shift observed after 6 h of BCG internalisation.

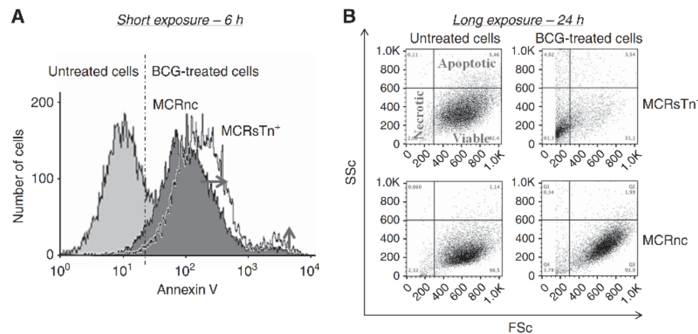


**Figure 4. Effect of BCG in the viability of bladder cancer cell lines expressing sTn.** (**A**) The apoptotic status of MCR cell lines was evaluated after 6 h exposure to BCG, by Annexin V staining and flow cytometry analysis. Flow cytometry histograms show MCRnc and MCRsTn$^+$ cells labelled with Annexin V before (light grey histograms) and after 6 h of BCG treatment (dark grey and open histograms, respectively). MCRsTn$^+$ cells showed higher Annexin V labelling ($MFI_{MCRnc}$ = 2560 to $MFI_{MCRsTn+}$ = 2640—horizontal arrow) and a higher percentage of cells with strong Annexin V labelling (4% to 7%—vertical arrow). (**B**) Analysis of size and granularity of cells exposed 24 h to BCG. Dot plot analysis of BCG-treated MCRsTn$^-$ revealed a marked decrease of both side-angle light scatter (SSc) and forward-angle light scatter (FSc) signals, which is consistent with massive cell death. By contrast, BCG treatment resulted in little changes of the dot plot pattern of MCRnc cells. Data are from one representative assay out of three independent experiments.

sections with a $\beta$-(1-3)-galactosidase removed the *O*-3-linked Gal residue exposing the sTn antigen, allowing its detection by immunohistochemistry with the same antibody used for sTn without significant time consumption. This approach may now be applied to estimate s6T expression in other solid tumours. Studies *in vitro* studies have shown that s6T expression influences tumour microenvironment similarly to sTn (Pinho *et al*, 2007). Therefore, both antigens were evaluated in the context of BCG immune response.

STn expression alone or in combination with that of s6T was associated with lower recurrence rates after BCG. Furthermore, patients expressing sTn and/or s6T presented longer RFS and these antigens were found to be independent predictive markers of reduced recurrence after BCG immunotherapy. Moreover, recurrences after treatment displayed a reduced expression of sTn antigens suggesting that BCG may be more effective against cells expressing these glycans. Thus, sTn-like *O*-glycans should be considered in a biomarker panel directed to predict BCG treatment outcome.

STn-expressing cells presented enhanced capacity for BCG adhesion and internalisation and higher BCG-mediated cell death *in vitro*. This strongly suggests that sTn expression favours BCG-mediated elimination of tumour cells, which may, in part, explain the high correlation between these glycans and treatment response. The exact mechanism underlying these observations remains unknown. However, BCG is known to bind fibronectin–$\alpha5\beta1$ integrin complexes promoting a rearrangement of cytoskeletal actin in host cells, which results in the phagocytosis of the pathogen (Chen *et al*, 2003; Alexandroff *et al*, 2010). Clement *et al* (2004) described that integrin $\beta1$ chains express sTn and that the antigen enhanced integrin-fibronectin adhesion. Thus, sTn may contribute to a more efficient binding of the bacillus to tumour cells and consequently a better response to BCG. The bacillus may also directly target cells in a fibronectin-independent manner (Schneider *et al*, 1994), namely by binding sTn or specific carbohydrates residues such as $\alpha$2,6 sialic acids. On the other hand, sTn is a product of incomplete *O*-glycosylation of proteins (Dall'Olio *et al*, 2012), a reduction in the structural complexity of

O-glycan may allow the bacillus to bind more efficiently to tumour cells. The expression of sTn also induces profound morphological changes in tumour cells (Clement *et al*, 2004; Pinho *et al*, 2007) that may further contribute to the bacillus attachment. A deeper understanding of these phenomena may provide new insights on the mechanism of action of BCG and ways to improve the therapeutics.

The efficiency of BCG therapy among sTn-positive tumours may also be related with the immunogenic properties of the antigen (Gilewski *et al*, 2007; Julien *et al*, 2009; Takamiya *et al*, 2013). sTn-based vaccines elicit strong immune responses against breast, ovarian and colorectal cancers in animal models (Gilewski *et al*, 2007; Julien *et al*, 2009). Still, using BCG as an immunologic adjuvant was considered essential for the development of both humoral and cellular immune responses against sTn (Miles *et al*, 1996; O'Boyle *et al*, 2006). Similarly, instillations with BCG may be enhancing immune responses against these tumour-associated glycans. On the other hand, we observed a higher internalisation of BCG by sTn-expressing cells. After BCG internalisation, malignant cells often act as antigen-presenting cells contributing to the immunologic cascade that leads to tumour clearance (Ratliff, 1992; Alexandroff *et al*, 2010). Therefore, one may also hypothesise that the expression of this particular glycan may increase the probability of generating BCG antigen-presenting cells in the tumour niche. Understanding these events may allow developing alternative carbohydrate-based immunotherapies for bladder cancer and should be addressed in future studies.

Although this is a retrospective study involving a limited number of cases, our results strongly suggest that BCG immunotherapy is efficient against sTn-positive tumours. Even though we have not determined the exact mechanisms underlying this event, we demonstrated that BCG adhesion and internalisation is higher for sTn-positive cells *in vitro*, further reinforcing tumour findings.

In conclusion, it has been demonstrated that sTn and s6T antigens correlated with a better response to this treatment. These glycans, in association with other BCG response-associated molecules, may allow the establishment of a predictive panel that can guide therapeutic decision.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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