DISCOVERY OF NOVEL BIOMARKERS IN GASTRIC CANCER BASED ON POST-TRANSLATIONAL MODIFICATIONS OF GLYCOPROTEINS

DESCOBERTA DE NOVOS BIOMARCADORES DE CANCRO GÁSTRICO BASEADA EM MODIFICAÇÕES PÓS-TRADUÇÃO DE GLICOPROTEÍNAS

CATARINA DE SENA BASTOS GOMES

PhD Thesis in Biomedicine

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"Winning means being unafraid to lose"
Fran Tarkenton

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Declaration

The results included in this thesis constitute research work of scientific articles and conference proceedings published in international journals.

Ao abrigo do Art. 8º do Decreto-Lei nº 388/70 fazem parte integrante desta Dissertação os seguintes trabalhos publicados e submetidos em revistas internacionais. Em cumprimento com o disposto no referido Decreto-Lei, declaro que participei ativamente na recolha e estudo do material incluído em todos os trabalhos.

Scientific papers

Catarina Gomes; Andreia Almeida; José Alexandre Ferreira; Luísa Silva; Hugo Santos-Sousa; João Pinto-de-Sousa; Lúcio L. Santos; Francisco Amado; Tilo Schwientek; Steven B. Levery; Ulla Mandel; Henrik Clausen; Leonor David; Celso A. Reis; Hugo Osório. Glycoproteomic analysis of serum from patients with gastric precancerous lesions. *J. Proteome Res.*, 2013, 12 (3), pp 1454–1466

Catarina Gomes; Hugo Osório; Marta Teixeira Pinto; Maria José Oliveira; Celso A. Reis. Expression of ST3GAL4 leads to SLe^x expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. *PLoS One*, 2013, 8 (6):e66737.

Conference proceedings

Catarina Gomes, Hugo Santos-Sousa, Tilo Schwientek, João Pinto-De-Sousa, Hugo Osório, Celso A. Reis. Serum Glycoprotein Biomarkers in Gastric Carcinoma Patients. abstract presented at annual conference of the Society for Glycobiology, St. Pete Beach, FL, USA, November 7 – 10, 2010. Glycobiology (2010) 20(11): 1488

Catarina Gomes, Maria Luísa Silva, João Pinto-de-Sousa, Hugo Santos- Sousa, Tilo Schwientek, Leonor David, Celso Albuquerque Reis, Hugo Osório. Glycan biomarkers in gastric lesions: tissue and serum characterization. Abstracted presented at GLYCO 21:XXI International Symposium on Glycoconjugate, Vienna, Austria, August 21-26, 2011. Glycoconj J (2011) 28:264

Catarina Gomes**,** Hugo Osório, Marta T. Pinto, Celso A. Reis. Role of SLe^a and SLe^x in gastric cancer cells. Abstract presented at GLYCOT Hannover 2012: 8th International Symposium on GLycosyltransfrases, Hannover, Germany, June 5-9th 2012. www.GLYCOT2012.org [8th International Symposium on Glycosyltransferases, Hannover, 5.6.-9.6.2012]

Catarina Gomes, Hugo Osório, Marta T. Pinto, Celso A. Reis. Overexpression of ST3Gal-IV induces activation of cell signaling pathways and alteration in gastric cancer cell line phenotype. Abstract presented at joint meeting of the Society for Glycobiology and American Society for Matrix Biology San Diego, CA, USA, November 11–14, 2012. Glycobiology (2012) 22 (11): 1645

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Outline

The present thesis has been divided into five different chapters.

The Chapter 1 and Chapter 2 consist of the scientific background and motivation of the work performed in this thesis regarding the discovery of cancer biomarkers based on alterations of glycosylation.

The Chapter 1 is a general introduction to the fields of gastric cancer, glycobiology and biomarkers in cancer.

The Chapter 2 summarizes the main objectives of the thesis.

The Chapter 3 reports the main results obtained during the work project. It consists of three main studies related to altered glycosylation observed in gastric cancer, and were subdivided in three parts:

The first study, Chapter 3.1, focuses on the identification of glycoproteins expressing truncated *O*-glycans in serum of patients as source for biomarker discovery. "Glycoproteomic analysis of serum from patients with gastric precancerous lesions. Published in *J. Proteome Res.*, 2013, 12 (3), pp 1454–1466."

The second study, Chapter 3.2, focuses on the biological role of SLe^x in gastric cancer cell behavior using a cell line model overexpressing ST3Gal IV. "Expression of ST3GAL4 leads to SLe^x expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. Published in *PLoS One*, 2013, 8 (6):e66737."

The third study, Chapter 3.3, focuses on the identification of the SLe^x expressing glycoproteins using a gastric cancer cell line model overexpressing ST3Gal IV, and validation in gastric carcinoma tissues as putative new biomarker in gastric cancer. "CEACAM5 carcinoembryonic antigen carries SLe^x in gastric carcinoma cells - implications for diagnosis improvement. Manuscript in preparation."

The Chapter 4 consists on a general discussion of the main findings and future perspectives, finishing with final conclusions.

The Chapter 5 includes other scientific contributions.

Abstract

All the cells that constitute epithelial surfaces are decorated by a large diversity of glycoconjugates that have been involved in numerous cellular processes, including cancer cell transformation. During malignant cancer cell transformation, cells express on their surfaces different glycan structures when compared with their normal counterpart. The expression of these altered glycans occurs mainly in glycoproteins and glycolipids and are characterized by the expression of abnormal truncated glycans (e.g. Tn, STn and T antigens) and increased sialylation in complex glycan structures (e.g. SLe^a and SLe^x antigens). The study of these glycan alterations common in cancer as well as the identification of proteins carriers of the altered glycans will contribute to the understanding of the carcinogenesis process and help in the finding of new cancer biomarkers.

The main objectives of this work were: to identify new serological biomarkers in gastric carcinogenesis, by the evaluation of T and STn expression in gastric carcinoma precursor lesions (gastritis and intestinal metaplasia) and in gastric carcinoma; to characterize the biological behavior of gastric carcinoma cells expressing SLe^x, to evaluate the molecular modulation of SLe^x expressing cells by tyrosine kinase receptors activation; and to identify protein carriers of SLe^x.

STn and T antigens were shown to be expressed in biopsies of individuals with gastritis, intestinal metaplasia and tissues from gastric carcinoma patients. The proteomic study of serum from individuals with gastritis, intestinal metaplasia and gastric carcinoma revealed the presence of these truncated glycans and lead us to identify some proteins such as acute phase proteins (mainly complement proteins), vitronectin and plasminogen. The detailed mass spectrometry analysis of glycan structures in serum plasminogen from intestinal metaplasia individuals allowed the validation of the STn structure. The overexpression of ST3Gal IV in gastric carcinoma cell line MKN45, induced the expression of type 2 sialylated Lewis structure, SLe^x, in cellular and secreted proteins. The SLe^x expression lead to a more aggressive *in vitro* phenotype of gastric cells, characterized by an increased cellular invasion with increased adhesion capacity to extracellular matrix proteins such as collagen IV and vitronectin. Using the chicken chorioallantoic membrane (CAM) as *in vivo* model for the evaluation of cancer cell invasion capacity we demonstrated that cells expressing SLe^x show increased capacity to infiltrate and invade CAM. From the evaluation of tyrosine kinase receptors activation we

observed increased activation of c-Met in SLe^x expressing cells, a receptor already described as playing a role in cellular invasion and metastization process. As follow consequence of c-Met activation we observed increased phosphorylation of Src, FAK and activation of RAC1, RhoA and Cdc42 GTPases contributing for the modulation of cellular biological behavior. The identification of SLe^x protein carriers, by a proteomic approach, lead us to identify the carcinoembryonic antigen (CEA). This result was further confirmed in cells by proximity ligation assay (PLA) and validated by immunoprecipitation. The expression of CEA and SLe^x and the presence of CEA/SLe^x was evaluated by immunohistochemistry and PLA in gastric carcinoma tissues, and revealed that 80.6% of the cases show CEA/SLe^x. This result was associated with the presence of venous invasion of the carcinomas.

Overall, our results give new insights for the application of both plasminogen STn and CEA SLe^x glycosylation pattern as serum biomarkers of gastric pathologies, and open new avenues for future targeted evaluation of these specific glycobiomarkers in additional immunoassay-based approaches. Moreover, we showed that SLe^x expression on the surface of malignant cells plays an important role in tumor invasion and metastasis.

Resumo

Todas as células que constituem as superfícies epiteliais são decoradas por diversos glicoconjugados que estão envolvidos em inúmeros processos celulares, inclusive na progressão para cancro. Aquando da transformação maligna, estas células passam a expressar, à sua superfície, diferentes estruturas de glicanos. A expressão desses glicanos alterados acontece maioritariamente em glicoproteínas e glicolípidos e é caracterizada pela expressão de formas truncadas anormais (antigénios Tn, STn e T), assim como aumento da sialilação de glicanos complexos (antigénio SLe^a, SLe^x). O estudo destas alterações comuns em cancro, assim como a identificação de proteínas portadoras destes glicanos alterados pode contribuir significativamente para a compreensão do processo de carcinogénese e da progressão tumoral assim como ajudar na descoberta de novos biomarcadores de cancro.

Este estudo teve como objetivos: identificar novos biomarcadores serológicos envolvidos na carcinogénese gástrica através do estudo da expressão de glicanos truncados como STn e T em lesões precursoras de carcinoma gástrico (gastrite e metaplasia intestinal) e em carcinoma gástrico; caracterizar o comportamento biológico de células de carcinoma gástrico que expressam SLe^x, avaliar a modelação molecular por ativação de recetores de tirosina cinase em células que expressam SLe^x; e identificar proteínas portadoras de SLe^x.

A expressão de estruturas STn e T foi observada em tecidos de biopsias de indivíduos com gastrite e metaplasia intestinal, assim como em casos de carcinoma gástrico. A análise proteómica do soro de indivíduos com carcinoma gástrico e lesões precursoras de carcinoma gástrico (gastrite e metaplasia intestinal) revelou a presença dessas mesmas estruturas, e levou à identificação de proteínas de resposta de fase aguda (maioritariamente proteínas do sistema de complemento), de vitronectina e plasminogénio. O estudo detalhado, por espectrometria de massa, da estrutura de glicanos do plasminogénio sérico levou à validação da presença da estrutura STn em indivíduos com metaplasia intestinal. A sobre-expressão, *in vitro*, da sialiltransferase ST3Gal IV numa linha celular de carcinoma gástrico, MKN45, induziu a expressão de estruturas Lewis sialiladas do tipo 2, SLe^x, em proteínas celulares e secretadas. A expressão de SLe^x levou a um comportamento mais agressivo das células *in vitro*, caracterizado pelo aumento da invasão celular assim como maior capacidade de adesão celular a proteínas de matriz como colagénio IV e vitronectina. A aplicação, como modelo *in vivo*, da membrana corioalantóica (CAM) de embrião de galinha para a avaliação da capacidade invasiva de células

que expressam SLe^x evidenciou uma maior capacidade destas células em penetrar e invadir a membrana corioalantóica. Da avaliação da ativação de recetores de tirosina cinase, observamos aumento de ativação de c-Met em células que expressam SLe^x, um recetor já descrito como estado envolvido no processo de invasão e metastização celular. Como efeito subsequente à ativação do c-Met, um aumento de fosforilação de Src, FAK e ativação de RAC1, RhoA e Cdc42 GTPases foi observado como participando na modulação do comportamento biológico celular. O estudo proteómico de identificação das proteínas transportadoras de SLe^x identificou o antigénio carcinoembrionico (CEA), e este resultado foi posteriormente confirmado por PLA em células e validado por imunoprecipitação. A presença da co-expressão de CEA e SLe^x foi também avaliada por imunohistoquímica e PLA em tecidos de carcinoma gástrico, e revelou que 80.6% dos carcinomas gástricos apresentam co-expressão de CEA com SLe^x. A co-expressão de CEA com SLe^x apresentou associação com invasão venosa dos carcinomas.

Os resultados obtidos poderão contribuir significativamente para a aplicação de alterações de glicosilação de proteínas, como plasminogénio com STn e CEA com SLe^x, como possíveis novos biomarcadores de patologias gástricas, e abrem novas portas para o desenvolvimento de novos testes sorológicos que avaliem estas alterações. Adicionalmente, este trabalho permitiu demonstrar que a expressão de SLe^x em células tumorais está associada com a invasão tumoral e com o processo de metastização.

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Chapter 1

General Introduction

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GENERAL VIEW OF CANCER INCIDENCE AND MORTALITY

Cancer is a leading cause of death worldwide (Twombly 2005) and a tremendous effort has been made to improve health by developing new approaches for early diagnosis, treatment and prevention.

In 2008, it was estimated an overall incidence of more than 12 million new cancer cases resulting in approximately 8 million cancer deaths (Ferlay *et al.,* 2010). Lung cancer remains the most frequent cancer worldwide, presenting both higher incidence and mortality rates. The ranking, in terms of incidence, is followed by breast cancer, the second most common cancer overall, colorectal cancer, stomach cancer, prostate cancer and liver cancer. Regarding mortality, gastric cancer is the second cause of cancer death worldwide followed by liver cancer, colorectal cancer and breast cancer.

GASTRIC CANCER - GENERAL ASPECTS

Epidemiological Perspective

The International Agency for Research on Cancer (IARC), a part of the World Health Organization (WHO), often estimates the cancer incidence and mortality in broad areas of the world and more recently it provides a more detail estimate comprising the country level through the GLOBOCAN series.

According to the last estimate, gastric cancer is one of the main cause of cancer death worldwide, especially in developing countries (Ferlay *et al.,* 2010). About one million new cases were estimated to have occurred (989,000 cases, 7.8% of the total), making it currently the fourth most common cancer malignancy in the world. This estimative differs significantly from the first in 1975, when stomach cancer was globally the most common neoplasm (Parkin *et al.,* 1984; La Vecchia *et al.,* 2010). Incidence of gastric cancer in developing countries represents more than 70% of total cases, and half of the world total cases occurs in Eastern Asia, mainly in China (Ferlay *et al.,* 2010). In addition, gastric cancer is the second leading cause of cancer death in both sexes worldwide and the highest mortality rates are estimated in Eastern Asia with Central and Eastern Europe, and Central and South America also present high mortality rates. Nonetheless, the lowest mortality rate is observed in Northern America.

Besides these GLOBOCAN perspectives, there is also estimates of cancer burden in Europe. Overall, it was estimated about 3 million new cancer cases diagnosed and 1.7 million cancer

deaths in Europe in 2006 (Ferlay *et al.,* 2007). This study estimated that overall, the most frequent cancer types were breast cancer, followed by colorectal cancers, lung cancer, prostate cancer and gastric cancer. In terms of mortality, lung cancer was the most common cause of death from cancer, followed by colorectal cancer, breast cancer, gastric cancer and prostate cancer. Higher incidence and mortality rates are still recorded in Eastern European countries. This European estimative differs from the GLOBOCAN worldwide estimative and fortunately, gastric cancer incidence and mortality are declining throughout Europe, in both men and women (Boyle *et al.,* 2005; Ferlay *et al.,* 2007).

The incidence of gastric cancer varies from country to country, probably as a result of genetic, epigenetic, and environmental factors. Gastric cancer has been associated with many factors known to contribute to the development and progression of the disease (Crew *et al.,* 2006). Some of the factors thought to be involved in the development of gastric cancer are sex, where gastric cancer occurs twice as often in men; age, where gastric cancer is more common in people over the age of 55 (Yamaoka *et al.,* 2009); race, in the United States of America some studies point for differences in incidence among the different populations (Schlansky *et al.,* 2011) and dietary factors in particular a high salt intake along with the use of nitrate for food preservation (Ramon *et al.,* 1993; Tsugane 2005; Peleteiro *et al.,* 2011). Other related risk factors are fruit and vegetables intake (Riboli *et al.,* 2003; Soerjomataram *et al.,* 2010), smoke habit (La Torre *et al.,* 2009), alcohol consumption (Duell *et al.,* 2011) and high body mass index (Hampel *et al.,* 2005; Kubo *et al.,* 2006). In addition to these environmental factors, Helicobacter pylori, a gram-negative bacterium that is thought to be present in the stomach of half the global population, has also been implicated in gastric carcinogenesis (Peek *et al.,* 2002; Lochhead *et al.,* 2007; Lochhead *et al.,* 2008).

In this regard, the decline in incidence and mortality rates observed in gastric cancer throughout Europe is generally attributed to better food preservation, enhanced nutrition and improved control of *Helicobacter pylori* infection.

Gastric Carcinogenesis

Based on epidemiologic evidence, the IARC classified in 1994 *H. pylori* as a class I carcinogen (IARC 1994). Since then, continuous increasing data supports the role of *H. pylori* in gastric carcinogenesis that was firstly established with a very elegant study with the Mongolian gerbil animal model demonstrating gastric cancer induction by *H. pylori* infection (Watanabe *et al.,* 1998).

H. pylori colonization is generally acquired during childhood (Malaty *et al.,* 2002; Rowland *et al.,* 2006) but if not treated the infection persists lifelong (Vincent 1995; Blaser *et al.,* 2004; Lehours *et al.,* 2007). The transmission infection mode is reported to be human to human (Graham *et al.,* 1991; Neale *et al.,* 1995), most probably by gastric-oral transmissions associated with gastroenteritis and vomiting and oral-oral or fecal-oral transmission (Leung *et al.,* 1999; Parsonnet *et al.,* 1999; De Schryver *et al.,* 2006; Perry *et al.,* 2006; Solnick *et al.,* 2006).

As referred above, *H. pylori* infection is considered a major risk factor that together with other environmental factors triggers a cascade of gastric lesions that cause alterations in the gastric mucosa resulting in atrophy of the mucosal barrier which may increase the risk of carcinogenesis in the underlying epithelial layer leading ultimately to gastric cancer (Uemura *et al.,* 2001; Correa *et al.,* 2007). In 1992, Pelayo Correa first described the gastric carcinogenesis pathway characterized by a multiple step process started by *H. pylori* infection (Correa 1992) (**Figure 1**).

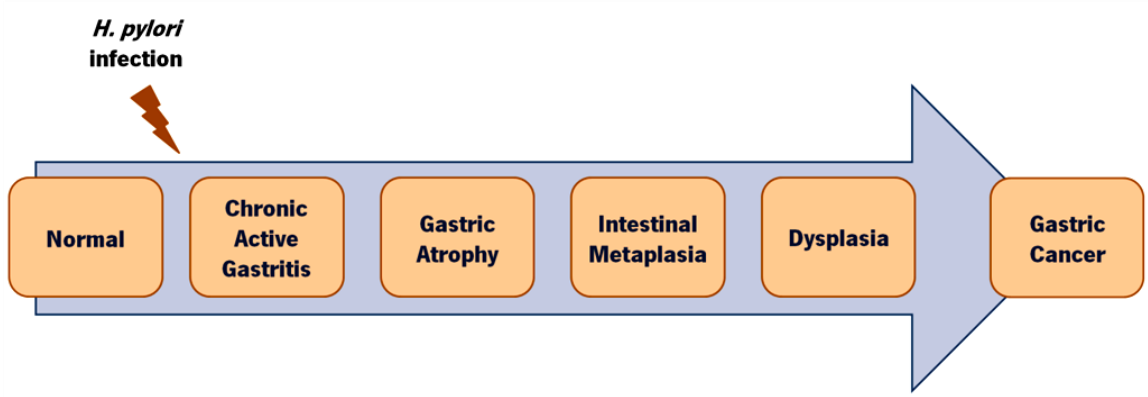

Figure 1: Gastric carcinogenesis pathway proposed by Pelayo Correa in 1992, characterized by a multi-step process started by *H. pylori* infection leading to gastric tissue inflammation that can evolve to atrophy, metaplasia, dysplasia and ultimately gastric cancer.

This multistep model propose that *H. pylori* infection, in association with other environmental factors, triggers a cascade of events that starts with a chronic inflammation or a **chronic gastritis** that can evolve to **atrophic gastritis** with focal loss of glands. As consequence of this atrophy, several changes can occur and the local gastric cells can be replaced by intestinal type cells conferring a tissue intestinal phenotype named **intestinal metaplasia**. The next step in the cascade is characterized by atypical changes in nuclear morphology and irregular tissue architecture and is called **dysplasia** (reviewed in Correa *et al.,* 2007). High-grade dysplasia commonly progress to **gastric cancer** of the more common intestinal subtype, the final step of the cascade (de Vries *et al.,* 2007).

Regarding this well established model, gastric mucosal atrophy is a major risk factor to develop gastric cancer. In addition, different patterns of atrophic gastritis are associated with

different risk of developing gastric cancer. A recent system of gastritis classification, the OLGA (Operative Link for Gastritis Assessment)-staging system, consider both the topography (antral and corpus mucosa) and the extent of gastric mucosa atrophy (score) to determine gastritis-associated gastric cancer risk (Rugge *et al.,* 2008). Taking in consideration this classification, several studies consistently associated increased risk of developing gastric cancer with OLGA stages III/IV (Rugge *et al.,* 2007; Rugge *et al.,* 2010; Rugge *et al.,* 2011; Marcos-Pinto *et al.,* 2013). More recently, a modification in the OLGA system of classification, OLGIM (Operative Link on Intestinal Metaplasia Assessment), was proposed to consider the assessment of intestinal metaplasia instead of atrophy and this new classification has been shown to be more accurate in determine gastric cancer risk (Capelle *et al.,* 2010). These classifications show to be important for the application of follow-up strategies for patient-specific clinico-pathological future evaluations (Dinis-Ribeiro *et al.,* 2012; Marcos-Pinto *et al.,* 2012).

All *H. pylori* strains have the capacity of promoting gastric inflammation, however most of the infected individuals show few or no symptoms and a very small percentage of *H. pylori* infected individuals will develop gastric carcinoma (Dooley *et al.,* 1989; Suerbaum *et al.,* 2002; Amieva *et al.,* 2008). Considering this reality, the outcome of *H. pylori* infection for each individual is difficult to predict since it depends on different aspects such as host genetic susceptibility, strain virulence characteristics and environmental factors.

Host Genetic Susceptibility

It is recognized the importance of host genetics in gastric cancer development and the association with *H. pylori* infection. The prevalence of a number of genetic polymorphisms in some inflammation-related genes have been demonstrated to be associated with risk of developing gastric lesions, mainly in *H. pylori* infected individuals.

It has been shown that human genetic pro-inflammatory polymorphisms within the genes interleukin (IL)-1B and IL-1RN are associated with risk for gastric cancer development (El-Omar *et al.,* 2000; Machado *et al.,* 2001; Figueiredo *et al.,* 2002). IL-1B gene encodes for a pro-inflammatory cytokine IL-1β that plays an important role in initiating and amplifying the inflammatory response upon *H. pylori* infection (Noach *et al.,* 1994). On the other hand, IL-1RN gene encodes for an interleukin 1 receptor antagonist IL-1ra, an anti-inflammatory cytokine, that competitively binds IL-1β receptors modulating the potentially damage effects of IL-1β (Arend *et al.,* 1998). The expression of IL-1B-511*T, IL-1B-31*C and IL-1RN*2 alleles have been associated

with increased IL-1β production (Pociot *et al.,* 1992; Danis *et al.,* 1995; Santtila *et al.,* 1998; Hwang *et al.,* 2002) and have been found to confer an increased risk for the development of gastric cancer (Machado *et al.,* 2001) chronic atrophic gastritis (Furuta *et al.,* 2002) and intestinal metaplasia (Figueiredo *et al.,* 2002; Zambon *et al.,* 2002).

Furthermore, polymorphism in the tumor necrosis factor α (TNF-α) gene and interferon gamma receptor 1 (IFNGR1) has also been associated with increased gastric cancer risk (El-Omar 2001; Machado *et al.,* 2003; Canedo *et al.,* 2008). The pro-inflammatory TNF-α cytokine, encoded by TNF-α gene, has been shown to be increased in gastric mucosa of patients infected by *H. pylori* (Noach *et al.,* 1994; Arend *et al.,* 1998), and the expression of TNF-α-308*A allele has been associated with increased risk for chronic atrophic gastritis and gastric cancer development (Machado *et al.,* 2003). IFNGR1 is a cytokine receptor for IFNϒ, a cytokine that has been described to play a pivotal role in promoting *H. pylori* induced mucosal inflammation when upregulated (Smythies *et al.,* 2000). The presence of IFNGR1-56*T polymorphism has been reported as a relevant host susceptibility factor for gastric cancer development (Canedo *et al.,* 2008).

In addition, other findings claimed that MUC1 polymorphism present different susceptibility for the development of gastric lesions (Silva *et al.,* 2001) and gastric carcinoma (Carvalho *et al.,* 1997). It was also reported that *H. pylori* adhesion to gastric cells depends on the size of the MUC1 variable number tandem repeats (VNTR) domain (Costa *et al.,* 2008).

Helicobacter pylori **Virulence Factors**

Although the final clinical outcome is dependent also on host and environmental factors, it was also observed that not every *H. pylori* strain resulted in similar damage for the host, leading to the distinction between high and low pathogenic strains. Thus, it was possible to identify bacterial features that confer increased risk to develop gastric disease (van Doorn *et al.,* 1998; Figueiredo *et al.,* 2005).

The presence of cytotoxin-associated gene pathogenicity island (cagPAI) was associated with more severe disease outcomes and was considered to be involved in the development of atrophic gastritis, peptic ulcer disease and gastric carcinoma (Blaser *et al.,* 1995; Kuipers *et al.,* 1995; Figueiredo *et al.,* 2002; Nomura *et al.,* 2002), a feature that has been well established in animal models (Ogura *et al.,* 2000; Wiedemann *et al.,* 2009). Bacterial strains that are cagPAI positive (cagPAI+, more virulent strains), are characterized by containing genes that encode a type IV

secretion system, which functions as a needle that permits the insertion of bacterial products into the host cells (Censini *et al.,* 1996; Tanaka *et al.,* 2003) as well as the gene encoding the CagA protein, one of the proteins delivered to epithelial cells through this system (Backert *et al.,* 2000). Once inserted in epithelial cells, CagA protein associates with proteins from the tight junction complexes, leading to loss of epithelial integrity (Amieva *et al.,* 2003) and its phosphorylation on tyrosine residues by Src family kinases promotes the interaction with several cell signal-transduction pathways resulting in cytoskeleton rearrangement, motility, proliferation and apoptosis (Segal *et al.,* 1999; Asahi *et al.,* 2000; Odenbreit *et al.,* 2000; Stein *et al.,* 2000). The presence of an intact type IV secretion system as well as CagA protein phosphorylation is also associated with the production of interleukin (IL)-8 in gastric cells (Brandt *et al.,* 2005; Figueiredo *et al.,* 2005; Lai *et al.,* 2011).

Several other virulent factors have been characterized and described such as the vacuolating cytotoxin gene (vacA) that encodes a toxin that induce epithelial cell damage by cell vacuolization, membrane channel formation, disruption of endosomal/lysosomal function, apoptosis, and immunomodulation (Cover *et al.,* 1992; Cover 1996; reviewed in Cover *et al.,* 2005).

Additional, *H. pylori* virulence factors can comprise genes that encodes for blood group antigen binding adhesin (BabA) (Ilver *et al.,* 1998) and sialic acid-binding adhesin (SabA) (Mahdavi *et al.,* 2002). The expression of these adhesin proteins in the surface of *H. pylori* strains are involved with the capacity of the bacteria to colonize gastric mucosa by adhering to epithelial cells through host antigens receptors. *H. pylori* adhesion to the gastric epithelial cells constitutes a crucial step for gastric mucosa colonization and establishment of a successful infection, because it provides protection from clearance mechanisms such as liquid flow, peristaltic movements or renewal of the mucous layer. The host antigens receptors involved in bacteria colonization are glycan structures present on proteins or glycolipids in the surface of the host gastric epithelial cells namely H-type 1 and Lewis b (Le^b) for Bab A adhesin (Boren *et al.,* 1993; Ilver *et al.,* 1998) and Sialyl Lewis X (SLe^x) and Sialyl Lewis A (SLe^a) for SabA adhesin (Johansson *et al.,* 2005; Walz *et al.,* 2005; Aspholm *et al.,* 2006). *H. pylori* has the capacity of modulate the expression of these two adhesins to achieve a long-term colonization, according to the host antigen receptors expression. For this reason, *H. pylori* take advantage of expressing SabA adhesin in inflamed gastric tissues that is characterized by increased expression of sialylated structures (Ota *et al.,* 1998; Mahdavi *et al.,* 2002). Infection with *H. pylori* that expresses BabA is associated with increased epithelial proliferation and inflammation, increased risk for duodenal

ulcer, gastric atrophy, intestinal metaplasia, and gastric adenocarcinoma (Gerhard *et al.,* 1999; Prinz *et al.,* 2001; Yu *et al.,* 2002).

Gastric Cancer Classification

Most of gastric cancers are adenocarcinomas that occur in the lining of the stomach and account for nearly 90% of the total cases. About 2-7% of the gastric malignancies are lymphoma of mucosa-associated lymphoid tissue (MALT) (Wotherspoon 1998), and the remainder percentage includes very rare cases of gastric stromal tumors (sarcomas) developed from the muscle or connective tissue of the stomach wall (Duffaud *et al.,* 2003), and carcinoid tumors (Zhang *et al.,* 2011).

Different stages of gastric adenocarcinoma are characterized by the capacity of invasion deeply into the stomach wall extending to nearby organs such as lymph nodes, abdominal cavity, liver, pancreas, esophagus or intestine (Washington 2010). Furthermore, gastric carcinoma cells released by the primary tumor into lymphatic and/or blood circulation can spread into distant target organs (lungs, brain, ovaries, bones) forming secondary tumors (a process also called metastization) (Kemp *et al.,* 2010; Ahn *et al.,* 2011; Yamanishi *et al.,* 2011).

Several classification systems have been proposed to aid in the description of gastric cancer based on microscopic pattern (Ming, Carneiro, and Goseki). The two most commonly used are the World Health and Organization (WHO) Lauren systems. According to WHO, gastric carcinomas are classified in adenocarcinoma, papillary carcinoma, tubular carcinoma, mucinous carcinoma and signet ring-cell carcinoma. However Lauren's classification is the predominant histological classification worldwide that divides gastric carcinoma in two major subtypes - intestinal type and diffuse type carcinomas (Lauren 1965) although considering an third subtype designated unclassified or mixed type carcinoma. Nevertheless, intestinal type and diffuse type carcinomas don't differ only by histological appearance, but also by epidemiology, pathogenesis, and genetic profiles differences (Shah *et al.,* 2011). Morphologically, these two types of gastric carcinoma differ in the intercellular adhesion molecules that are well preserved in intestinal-type tumors and defective in diffuse carcinomas. In intestinal-type carcinoma, cells are still attached in tubular or glandular formations (similar to adenocarcinomas arising in the intestinal tract - hence their designation as intestinal-type); in contrast to the lack of adhesion molecules in diffuse carcinomas allows individual tumor cells to grow and invade neighboring tissues without the formation of tubules or glands. Moreover, the molecular basis of these two carcinoma types is also different. In the diffuse-type carcinoma the main carcinogenic event is the loss of function of E-cadherin, a key

cell surface protein for intercellular adhesion and maintenance of the organization of epithelial tissues. Impairment of E-cadherin function occurs through biallelic inactivation of its related gene CDH1 via germline or somatic mutation, allelic imbalance events (loss of heterozygosity), epigenetic silencing of gene transcription through aberrant methylation of the CDH1 promoter (Oliveira *et al.,* 2006; Carneiro *et al.,* 2008; Yamamoto *et al.,* 2011; Corso *et al.,* 2012), or by modulation of E-cadherin *N*-glycosylation (Pinho *et al.,* 2011). In the intestinal-type carcinoma the main carcinogenic event is *H. pylori* infection followed by a cascade of events where several molecular changes occur leading to transformation of gastric cells into intestinal type cells (as mentioned above).

Although *H. pylori* associated preneoplastic lesions are a feature of intestinal-type gastric cancer, some evidences claimed that there is an association between *H. pylori* infection and diffuse-type gastric cancer (Handa *et al.,* 1996; Uemura *et al.,* 2001). The association of *H. pylori* infection and diffuse-type gastric cancer is observed during the progression of atrophic gastritis in individuals presenting active gastritis (Sipponen *et al.,* 1992; Solcia *et al.,* 1996). Conversely, in hereditary diffuse gastric cancer this association is not observed (Carneiro *et al.,* 2004).

Besides gastric carcinoma, *H. pylori* infection in gastric mucosa is also associated with the development of mucosa-associated lymphoid tissue (MALT), increasing the risk of gastric MALT lymphoma (Parsonnet *et al.,* 1994; Sagaert *et al.,* 2010). This involvement is support by the fact that most of the patients with low-grade MALT-lymphoma are infected with *H. pylori*, and that, in most of these cases, eradication of *H. pylori* infection results in tumor regression (Thiede *et al.,* 1997; Zullo *et al.,* 2010; Kuo *et al.,* 2012).

Briefly, there are several factors that contribute for gastric disease development. As mentioned above, also glycan antigen receptors expressed in the surface of gastric epithelial cells contribute for the development of gastric diseases.

GLYCANS: WIDESPREAD MOLECULES THAT DECORATE CELL SURFACES

It is amazing to observe that all living cells present a vast collection of glycan structures that decorate cell surfaces (Varki *et al.,* 2009a; Varki 2011). Glycan expression on intracellular and cell surfaces proteins and lipids are implicated in numerous biological functions (Varki 1993). Oligosaccharide moieties are involved in cell-cell and cell-matrix interactions and recognition, intra-

and intercellular protein trafficking, pathogen infection, immuno responses and recognition of self, modulation of protein function and signaling, as well as altering the dynamics of glycoprotein endocytosis and regulation of proper maturation and folding of newly synthesized proteins (Varki 1993; Moremen *et al.,* 2012)

Glycosylation

Glycosylation is characterized by the enzymatic covalent attachment of a carbohydrate to a polypeptide, lipid, polynucleotide, carbohydrate, or other organic compound catalyzed by glycosyltransferases using specific sugar nucleotide donor substrates; in opposition to the non-enzymatic chemical reaction of glycation.

The synthesis of a glycan structure is a very complex and organized process that involves a huge number of genes including those that code for glycosyltransferases, glycosidases and chemical chaperons, as well as the enzymes responsible for nucleotide sugars synthesis and transport (Rini *et al.,* 2009). Furthermore, the availability and localization of the nucleotide sugar donors can compromise glycosyltransferases activity in the synthesis of a glycan structure.

The human genome contains about 250 to 500 genes that are involved in the glycan assembly, like those that code for proteins involved in the synthesis and degradation of glycans or transport of sugar donors, accounting for approximately 2% of the total human genome (Schachter *et al.,* 2009). Generally the enzymatic attachment of glycans occurs in non-carbohydrate molecules forming the glycoconjugates. Glycoconjugates comprise a different class of molecules such as glycosphingolipids, glycosaminoglycans present as free polysaccharides or as part of proteoglycans, glycoproteins and glycosylphosphatidylinositol-linked proteins (**Figure 2**).

Glycan structures can be attached to a lipid structure forming glycolipids. Almost all glycolipids in vertebrates are **glycosphingolipids** (**GSLs**) that belongs to the sphingolipid family (Schnaar *et al.,* 2009). GSLs are characterized by the linkage of a glycan structure to an sphingolipid core structure called ceramide. The biosynthesis of GSLs occurs by a stepwise addition of sugars first to ceramide, typically in a β-linked galactose (galactosylceramide-GalCer) or glucose (glucosylceramide-GlcCer), and then to the arising glycan. GlcCer synthesis starts in the cytoplasmic face of the endoplasmic reticulum (ER) and early Golgi apparatus and is further elongated by a series of glycosyltransferases in the Golgi lumen. Conversely, GalCer synthesis occur on the ER lumen and then go through the Golgi, where it may be sulfated to form sulfatide. The elongation process leads to a variety of different combinations that subclassify GSLs as

neutral (no charged sugars or ionic groups), sialylated (having one or more sialic acid residues) generally known as gangliosides, or sulfated. GSLs are found embedding in the cell membranes, due to hydrophobic properties of the lipid tail, contributing for the maintenance of cell membranes integrity and clustered in lipid rafts contributing for cell signaling processes (Schnaar *et al.,* 2009).

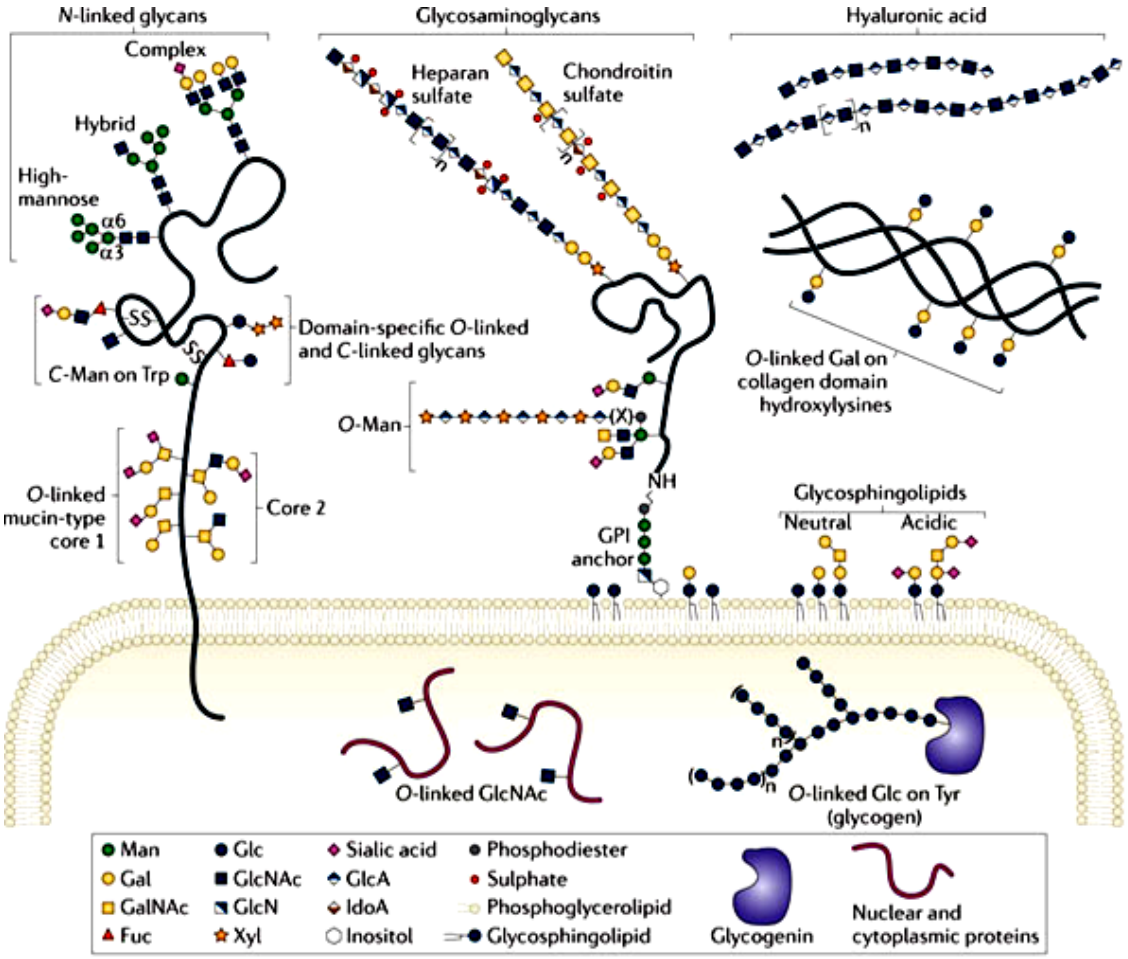

Figure 2: Common classes of animal glycan structures. The major classes of animal glycans are shown, with an emphasis on typical vertebrate sugar chains. Most glycans on membrane and secreted proteins are found in *N*-linkage to Asn or in *O*-linkage to Ser/Thr. *O*-linked glycans are classified by their initiating monosaccharide. Addition of GalNAc initiates mucin-type *O*-linked glycans and extension with Gal, GlcNAc or GalNAc produces eight different core structures. Man linked initiates another class of *O*-linked glycan (*O*-Man glycans), as well as Fuc residues (*O*-Fuc glycans). *O*-linked GlcNAc is found on the extracellular domains of some proteins and on numerous cytosolic and nuclear proteins. Furthermore, *O*-linked glycan structures can be found attached to other amino acids, including Glc residue to Ser and Tyr residues of glycogenin, and Gal on hydroxylysine of collagen domains. Also glycosaminoglycans (GAGs) are *O*-linked glycans initiated by a conserved tetrasaccharide (GlcA-β1,3-Gal-β1,3-Gal-β1,4 Xyl-β) and classified by the composition of their disaccharide repeat that are usually found attached to proteins forming proteoglycans. A GAG-like polymer that forms hyaluronic acid is the only glycan that is not linked to a protein or lipid. In addition to proteins, sphingolipids can be modified by glycosylation, which are ceramide-linked glycans.The monosaccharide abbreviations are Man, Mannose; Gal, Galactose; GalNAc, N-acetylgalactosamine; Fuc, Fucose; Glc, Glucose; GlcNAc, N-acetylglucosamine; GlcN, Glucosamine; Xyl, Xylose; GlcA, Glucuronic acid; and IdoA, Iduronic acid. Adapted from (Moremen *et al.,* 2012).

Glycosaminoglycans (**GAGs**) are larger linear polysaccharides, formed by repeated disaccharide building blocks composed of an amino sugar (N-acetylglucosamine-GlcNAc, glucosamine-GlcN that is variously N-substituted, or N-acetylgalactosamine-GalNac) and either an uronic acid (glucuronic acid-GlcA or iduronic acid-IdoA) or galactose (Gal). GAGs can be found as free polysaccharides, such as hyaluronic acid, or as part of proteoglycans. Proteoglycans are characterized by one or more GAGs covalently attached to a protein core. GAGs are attached to proteins by a conserved tetrasaccharide (GlcA-β1,3-Gal-β1,3-Gal-β1,4 Xyl-β) to Ser residues in an *O*-linkage that will be further elongated with disaccharide repeats giving rise to two different classes: chondroitin sulfate and heparan sulfate (Esko *et al.,* 2009). Besides proteoglycans, GAGs can also be found as a free polysaccharide such as hyaluronic acid. Hyaluronic acid is the only glycosaminoglycan synthesized in the plasma membrane, with the growing polymer being secreted into the extracellular environment. The synthesis of hyaluronic acid is catalyzed by hyaluronan synthases (HAS) and consists of repeating disaccharides composed of GlcNAc and GlcA. Hyaluronic acid is the only glycan that is not linked to a protein or lipid (Hascall *et al.,* 2009). Proteoglycans and glycosaminoglycans can be found on the cell surface, inside the cell, and in the extracellular matrix (ECM) and account for many functions like promoting cell adhesion to ECM; binding of cytokines, chemokines and growth factors; acting as receptors for proteases and as coreceptors for various and tyrosine kinase growth factors receptors (Esko *et al.,* 2009).

As mentioned above, proteins can also be glycosylated giving rise to **glycoproteins**. Glycoproteins show a huge heterogeneity in its glycan structure that can be usually attributed to a non template driven biosynthetic process in endoplasmic reticulum and Golgi compartment, and a lack of any proofreading machinery. In addition, the composition of the final glycan structure relies on the polypeptide backbone as well as a number of variable factors such as the expression levels of glycosidases and glycosyltransferases and the availability of substrates, which fluctuate during cell growth, differentiation and development (Schwientek *et al.,* 2002; Varki *et al.,* 2009b; Du *et al.,* 2010; Fernandez-Valdivia *et al.,* 2011). There are several types of glycan linkage on proteins that present different glycan biosynthesis and composition wich will be discussed with detail in the next section.

GLYCOSYLATION THE MOST DIVERSE POST-TRANSLATIONAL MODIFICATION OF PROTEINS

Newly formed proteins synthesized in the ER, can be further decorated with one or more biochemical moieties, a process so called post-translational modification (PTM), giving rise to a huge protein heterogeneity. Several types of modifications can occur in proteins and the understanding of the extent and pattern of these PTMs gives insight into the function and dynamics of the proteome.

PTM can be classified in two main categories: the first include the covalent addition of chemical groups by enzymatic catalysis, the second comprises the cleavage of peptide backbones by the action of proteases or autocatalysis. Many modifications resulting in the addition of chemical group to an amino acid residue can be found in proteins, and the major types of protein covalent modifications are phosphorylation, acetylation, glycosylation, methylation, and ubiquitylation. These covalent addition are classified according to the type of amino acid side residue involved, the class of the enzyme implicated in the process and the degree of reversibility (Walsh *et al.,* 2005). Among these major types of PTMs, glycosylation is the most diverse and complex modification that occurs in proteins, with at least one half of the known proteins estimated to be glycosylated (Apweiler *et al.,* 1999). This type of modifications strongly influences many of the protein functional aspects, including cellular localization, turnover and protein quality control (Fukuda *et al.,* 1989; Parodi 1999; Arnold *et al.,* 2007). Protein glycosylation can be classified into several types according to the glycan linkage site: ***N*-linked** glycosylation, ***O*-linked** glycosylation, ***C*-linked** glycosylation (C-mannosylation), **Phospho-linked** glycosylation **or phosphoglycosylation** and **glycophosphatidylinositol (GPI)-anchored** glycosylation or **glypiation** (Spiro 2002; Moremen *et al.,* 2012) (**Figure 2**).

Proteins at the cell surface can be integrated in the cell membrane by a **GPI-anchored** moiety, called **GPI-anchored proteins** firstly described in 1985 (Ferguson *et al.,* 1985). Proteins are attached to GPI via their carboxyl termini through a phosphodiester linkage of phosphoethanolamine to a trimannosyl-non acetylated glucosamine (Man3-GlcN) core (Man(α1–2)Man(α1–6)Man(α1–4)GlcN), and can be found in the outer leaflet of the lipid bilayer facing the extracellular environment. The reducing end of GlcN is linked to phosphatidylinositol (PI) which is then anchored by another phosphodiester linkage to the cell membrane through its hydrophobic region and the distal, nonreducing mannose residue is attached to the protein via an

ethanolamine phosphate (EtNP) bridge between the C-6 hydroxyl group of mannose and the α-carboxyl group of the carboxy-terminal amino acid. The Man3-GlcN core oligosaccharide core can suffer various modifications during all the process of secretion from the cell (Ferguson *et al.,* 2009). GPI-anchored proteins have been described as having a critical role in a variety of receptor mediated signal transduction pathways, adhesion, and antigenicity (Maeda *et al.,* 2011).

Another type of protein glycosylation is the **phosphoglycosylation**, the most abundant protein glycosylation in parasites. This type of glycosylation is characterized by the enzymatic addition of sugar residues to serine (Ser) residues in the polypeptide chain, through a phosphodiester linkage. The presence of phosphoglycans in proteins, more specifically GlcNAcα-1-P, was firstly described in 1980 by Gustafson and Milner in endopeptidase Proteinase I isolated from Dictyostelium discoideum (Gustafson *et al.,* 1980), but it was only in 1995 that this linkage was demonstrated to be in Ser residues, catalyzed by UDP-GlcNAc:Ser protein *N*-acetylglucosamine-1-phosphotransferase (Ser:GlcNAc phosphotransferase) and the process called phosphoglycosylation (Freeze *et al.,* 1995). Since that, other sugar residues were found to be linked to Ser residues by a phosphodiester linkage such as Mannose (Man) residues (Ilg *et al.,* 1994) or Xylose (Xyl) (Haynes *et al.,* 1996). A specific role for phosphoglycoproteins is not yet elucidated, however some studies associate phosphoglycoproteins with the immunogenicity of the parasites (Cooper *et al.,* 1993; Ilg *et al.,* 1993).

Moreover, mannose (Man) carbon-carbon (C-C) linkages to the C2 position of tryptophan (Trp) residues were also described and named ***C*-mannosylation** (Hofsteenge *et al.,* 1994; de Beer *et al.,* 1995; Loffler *et al.,* 1996). This type of protein glycosylation can be found in cells from a variety of mammals (Krieg *et al.,* 1997), and involves a protein *O*-mannosyltransferase (POMT) that uses dolichyl-phosphate-mannose as a precursor (Doucey *et al.,* 1998), know to occur in a consensus sequence -W-x-x-W/F- (x could be any amino acid) in which the first Trp residue becomes mannosylated. Sequences presenting alternative phenylalanine (Phe) to Trp residue show a reduced efficiency of 3.5-fold in C-mannosylation (Krieg *et al.,* 1998).

In addition to the above described types of glycosylation, there are two main types of protein glycosylation in eukaryotes, the ***N*-** and the ***O*-linked glycans**. ***N*- glycosylation** is described by the addition of a sugar precursor to a nitrogen group of an asparagine (Asn) amino acid. In contrast, ***O*-glycosylation** is characterized by the addition of monosaccharides to a hydroxyl group of Ser, threonine (Thr) and tyrosine (Tyr) residues; and, to a lesser extent, to hydroxyproline and hydroxylysine (Spiro 2002). The different classes of *O*-glycans are known to be dependent on

the first added sugar (Brockhausen *et al.,* 2009), including α- or β-linked *O*-Gal (Seyer *et al.,* 1977), β-linked *O*-GlcNAc (Hart 1997), α-linked *O*-Man (Endo 1999), α-linked *O*-Fuc (Harris *et al.,* 1993; Hofsteenge *et al.,* 2001), α- or β-linked *O*-Glc (Harris *et al.,* 1993), β-linked *O*-Xyl (GAGs) (Kresse *et al.,* 1994; Lin 2004) and the most abundant form of *O*-linked glycosylation in higher eukaryotes α-linked *O*-GalNAc also known as mucin type *O*-glycans (Van den Steen *et al.,* 1998). The structural complexity of the chains initiated by *O*-linked GalNAc is very high, exceeding that of other *O*-linked and *N*-linked chains. In addition, there is the *O*-GlcNac glycosylation that is the only type of protein glycosylation, mainly found on intracellular nucleocytosolic proteins. This type of glycosylation is characterized by a reversible process comprising a single GlcNAc residue added to Ser and/or Thr, and is important in the modulation of the biological activity of intracellular proteins (Holt *et al.,* 1986), often competing with phosphorylation (Wang *et al.,* 2008).

The Biosynthetic Pathway of *N*-glycosylation

In all eukaryotes, the core pathway for the establishment of *N*-linked glycosylation is well conserved (Stanley *et al.,* 2009b). This process mainly takes place in the endoplasmic reticulum (ER) and further processing and rebuilding of the *N*-glycans occurs in the Golgi compartment generating a large diversity of possible structural outcomes (Dennis *et al.,* 2009; Varki *et al.,* 2009c). *N*-glycosylation is considered an important protein posttranslational modification in eukaryotic cells given the high occurrence of *N*-glycans in glycoproteins. It is estimated that about 90 % of all glycoproteins carry *N*-linked glycans with an average of 1.9 *N*-linked glycans per polypeptide chain (Apweiler *et al.,* 1999).

Sugars that constitute *N*-glycan structures are covalently attached to the proteins at asparagine (Asn) residues by an *N*-glycosidic bond, and five different *N*-glycan linkages have been reported, of which N-acetylglucosamine to asparagine (GlcNAcβ1-Asn) is the most common (Stanley *et al.,* 2009b). This *N*-glycosylation process starts in the ER and is characterized by a linkage to Asn residue, via a common trimannosyl chitobiosyl pre-assembled core sugar structure. The assembled of this core structure can occur whenever a consensus sequence Asn-X-Ser/Thr is present in the nascent protein, where X can be any amino acid with the exception of proline. Less commonly, the sequence Asn-X-Cys can also be used to construct *N*-glycan structures (Gavel *et al.,* 1990; Sato *et al.,* 2000).

The biosynthesis of the core trimannosyl chitobiosyl structure, starts in the cytosolic surface of the ER membrane, and is characterized by a stepwise addition of two N-acetylglucosamines (2

GlcNAc) and five mannoses (5xMan) to dolichylphosphate (Dol-P) giving rise to Dol-P-P-$GlcNAc_2Man_5$ (Helenius *et al.,* 2001; Helenius *et al.,* 2004). Thereafter, the Dol-P-P-$GlcNAc_2Man_5$ structure is "flipped" to the luminal side of ER membrane where four mannose and three glucose additional residues are added forming the final core structure Dol-P-P-GlcNAc2Man9Gluc3. The substrate donors for the addition of the last four Man and three Glc are the Dol-P-Man and Dol-P-Glc, also made on the cytoplasmic face of the ER and "flipped" onto the luminal face. Each of the sugar additions is catalyzed by a specific glycosyltransferase located on both sides of the ER membrane (Helenius *et al.,* 2001; for a review see Stanley *et al.,* 2009b).

The transfer of the complex core structure from the Dol-P-P-$GlcNAc_2Man_9Gluc_3$ donor to the nascent polypeptide backbone takes place in the ER lumen and is catalyzed by the oligosaccharyltransferase (OST) enzyme complex (Helenius *et al.,* 2001; Helenius *et al.,* 2004; Stanley *et al.,* 2009b). Following the covalent attachment of the core oligosaccharide to Asn residues, a series of processing reactions trims the *N*-glycan in the ER. First, the three Glc residues (the terminal α1–2Glc and the two inner α1–3Glc) are removed sequentially by α-glucosidases I and II, followed by one mannose removal by ER α-mannosidade which specifically removes the terminal α1–2Man from the central arm of $Man_9GlcNAc_2$. These initial steps are known to be important in regulating glycoprotein folding, a process that is mediated by the interaction between enzymes and chemical chaperons in the ER that recognize specific features of the trimmed glycan. At this point, misfolded proteins can be recognized and targeted for ER degradation by two different quality control processes, the calnexin/calreticulin system and the ER degradation-enhancing α-mannosidase I–like protein (EDEM). Calnexin (membrane-bound) and calreticulin (soluble) sequester the newly synthesized glycoprotein and act as molecular chaperones by a deglucosylation reglucosylation cycle promoting correct folding, preventing aggregation of folding intermediates, blocking premature oligomerization, and by facilitating formation of native disulfide bonds (Stanley *et al.,* 2009b). Another group of proteins that are believed to interact with the deglucosylated chain and help in the correct folding of glycoproteins are EDEMs. Little is known about EDEMs functions, however evidence suggests that EDEMs have catalytic activity and that overexpression enhances misfolded glycoprotein degradation (Freeze *et al.,* 2009). During this quality control process, if glycoproteins fail to fold or oligomerize properly they are eventually retrotranslocated to the cytoplasm and destroyed by *N*-deglycosylation and proteasomal degradation, a process called ER-associated degradation (ERAD).

When correctly folded, glycoproteins leave the ER and travel through the Golgi compartment where several steps of removal and addition of sugars occur by the action of several glycosidases and glycosyltransferases, creating a variety of glycan structures. In the majority of the multicellular organisms, glycoproteins entered in the cis-Golgi compartment and trimming of α1–2Man residues is carry on by the action of α1–2 mannosidases, originating the $Man_5GlcNAc_2$ intermediate glycan structure for the synthesis of hybrid and complex *N*-glycans. Further processing reactions for the biosynthesis of hybrid and complex *N*-glycans are initiated in the medial-Golgi and are pursued in the trans-Golgi (Helenius *et al.,* 2001). In the end, *N*-glycan structures share a common pentasaccharide core region (Manα1–6(Manα1-3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn) that can be classified into three main classes: **high-mannose type**, **complex type** and **hybrid type** (**Figure 3**).

The biosynthesis of these three different glycan structures occurs in a stepwise manner by the action of several enzymes such as *N*-acetylglucosaminyltransferases, galactosyltransferases, sialyltransferases and fucosyltransferases, in a very well-orchestrated fashion (Stanley *et al.,* 2009b). In brief, the **high mannose type** structure is characterized by the presence of only mannose residues attached to the pentasaccharide core structure; **complex type** has two antennae or branches initiated by the addition of two GlcNAc residues catalyzed by *N*-acetylglucosaminyltransferases (GnTs); and **hybrid type** in which mannose residues are attached to the Manα1–6 arm and a GlcNAc residue to the Manα1–3 arm of the core structure (Helenius *et al.,* 2001; Helenius *et al.,* 2004; Stanley *et al.,* 2009b).

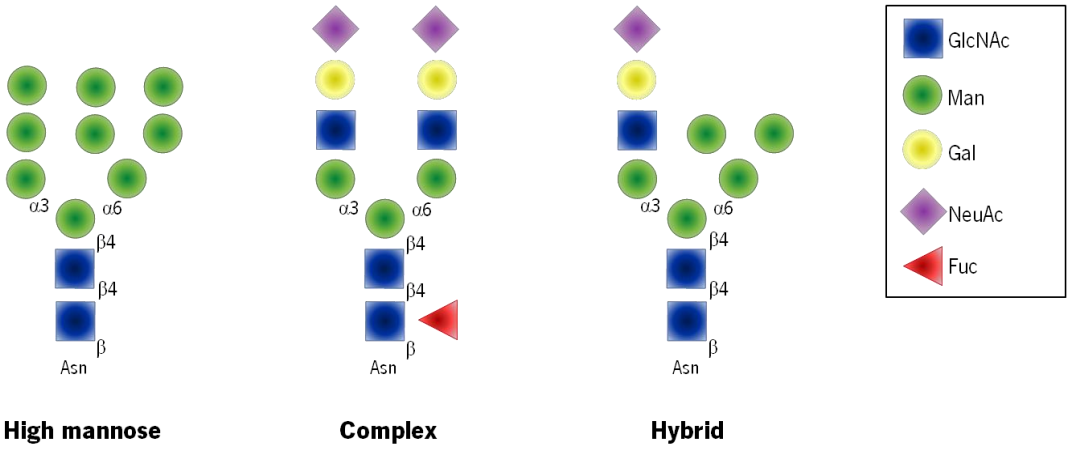

Figure 3: Types of N-glycans. *N*-glycans added to protein at Asn-X-Ser/Thr sequons, that shear a common core $Man_3GlcNAc_2Asn$, are of three general types in a mature glycoprotein: High mannose, Complex, and Hybrid.

The enzymes responsible for the synthesis of complex structures in *N*-glycans are common to the elongation process of the mucin type *O*-glycosylation, and occur both in the Golgi compartment. For this reason, the terminal protein glycosylation can be very similar between *N*- and *O*-glycans.

Biosynthesis of Mucin Type *O*-glycosylation

In contrast to *N*-glycosylation, the *O*-glycosylation is a stepwise process with monosaccharide added incrementally, beginning with the addition of a single sugar residue to either a Ser or Thr amino acids in the protein backbone and then to the nascent sugars in a process called elongation. *O*-linked glycosylation is a relatively late-stage event in the protein maturation, and involves a large set of enzymes localized at the Golgi compartment (Hanisch 2001).

The most ubiquitous type of initial *O*-glycosylation is the one formed by the addition of GalNAc (*O*-GalNAc) to Ser or Thr, also described as mucin type glycosylation. The addition of the first monosaccharide residue on protein backbones is mediated by a family of **UDP-GalNAc:polypeptide GalNAc-transferases** (**ppGalNAc-Ts**) and occurs in the Golgi compartment (Roth *et al.,* 1994; Clausen *et al.,* 1996; Rottger *et al.,* 1998; Bennett *et al.,* 2012; Gerken *et al.,* 2013). Although most protein glycosylation events are controlled by one or two genes encoding the enzymes responsible for the initiation step of glycosylation, human mucin-type *O*-glycosylation is controlled by a large family of up to 20 homologous genes encoding ppGalNAc-Ts, fifteen of which were confirmed to be expressed and functionally active (Clausen *et al.,* 1996; Ten Hagen *et al.,* 2003; Tian *et al.,* 2009; Bennett *et al.,* 2012). The addition of GalNac residues by ppGalNAc-Ts is not dependent on a defined consensus recognition sequence and in theory, any Ser or Thr residues can be *O*-glycosylated. However, some aspects have been identified that allow an improved prediction of the polypeptide *O*-glycosylation sites (Hansen *et al.,* 1995; Julenius *et al.,* 2005; Gerken *et al.,* 2011). Some authors claimed that the initiation of GalNAc glycosylation by ppGalNAc-Ts is ruled by the sequence context of putative *O*-glycosylation sites. Each ppGalNAc T isoform may be uniquely sensitive to peptide sequence and overall charge, which together dictates the substrate sites that will be glycosylated (Clausen *et al.,* 1996; Kato *et al.,* 2001; Gerken *et al.,* 2006; Wandall *et al.,* 2007; Gerken *et al.,* 2011). Moreover, there are evidences for a dynamic regulatory mechanism of the initial GalNAc addition, either by competition for different substrate sites or by competition of ppGalNAc-Ts with the glycosyltransferases responsible for the formation of core glycans (Hanisch *et al.,* 1999; Gill *et al.,* 2011). Also very interesting is that tissues show specific ppGalNAc-Ts enzyme expression

(Bennett *et al.,* 1998; Bennett *et al.,* 1999; Mandel *et al.,* 1999; Gomes *et al.,* 2009). Overall, the cellular repertoire of glycosyltransferases, with their distinct donor and acceptor sugar specificities, their localizations in sub-compartments of the Golgi and their sequential action will dictate the cell's specific *O*-glycosylation profile.

In mucin type glycosylation the addition of the first monossacharide residue by ppGalNAc-Ts, which transfer a GalNAc residue from a sugar donor UDP-GalNAc to Ser/Thr residues of the acceptor protein, gives rise to the formation of the Tn antigen (GalNAc-Ser/Thr) (**Figure 4**). A two-step model of *O*-GalNAc biosynthesis is proposed, where a subset of GalNAc-Ts add GalNAc at low density to the polypeptide chain, preferentially with unglycosylated Ser/Thr residues or those containing GalNAc-Ser/Thr flanking the active site; and another subset of GalNAc-Ts that catalyze the addition of GalNAc residues to Ser/Thr adjacent to the existing GalNAc-Ser/Thr sites (reviewed in Gill *et al.,* 2011). This initial step regulates the site and level of occupancy of total *O*-glycan modification in the target proteins, which will be then further elongated giving rise to the global final glycan chain structure. Extension of the GalNAc residue can generate eight different cores (**Figure 4**) and cores 1 to 4 are the most common in humans. Elongation of the GalNAc residue can be employed by C1GalT enzyme or T synthase, originating the core 1 structure, also known as T antigen (Galβ1-3GalNAc-Ser/Thr); or by C3GnT making the core 3 structure (GlcNAcβ1-3GalNAc-Ser/Thr) (**Figure 4**). Further elongation of these core 1 and core 3 structures is performed by C2GnT family of enzymes that catalyze the specific addition of a β1,6GlcNAc originating the corresponding core 2 and core 4 structures (**Figure 4**). Therefore, the C2GnT1 and C2GnT3 function to synthesize the core 2 structure, whereas C2GnT2 catalyzes the core 4 synthesis (reviewed in Gill *et al.,* 2011).

Additional core extension can occur in the GlcNAc moiety of the core 2 and core 4 *O*-glycans in two different ways: addition of a simple Gal alone by β3/4GalT enzymes or by addition of poly-lactosamine repeats (Gal-GlcNAc) through the concerted action of β3/4GalT and β3/4GnT enzymes. Conversely, the core 3 GlcNAc moiety is only extended through the addition of Gal by β4GalT4 or β3GalT5. The extension of core structures by the action of β3/4 Gal-Ts and β3/4 Gn-Ts leads to type 1 and type 2 chains formation, where type 1 chains are characterized by the β1,3 linkage of the Gal residue to the GalNAc, while in type 2 chains this linkage is β1,4. Both chains are frequently terminated by Lewis-type blood group-related antigens. Finally, all *O*-glycan are capped to terminate *O*-glycosylation through addition of sialic acid by ST3Gal- and ST6Gal-sialyltransferases.

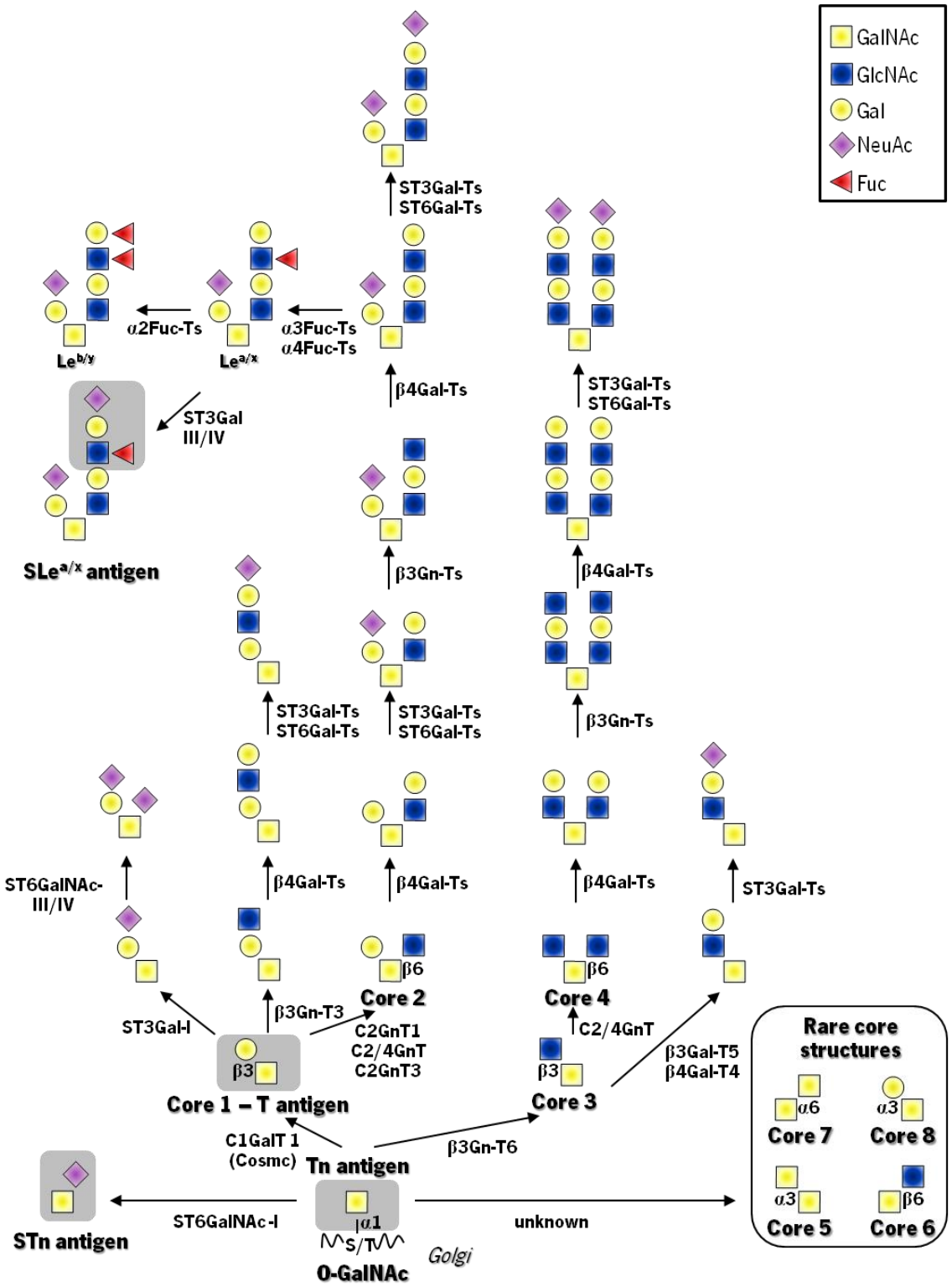

Figure 4: Schematic representation of mucin type *O*-glycosylation biosynthesis pathway. The image depicts the biosynthetic pathway of mucin type glycosylation that can generate eight different cores structures (with cores 1 to 4 the most common in humans), and are extended giving rise to a vast diversity of structures. For image simplicity only representative enzymes were included. Gray boxes correspond to cancer-associated antigens.

As mentioned above, there are four additional core *O*-glycans structures (core 5–8) that have been biochemically characterized in tissues. They are thought to be generated through direct

modification of the nascent GalNAc *O*-glycan but the enzymatic machinery necessary to generate these *O*-glycans is not known.

Furthermore, some of these branching reactions, like the formation of Type 1 and Type 2, are common to other *O*- and *N*-glycans and glycolipids. For instance, once *N*-glycans are produced in the ER and early Golgi, and *O*-glycan and glycosphingolipids core structures are generated within the Golgi apparatus, each can be modified with outer extensions, which can be highly similar among these three classes of glycans (Stanley *et al.,* 2009a). Moreover, the same glycosyltransferases can compete for extending the termini of these three types of glycan chains, highlighting the importance of the Golgi compartment in generating glycan diversity (Varki 2011).

More recently, it has been suggested a new alternative model of GalNAc addition based on the subcellular localization of GalNAc-Ts (Gill *et al.,* 2010). Despite the fact that many studies reported that enzymes responsible for *O*-GalNAc addition are expressed in the Golgi compartment, this work clearly demonstrates the possible localization of GalNAc-Ts in the ER depending of physiological status of the cells. It was shown that GalNAc-Ts from the Golgi compartment can be relocated to the ER by COP-I coat vesicles retrograde trafficking, a process that is regulated by the Src tyrosine kinase, driving a significant increase in *O*-glycosylation initiation. This increase in *O*-GalNAc addition impairs the activity of downstream enzymes resulting in increased short glycan synthesis, such as STn, Tn and T (reviewed in Gill *et al.,* 2011). It is important to note that Src is a proto-oncogene often implicated in cancer (Guarino 2010), and concurrently cancer cells commonly express short truncated glycans, including Tn, sialyl-Tn and T antigens, that are usually absent in normal tissues (Springer 1984; David *et al.,* 1992; Werther *et al.,* 1996; Baldus *et al.,* 2000; Leivonen *et al.,* 2001).

ABERRANT GLYCOSYLATION IN CANCER

Glycosylation is a feature that enhances the functional diversity of proteins and influences their biological activity. Dysfunction of glycans at the cell surface or in secreted proteins is recognized as a factor that can cause or contribute to the development of several diseases, with alterations in glycosylation being frequently observed in patients suffering from congenital diseases, immunodeficiency and cancer (Hakomori 2002; Ohtsubo *et al.,* 2006; Drake *et al.,* 2010; Tabak 2010). Particularly during carcinogenesis, glycans are recognized to be involved in different

stages of tumor progression, being key players in the proliferation, invasion, metastasis and angiogenesis processes (Fuster *et al.,* 2005).

In cancer, glycans structures are different from their normal counterpart, making glycosylation changes a hallmark of malignant cell transformation. The most frequently reported tumor-associated glycosylation alterations are the increased sialylation and polysialic acid synthesis, the appearance of sialylated Lewis antigens in glycolipids and glycoproteins, the formation of truncated *O*-glycan chains and the increase in branching of *N*-glycans (David *et al.,* 1992; Reis *et al.,* 2010; Kang *et al.,* 2011; Pinho *et al.,* 2011; Dall'Olio *et al.,* 2012). Some of the glycosylation changes that are frequently observed in cancer are summarized in Figure 4 grey boxes.

One of the underlying causes of glycosylation changes observed in cancer is the deregulated expression or localization of glycosyltransferases and associated proteins within the tumor cell (Hakomori 2002; Gill *et al.,* 2011; Meany *et al.,* 2011; Harduin-Lepers *et al.,* 2012). Several different glycosyltransferases have been described to present altered expression during the carcinogenesis process. Among the best characterized glycosyltransferases are N-acetylglucosaminyltransferase V (GnT-V), which is overexpressed through regulation by the Ets-1 transcription factor in malignant cancer cells (Ko *et al.,* 1999) and is responsible for the increased expression of branched *N*-glycans (Pinho *et al.,* 2009; Taniguchi *et al.,* 2011; Pinho *et al.,* 2012); and sialyltransferases (Dall'Olio *et al.,* 2001; Harduin-Lepers *et al.,* 2012), which are at least partially responsible for the generation of the polylactosamine residues, polysialic acid, terminal and truncated sialylated structures and some gangliosides.

Altered expression of sialyltransferases and their associated products have been widely studied in cancer (Harduin-Lepers *et al.,* 2012). Sialylated glycan structures, that result from the increased expression of sialyltransferases (STs) (Harduin-Lepers *et al.,* 2012), include sialyl Lewis antigen (SLe) and sialyl-Tn (STn). These structures are commonly observed on glycoproteins and gangliosides in various cancers, which are frequently associated with poor prognosis in patients with breast (Cazet *et al.,* 2010b), colon (Itzkowitz *et al.,* 1990) and stomach (David *et al.,* 1992; Ma *et al.,* 1993; Marcos *et al.,* 2011) cancer. Up to 20 different sialyltransferases have been described to catalyze the transfer of sialic acid residues from a donor substrate CMP-sialic acid to the oligosaccharide side chain of the glycoconjugates. This sialic acid generally occupies the terminal non-reducing position on glycan chains (Harduin-Lepers *et al.,* 2005). Different sialyltransferases show a cell and tissue specific expression pattern and differ in substrate specificities and types of linkage formed (Harduin-Lepers *et al.,* 2005). Depending on these

characteristics, STs are classified in four families - ST3Gal, ST6Gal, ST6GalNAc and ST8Sia. ST3Gal transferases are α2,3-STs and are partially responsible for the biosynthesis of sialylated Lewis structures since they can act on both type 1 (Gal β1,3 GlcNAc) or type 2 (Gal β1,4 GlcNAc) disaccharide sequences. The sialyltransferase ST3Gal III preferentially acts on type 1 rather than on type 2 disaccharides and is involved in the synthesis of SLe^a (Kitagawa *et al.,* 1993). Alternatively, ST3Gal IV and ST3Gal VI mainly catalyze the α2,3 sialylation of type 2 disaccharides, leading to the biosynthesis of SLe^x (Okajima *et al.,* 1999; Ellies *et al.,* 2002; Conze *et al.,* 2010; Colomb *et al.,* 2012; Yang *et al.,* 2012). In cancer, the increased expression of sialylated Lewis-type blood group antigens, such as SLe^a and SLe^x, mimics their normal expression on blood cells (monocytes and neutrophils) potentiating cancer cell migration through binding to endothelial cell selectins (Varki 1994; Fuster *et al.,* 2005). Therefore, SLe^a and SLe^x overexpression are a common feature of several carcinomas (e.g., lung, colon, gastric and pancreas) and are associated with increased metastatic capacity (Fukuoka *et al.,* 1998; Kim *et al.,* 1998; Tatsumi *et al.,* 1998; Borsig *et al.,* 2002; Perez-Garay *et al.,* 2013) and poor overall patients' survival (Nakamori *et al.,* 1997; Amado *et al.,* 1998; Baldus *et al.,* 1998; Nakamori *et al.,* 1999; Grabowski *et al.,* 2000).

Besides ST3Gal transferases, ST6GalNac transferases also show different expression in cancer. The expression of ST6GalNAc transferases, in particular ST6GalNAc I (Marcos *et al.,* 2004; Sewell *et al.,* 2006; Marcos *et al.,* 2011; Julien *et al.,* 2012; Ferreira *et al.,* 2013), have been associated with the expression of STn antigen in cancer. The STn antigen (Neu5Acα2-6GalNAcα-*O*-Ser/Thr), is a simple mucin-type carbohydrate antigen that shows limited expression in normal tissues but is highly expressed in most gastric (David *et al.,* 1992; Victorzon *et al.,* 1996; Baldus *et al.,* 2000), colorectal (Itzkowitz *et al.,* 1990), ovarian (Kobayashi *et al.,* 1992), breast (Yonezawa *et al.,* 1992; Leivonen *et al.,* 2001; Sewell *et al.,* 2006), pancreatic (Kim *et al.,* 2002) and bladder (Ferreira *et al.,* 2013) carcinomas. The expression of STn is an established indicator of poor prognosis in patients with gastric (Werther *et al.,* 1994; Werther *et al.,* 1996; Nakagoe *et al.,* 2002), colorectal (Itzkowitz *et al.,* 1992; Karlen *et al.,* 1998), and ovarian cancer (Holmberg *et al.,* 2000) and contributes to the aggressive phenotype of carcinoma cells by altering their behavior (Clement *et al.,* 2004; Julien *et al.,* 2005; Julien *et al.,* 2006; Pinho *et al.,* 2007; Ferreira *et al.,* 2013). Expression of STn is also observed in precursor and early lesions of carcinomas of the gastrointestinal tract, such as intestinal metaplasia of the stomach (David *et*

al., 1992; Ferreira *et al.,* 2006), adenomatous polyps (Itzkowitz *et al.,* 1992), chronic ulcerative colitis (Karlen *et al.,* 1998) and pancreatic intraepithelial neoplasias (Kim *et al.,* 2002).

Moreover, expression of truncated *O*-glycans such as STn and Tn antigens in cancer hs also been associated to dysfunction of proteins associated to glycosyltranferases and all the glycosylation process. One example is the deficiency in Cosmc chaperon protein that was demonstrated to lead to the synthesis of Tn and STn antigen (Ju *et al.,* 2008b; Wang *et al.,* 2010). Cosmc protein is known to stabilize T synthase, the enzyme responsible for Core 1 synthesis. T synthase enzyme is present in most cell types, and in vertebrates it requires Cosmc specific chaperone to be correctly exported from endoplasmic reticulum to the Golgi compartment ensuring its fully activity (Ju *et al.,* 2008a; Aryal *et al.,* 2010; Ju *et al.,* 2011; Sun *et al.,* 2011). As consequence of the presence of these truncated *O*-glycans at the cell surface of cancer cells, antigenic peptide backbones that are not accessible in normal cells are exposed (Burchell *et al.,* 2001).

Mucins are the major carriers of cancer-associated carbohydrates, including truncated oligosaccharides forms (immature) and sialylated terminal structures. There are many descriptions about mucin aberrant glycosylation in cancer. For example, some adenocarcinoma cells, such as the case of breast, over-express the cell-membrane mucin MUC1 with truncated non-branched *O*-glycans consisting of Tn and STn antigens as well as of Core 1 glycans such as T and sialyl-T (ST) antigens, whereas in the normal cell counterpart branched *O*-glycans of Core 2 and to a lesser extent of Core 3 are typical found in glycoproteins (Lloyd *et al.,* 1996; Brockhausen 2006; Singh *et al.,* 2007; Cazet *et al.,* 2010a). In gastric cancer it was described the presence of T antigen in MUC1 protein (Santos-Silva *et al.,* 2005) and also STn antigens in MUC2 protein, and this last is also found in intestinal metaplasia, a gastric precursor lesion (Ferreira *et al.,* 2006; Conze *et al.,* 2010). More recently, Pinto *et al*. demonstrated that, in a panel of different carcinoma tissues, several different mucins were abnormally glycosylated (Pinto *et al.,* 2012).

Aberrant Glycosylation In Gastric Cancer

Many different glycan alterations have been described in gastric lesions and cancer. Recently, differences have been observed in the glycosylation of gastric mucins in different gastric lesions (Conze *et al.,* 2010; Pinto *et al.,* 2012). As described previously in this chapter, gastric carcinogenesis, in most of the cases, is characterized by the development of a chronic inflammation upon *H. pylori* infection that can evolve to gastric cancer. During the gastric

carcinogenesis pathway many different alterations occur and interestingly this process is accompanied by changes on the gastric mucosa glycosylation profile of glycoconjugates.

Human gastric tissues express several different mucins representing a very broad family of polydisperse high molecular weight glycoproteins that are part of the gastric mucosa and glycocalyx. From the more than 20 mucins already described (Rose *et al.,* 2006), MUC1, MUC4, MUC5AC, and MUC6 are expressed in gastric tissue (Audie *et al.,* 1993; De Bolos *et al.,* 1995; Ho *et al.,* 1995; Byrd *et al.,* 1997; Reis *et al.,* 1997; Reis *et al.,* 1998; Reis *et al.,* 2000), with MUC1, MUC5AC, and MUC6 being the most prominent (Ho *et al.,* 1995). Gastric mucins are highly glycosylated and from one single several hundred of carbohydrate chains arise. The carbohydrate chains covering gastric mucins are extremely diverse contributing to the complexity of these molecules. The biosynthesis of the carbohydrate chains present on proteins and lipids on cell membranes involve many different glycosyl- and sulfotransferases, resulting in a myriad of final sugar structures. In the gastrointestinal tissues, the high levels of C3GnT enzyme favor the biosynthesis of the core 3 structures and the downstream core 4 *O*-glycans (Iwai *et al.,* 2002). Thus, the normal gastric mucosa expresses mostly neutral glycans, but upon *H. pylori* infection and inflammation, there is *de novo* expression of charged sialylated Lewis antigens, including SLe^x and SLe^a (Ota *et al.,* 1998; Mahdavi *et al.,* 2003; Kobayashi *et al.,* 2004; Marcos *et al.,* 2008). Besides the *de novo* expression of these sialylated Lewis antigens in gastric lesions, it is also described the expression of STn antigen in MUC 2 present in goblet cells in intestinal metaplasia tissues (Ferreira *et al.,* 2006; Conze *et al.,* 2010). Briefly, two main types of intestinal metaplasia have been described so far according to the mucin expression profile. The complete type, that is characterized by loss of expression of the typical gastric mucins MUC1, MUC6 and MUC5AC and the de novo expression of MUC2; and the incomplete type that co-expresses gastric mucins MUC1, MUC6 and mostly MUC5AC with the intestinal MUC2 mucin (Reis *et al.,* 1999). In addition to gastric precursor cancer lesions, it is also observed overexpression of sialylated antigens in gastric tumor cells (Baldus *et al.,* 1998), and expression of dimeric SLe^x was associated with venous invasion and poor disease prognosis (Amado *et al.,* 1998). In addition to sialylated glycans, most of the gastric tumors also show high expression of the simple mucin type antigens Tn and STn (David *et al.,* 1992; Baldus *et al.,* 2000) and expression of MUC2 (Pinto-de-Sousa *et al.,* 2002; Mesquita *et al.,* 2003).

In gastric tissues ST6GalNAc-I was proposed as a major enzyme responsible for controlling STn expression (Marcos *et al.,* 2011), and also ST3Gal IV (Carvalho *et al.,* 2010; Jun *et al.,*

2012), FUT IV (Petretti *et al.,* 1999) and β3GnT5 (Marcos *et al.,* 2008) have been described to be involved in SLe^x antigen expression. In gastric carcinoma tissues, the increased expression of ST3Gal IV (Petretti *et al.,* 1999; Jun *et al.,* 2012) is associated with increased expression of α2,3 sialic acids and sialyl Lewis antigens and correlated with poor prognosis and increased metastatic capacity (Amado *et al.,* 1998). These reports highlight the role of sialyltransferases and demonstrate that the expression of crucial glycan determinants, such SLe^x, plays an important role in gastric tumor progression. However, the molecular mechanisms underlying this altered enzyme expression and aggressive behavior of gastric cancer cells expressing these glycan determinants are not fully understood.

More recently, some studies pointed out the role of the inflammation process and interleukin (IL) production in the expression of several glycosyltransferases involved in SLe^x biosynthesis (Blander *et al.,* 1999; Higai *et al.,* 2006; Marcos *et al.,* 2008; Trinchera *et al.,* 2011). Besides sialyltransferases, CMP-sialic acid donors are also transcriptionally regulated during inflammation, leading to SLe^x glycan biosynthesis (Huopaniemi *et al.,* 2004). Blander and co-workers demonstrated that ST3Gal IV and FUT VII are up-regulated upon CD4 T cell activation by IL-12 or IL-4, and this activation leads to SLe^x expression on Th1 activated cells (Blander *et al.,* 1999). In addition, IL-1β was reported to induce SLe^x via enhanced expression of ST3Gal IV and FUT VI gene on hepatocellular carcinoma HuH-7 cells (Higai *et al.,* 2006). In gastric carcinoma cells, IL-1βand IL-6 were reported to induce significant increases in the mRNA levels of FUT1, FUT2 and FUT4 with no alterations in ST3Gal IV expression (Padro *et al.,* 2011). Thus, this increased expression of FUT enzymes with no alterations in ST3Gal enzymes leads to decreased expression of SLe^x in a mouse xenograph model, in opposition to its expression on gastric intestinal type carcinoma tissues (Padro *et al.,* 2011). This fact highlights the already described association of increased expression of ST3Gal IV and induction of SLe^x in gastric carcinoma tissues (Petretti *et al.,* 1999; Jun *et al.,* 2012), that was not observed in this IL-1β and IL-6 inductive cellular model. Conversely, Rudd and co-workers described the expression of SLe^x in acute phase proteins from serum of individuals with gastric carcinoma as a possible result of increased IL-6 signaling (Bones *et al.,* 2011).

Furthermore, the role of tyrosine kinase receptor activation and downstream effectors have been reported in some ST overexpression studies (Cazet *et al.,* 2009; Cazet *et al.,* 2010b; Cazet *et al.,* 2012). In different studies, Cazet and co-workers demonstrated that expression of GD2

ganglioside, due to overexpression of ST8SIA1, induces c-Met activation enhancing breast cancer cell increased proliferation and migration (Cazet *et al.,* 2009; Cazet *et al.,* 2012).

Aberrant glycosylation is a functional marker that can be used to estimate the clinic-pathogenic process in cancer, and is a good starting point for cancer biomarker discovery.

BIOMARKERS FOR CANCER DETECTION

By definition biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention" (Glassman *et al.,* 2009).

In medicine the use of biomarkers lays on the principle that an observation or measurement can be used to establish a biological process and as an indicator that a specific disease is present. Most recent biomarker related studies, especially those for cancer biomarkers, have largely reported the incapability to validate the biomarker for clinical use, rather than successful validation (Diamandis 2010). Moreover, the majority of cancer biomarkers currently used in clinical practice are mainly recommended for monitoring response to treatment among patients with advanced disease and are not suitable for population screening or for early diagnosis.

In the last decades, many new studies have reported a handful of new cancer biomarkers. Still, very few are validated as efficient cancer biomarkers suitable for screening and early diagnosis (Manne *et al.,* 2005). The reason behind the ineffective validation of new cancer biomarkers cannot be attributed to the lack of pathophysiological knowledge, powerful techniques, or investment of funds but in inherent difficulties that are associated with biomarker discovery which have been underestimated. Deficiencies in the study design are a major reason for the difficulties found during biomarkers discovery and validation contributing for the short life span of many "newly discovered" biomarkers (Diamandis 2010). Some of the problems within the study design include aspects that may play a role before sample analysis (pre-analytical phase), during sample analysis (analytical phase) and/or after sample analysis (post-analytical phase). Prior to sample analysis, in the pre-analytical phase, aspects such as examination of various individual characteristics (such as sex, age and drugs) or sample collection and storage could independently affect biomarker levels. Moreover, in the analytical phase all the methodology employed in the study should be highly specific, sensitive and accurate to avoid introducing measurements bias or artifacts. In addition, in the post-analytical phase the important aspect to take in consideration is

the data interpretation, so the results can be extended to general population and replicated by independent studies. Therefore, careful validation in independent datasets by independent investigators and publication of the findings are probably the best way to identify a good biomarker.

Finally, in order to validate a tumor biomarker for clinical practice some requirements are needed such as: 1) a molecule to be effective in early diagnose must be released into circulation in appreciable amounts by a small asymptomatic tumor; 2) the biomarker should be highly specific for the tissue of origin; 3) the level of a biomarker should not be affected by a non-cancer disease. To overcome most of these problems, post-translational modifications such as glycosylation, can contribute to distinguish molecules that are only expressed in cancer tissues when compared with normal tissues or other diseases.

Glycoprotemic Advances in Gastric Cancer Biomarker Discovery

In the last two decades, several different proteomic approaches have been developed leading to new glycoproteomic advances, mainly in serum glycoproteome.

Many different biological matrices have been used in different studies, and in the case of gastric cancer biomarker discovery the majority of reported proteomic investigations used gastric tissues (Lee *et al.,* 2012), serum (Qiu *et al.,* 2009; Bones *et al.,* 2011; Liu *et al.,* 2012), saliva (Wu *et al.,* 2009) and gastric juice (Lee *et al.,* 2004; Hsu *et al.,* 2007). In addition, cell culture (Tomlinson *et al.,* 2002; Takikawa *et al.,* 2006) and mouse xenograft (Chong *et al.,* 2010; Florou *et al.,* 2010; Zhang *et al.,* 2010) models have also been employed in order to obtain some insight into new cancer biomarker finding.

Different strategies have been used to assist in the identification of new biomarkers such as several serological protein depletion approaches (Andersen *et al.,* 2010; Holewinski *et al.,* 2013), reduction of protein concentration range with equalization methods (Thulasiraman *et al.,* 2005), lectin affinity chromatography enrichments (Abbott *et al.,* 2010), antibody microarrays (Haab 2003), immunohistochemistry analysis (Ansari *et al.,* 2011; O Leary *et al.,* 2013), ELISA protocols (Scholler *et al.,* 2006; Zhou *et al.,* 2012), two-dimensional (2-D) gel electrophoresis (Rabilloud 2002), dimensional difference in-gel electrophoresis (2D-DIGE) (Kondo *et al.,* 2006), several mass spectrometry technologies SELDI-TOF-MS, MALDI-TOF-MS, LC-MS (Srinivas *et al.,* 2002), and many others.

Despite all of these new advances in the glycoproteome analysis, to date very few studies were able to identify new cancer biomarkers regarding alterations in the glycome and glycoproteome of patients bearing stomach cancer. Most of the studies that report the role of glycans in gastric

carcinogenesis are focused on the involvement of carbohydrate determinants in the interaction and binding of *H. pylori* to stomach epithelial cells (Peek *et al.,* 2002; Marcos *et al.,* 2008; Kobayashi *et al.,* 2009; Magalhaes *et al.,* 2009). In addition, identification of glycan alterations in gastric cancer has been limited to few descriptions on mucin glycoproteins (Conze *et al.,* 2010) or in glycosyltransferases and their associated glycans that show increased expression in cancer (Gretschel *et al.,* 2003; Marcos *et al.,* 2003; Kim *et al.,* 2004; Marcos *et al.,* 2004; Conze *et al.,* 2010; Marcos *et al.,* 2011; Jun *et al.,* 2012).

Although serum biomarker discovery has attracted increased attention in the last years, few studies have succeeded in the detection of alterations in serum proteins' glycosylation and identification of new biomarkers in stomach cancer. In 1998, Goodarzi and Turner characterized the *N*-glycosylation pattern of haptoglobin in a set of different diseases and verified that in gastric cancer that is a difference in the glycosylation pattern of this protein, claiming a potentially clinical application of haptoglobin glycosylation changes in patient's samples (Goodarzi *et al.,* 1998). More recently, Bones et al. showed that an increase in the levels of SLe^x present on triantennary glycans and also increased levels of core fucosylated agalactosyl biantennary glycans present on IgG, were associated with increasing disease pathogenesis (Bones *et al.,* 2011). Further, 2D gel electrophoresis analysis of the depleted serum showed a number of differentially expressed protein candidates (e.g. clusterin, leucine-rich-R2-glycoprotein, and kininogen-1), and some were validated to carry triantennary SLe^x using subsequent glycoproteomic approaches (Bones *et al.,* 2011). Prior to this finding, Bones et al. was able to demonstrate a new ultra performance liquid chromatography (UPLC) technology for fast and efficient identification of cancer associated alterations in the serum *N*-glycome of patients bearing stomach adenocarcinoma (Bones *et al.,* 2010). In this study the authors evaluated the contribution of the glycosylation present on four highly abundant serum glycoproteins IgG, haptoglobin, transferrin, and α1-acid glycoprotein and they found an increase in sialylation of haptoglobin, transferrin, and α1-acid glycoprotein and increased levels of core fucosylated biantennary glycans and decreased levels of monogalactosylated core fucosylated biantennary glycans on IgG with progressing cancer state (Bones *et al.,* 2010).

Despite the association between glycan expression alterations in tumor tissues and clinical prognosis has been documented, its application in the clinical setting has been limited to serological assays that detect glycoconjugates shed by the tumors into circulation.

Glycan-Based Serological Biomarker Assays In Cancer

Glycosylation changes on glycoconjugates either present on the surface or secreted by cancer cells are a major potential source of cancer biomarkers. Presently, most serological assays used for cancer detection, prognosis and monitoring are based on quantifying glycoconjugates in the serum of patients with cancer. These serological assays detect carbohydrate antigens such as SLe^a (CA19-9) and STn (CA72-4) or mucin glycoproteins such as MUC1 (CA15-3) and MUC16 (CA125) (Nustad *et al.,* 1996; Yin *et al.,* 2001; Yin *et al.,* 2002; Drake *et al.,* 2010; Reis *et al.,* 2010).

The use of these biomarkers for cancer screening limits the identification of the organ of origin since their broad expression in various different cancer types (Chang *et al.,* 2004; Baskic *et al.,* 2007). In addition, the presence of these biomarkers can also be found in some non-neoplastic and inflammatory diseases (Sevinc *et al.,* 2007), reducing the specificity of the assays for cancer screening (Harris *et al.,* 2007). Therefore, currently the clinical application of these makers is mostly for monitoring treatment and relapses with no consensual application in cancer diagnosis.

Nevertheless, sound data support the use of the CA125 assay for detection of ovarian cancer. The CA125 is detected in 80% of patients with ovarian cancer (Nustad *et al.,* 1996) and preoperative evaluation of CA125 has been shown to aid in the evaluation of prognosis for patients with ovarian cancer (Gostout *et al.,* 2006). Moreover, increased CA125 levels were found in 50% of patients with stage I ovarian cancer and in 25% of serum samples collected 5 years before diagnosis of ovarian cancer, stressing a possible application in early detection of cancer (Zurawski *et al.,* 1988). Similarly to CA125, increased levels of aberrant glycosylated MUC1 mucin, which is produced by cancer cells and shed into the circulation to be detected by the CA15-3 assay, have also been shown to be useful for prognosis evaluation in early stage breast cancer and for monitoring the course of the disease (Ebeling *et al.,* 2002; Kumpulainen *et al.,* 2002; Uehara *et al.,* 2008).

In addition to mucin expression in cancer, the aberrant expression of other carbohydrate antigens on glycoconjugates has also been shown to be useful for evaluating prognosis and for monitoring purposes in cancer. The CA19-9 serological assay detects the expression of SLe^a on glycolipids and glycoproteins and is usually used to evaluate the clinical response to therapy in patients with various cancer types such as pancreatic, colorectal, gastric or biliary cancer (Safi *et al.,* 1997; Locker *et al.,* 2006; Harder *et al.,* 2007; Pan *et al.,* 2011). In colon cancer, CA19-9 is a prognostic marker since patients presenting increased CA19-9 concentrations before surgery

had a fourfold increase in death rate at three years (Diez *et al.,* 1994). In gastric carcinoma, preoperative CA19-9 concentration remains one of the best prognostic factors (Reiter *et al.,* 1997; Ychou *et al.,* 2000), and preoperative positivity for CA19-9 is an independent risk factor for recurrence of gastric carcinoma (Marrelli *et al.,* 1999). Another carbohydrate antigen, STn, which is expressed in glycoproteins such as mucins, can be detected by the CA72-4 assay. Increased concentration of CA72-4 has been shown in patients with gastric, colorectal and pancreatic carcinomas (Ychou *et al.,* 2000; Gaspar *et al.,* 2001; Carpelan-Holmstrom *et al.,* 2004). In gastric carcinoma, CA72-4 assay can be used for monitoring disease recurrence (Ychou *et al.,* 2000) and has been shown to be an independent prognostic factor since patients positive for CA72-4 show a 3.8-fold higher risk of death (Louhimo *et al.,* 2004).

Besides mucins and aberrant expression of carbohydrate antigens, several other markers are used in clinical practice such as the case of prostate specific antigen (PSA) and carcinoembryonic antigen (CEA). CEA is highly expressed in colorectal, stomach, and several other cancers, and shed into the circulation (Bakalakos *et al.,* 1999; Goldstein *et al.,* 2005). Also, patients with prostate cancer show high levels of PSA in serum, making PSA the golden standard for prostate cancer diagnose and monitoring (Gann *et al.,* 1995; Draisma *et al.,* 2003; Hong *et al.,* 2012). However increase in serum concentration of both CEA and PSA can also have non-cancer-related causes (Yi *et al.,* 2013). The differential diagnostic application of benign and malignant lesions remains a hurdle that can be eventually overcome by taking into consideration the type and level of glycosylation. This feature has been previously observed in colonic tissues, where normal colonic mucosa and preneoplastic lesions display CEA molecules with different molecular weights due to glycosylation when compared to colon cancer cells (Garcia *et al.,* 1991). More recently, CEA was reported to display abnormal glycosylation in colon cancer (Saeland *et al.,* 2012). Regarding PSA, several reports have demonstrated altered PSA glycosylation in benign and malignant prostate lesions, highlighting the importance in considering the glycan composition of this biomarker for the clinical diagnosis of the disease (Peracaula *et al.,* 2003; Tajiri *et al.,* 2008; Meany *et al.,* 2009; White *et al.,* 2009; Dwek *et al.,* 2010; Li *et al.,* 2011; Gilgunn *et al.,* 2012; Vermassen *et al.,* 2012).

The glycosylation alterations observed in cancer should be taken in consideration, and constitute a key target for the development of novel serological- based assays for early cancer detection with major screening and clinical implications.

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Chapter 2

Aims and Objectives

Cancer is a leading cause of death worldwide and the development of new approaches for early diagnosis, treatment and prevention is a main concern to improve health.

Gastric cancer is the fourth most common cancer worldwide, being the second cause of cancer related deaths. The high mortality rate of gastric carcinoma is mainly due to the lack of early diagnosis of the disease. The failure in the early diagnosis of gastric cancer is closely associated with the lack of specific symptoms, with patients most of the times being diagnosed in advanced stages of disease where the five year survival rate is less than 15%. In clinical practice, endoscopy remains the gold standard for diagnosis of gastric cancer that fails the early diagnose since patients that do not display symptoms are generally not selected for endoscopic investigation. In addition, the clinical serological assays used nowadays also lack the specificity and sensitivity necessary for patient screening, being used mostly for monitoring disease. Therefore, the discovery of new biomarkers for detection of cancerous lesions in the stomach at the early developmental stage of the tumor is a main goal for a better outcome of gastric carcinoma.

Glycan alterations are a common feature of cancer, and during gastric carcinogenesis many different alterations occur, in particular changes on the gastric mucosa glycosylation profile of glycoconjugates. In addition, an increased body of evidence supports the role of glycan alterations in cancer, principally in cancer cell biology.

The general aim of the present work was to identify proteins carriers of cancer associated glycan antigens that can be possible new biomarkers in gastric cancerous lesions, and characterize the role of these glycans in gastric cancer cell biology.

Specific objectives

1. To identify glycoproteins, presenting cancer associated *O*-glycan structures, in serum from individuals with gastric lesions including gastric cancer, as potential new biomarkers.

The use of serum for the identification of cancer biomarkers is an extremely important goal since sampling is minimally invasive, and the ease with which assays could be translated to the clinic should mean a rapid take up. Most biomarkers are based on the expression of novel antigens by cells. Cancer cells have frequent alterations in the glycosylation pattern, which

normally results in expression of truncated *O*-glycans attached to the protein. The detection, in the serum, of these glycoproteins depends on the production by cancer cells and shedding into the blood stream. Nowadays, many of the clinical biological assays used in cancer diagnose and monitoring are based on glycoproteins expressing different glycan structures that are released into serum. In light of these we first aimed to assess the expression of truncated glycans, such as STn and T antigens, in tissues and serum of patients with gastric lesions including cancer, and identify the proteins carriers of these glycan structures. In Chapter 3.1, we present a glycoproteomic approach that was developed in order to identify proteins carriers of truncated glycans produced in different gastric pathological conditions and released into circulation. We further validated the glycan structure and glycosylation sites present in the identified glycoproteins by de-*O*-glycosylation with reductive β-elimination followed by nano-HPLC-MALDI-TOF/TOF, and by sialoglycopeptides enrichment with titanium dioxide chromatography and mass spectra data crossing with MSBridge software.

2. To disclose the biological role of ST3Gal IV and its associated carbohydrate determinant SLe^x in gastric cancer cell behavior.

Expression of cancer associated glycans relays in the altered expression of glycosyltransferases. Many different glycosyltransferases are deregulated in cancer, in particular sialyltransferases. Increased expression of sialyltransferases, for instance ST3Gal III and ST3Gal IV have been associated with increased expression of sialylated Lewis-type blood groups such as SLe^a and SLe^x. In addition, the increase expression of SLe^a and SLe^x in cancer is known to mimic their normal expression on blood cells (monocytes and neutrophils) potentiating cancer cell migration through binding to endothelial cell selectins. Therefore, SLe^a and SLe^x overexpression are a common feature of several carcinomas, and in gastric cancer have been associated with increased metastatic capacity and poor overall patients survival. In chapter 3.2, we aimed to unveil the role of ST3Gal III and ST3Gal IV in the biosynthesis of SLe^x antigens in a gastric carcinoma cell line MKN45. Furthermore, we intended to evaluate the biological role of SLe^x expression on gastric cancer cell behavior *in vitro* and *in vivo* using the chicken chorioallantoic membrane (CAM) model.

3. To evaluate the role of tyrosine kinase receptor activation and downstream effectors in cancer cell behavior of cells expressing SLe^x.

The expression of SLe^x antigens, due to overexpression of sialyltransferases, plays an important role in gastric tumor progression and behavior. The molecular mechanisms underlying this altered enzyme expression and aggressive behavior of gastric cancer cells expressing these glycan determinants is a hurdle that still need further investigations. The involvement of signaling pathways with activation of receptors and downstream effectors that enhance cell biological functions have been reported as possible effect to expression of such cancer glycan determinants. In Chapter 3.2, we aimed to assess the role of tyrosine kinase receptor activation and downstream evaluation of effectors in gastric cancer cells expressing SLe^x antigens.

4. To identify the SLe^x expressing glycoproteins in a gastric carcinoma cell line and to validate in gastric carcinoma tissues.

Aberrant glycosylation is a functional marker that can be used to estimate the clinic-pathogenic process in cancer, and is a good starting point for cancer biomarker discovery. Increased expression of ST3Gal IV and SLe^x antigen have been reported in gastric carcinoma tissues and also in association with venous invasion and poor prognosis for the patients. One of the current clinical biomarker used for diagnose of gastric cancer is the carbohydrate antigen SLe^a (CA19-9). Nevertheless, this serological assay lacks the levels of sensitivity and specificity required to make it clinically useful for the early detection of gastric carcinoma and is mainly used to monitor the disease after treatment, rather than diagnostic purposes. In chapter 3.3, we intended to identify the proteins carriers of SLe^x in the gastric carcinoma cell line overexpressing ST3Gal IV and validate the protein target and the molecular complex protein/SLe^x in gastric carcinoma tissues as a potential new cancer biomarker.

Chapter 3

Results

Content

“The good thing about science is that it's true whether or not you believe in it.”
Neil deGrasse Tyson

3.1

Glycoproteomic Analysis of Serum From Patients with Gastric Precancerous Lesions

Content

3.1 Glycoproteomic analysis of serum from patients with gastric precancerous lesions.

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ABSTRACT

Gastric cancer is preceded by a carcinogenesis pathway which includes gastritis caused by *Helicobacter pylori* infection, chronic atrophic gastritis that may progress to intestinal metaplasia (IM), dysplasia and ultimately gastric carcinoma of the more common intestinal subtype. The identification of glycosylation changes in circulating serum proteins in patients with precursor lesions of gastric cancer is of high interest and represents a source of putative new biomarkers for early diagnosis and intervention.

This study applies a glycoproteomic approach to identify altered glycoproteins expressing the simple mucin-type carbohydrate antigens T and STn in the serum of patients with gastritis, IM (complete and incomplete sub-types) and in control healthy individuals. The immunohistochemistry analysis of the gastric mucosa of these patients showed expression of T and STn antigens in gastric lesions, with STn being expressed only in IM. The serum glycoproteomic analysis using 2D-gel electrophoresis, Western blot, and MALDI-TOF/TOF mass spectrometry led to the identification of circulating proteins carrying these altered glycans. One of the glycoproteins identified was plasminogen, a protein that has been reported to play a role in *H. pylori* chronic infection of the gastric mucosa and is involved in extracellular matrix modeling and degradation. Plasminogen was further characterized and showed to carry STn antigens in patients with gastritis and IM.

These results provide evidence of serum proteins displaying abnormal *O*-glycosylation in patients with precursor lesions of gastric carcinoma and include a panel of putative targets for the non-invasive clinical diagnosis of individuals with gastritis and IM.

INTRODUCTION

The development of gastric cancer is associated with a long carcinogenesis pathway that is initiated by *Helicobacter pylori (H. pylori)*, a Gram-negative bacterium that causes gastritis, and may lead to the development of a chronic atrophic gastritis, intestinal metaplasia (IM) and ultimately gastric adenocarcinoma (Parsonnet *et al.,* 1991; Correa 1992; Suerbaum *et al.,* 1998; Wang *et al.,* 1998; Mesquita *et al.,* 2006; Correa *et al.,* 2007). *H. pylori* infects more than 70% of the population in some countries (Atherton 2006) but only a fraction of those individuals develop more severe gastric conditions, such as atrophic gastritis and IM, a pre-cancerous lesion (Correa 1992; Mesquita *et al.,* 2006). The current diagnosis for these precursor lesions relies almost exclusively in endoscopy followed by biopsy, which is both invasive and costly to apply for screening strategies. Therefore, biomarkers which can aid in the screening and identification of individuals with silent gastric pathologies are highly needed.

Glycosylation is a common post-translational modification of proteins with more than 50% of eukaryotic proteins thought to be glycosylated (Apweiler *et al.,* 1999). The pattern of protein glycosylation is cell and tissue specific and closely reflects the physiological status of the cell. Thereby, changes in glycan expression are frequently observed in several pathological conditions (Reis *et al.,* 2010) and in particular in the gastric context (David *et al.,* 1992; Silva *et al.,* 2002; Ferreira *et al.,* 2006). In gastric pathologies the glycosylation alterations include aberrant expression of simple mucin type carbohydrate antigens, namely T (Galβ1–3GalNAcα-*O*-Ser/Thr), Tn (GalNAcα-O-Ser/Thr), and sialyl-Tn (Neu5Acα2-6GalNAcα-O-Ser/Thr) (David *et al.,* 1992; Ferreira *et al.,* 2006; Conze *et al.,* 2010; Marcos *et al.,* 2011). An over-expression of sialylated Lewis antigens (Amado *et al.,* 1998) and the decreased expression of terminal α1,4-linked N-acetylglucosamine residues (αGlcNAc) has also been reported (Zhang *et al.,* 2001; Ferreira *et al.,* 2006; Karasawa *et al.,* 2012).

The biosynthesis of the carbohydrate structures in glycoproteins relies on a number of competitive and concerted processes involving several glycosyltransferases. Mucin (GalNAc)-type *O*-glycosylation (hereafter called *O*-glycosylation) is one of the most common types of glycosylation found in glycoproteins and consists of a glycan *O*-linked to a serine or a threonine residue. The

first step in *O*-glycosylation is the transfer of GalNAc from a sugar donor UDP-GalNAc to a serine or threonine residue and is controlled by UDP-GalNAc: polypeptide N-acetylgalactosaminyltransferases (GalNAc-Ts) (Clausen *et al.,* 1996; Bennett *et al.,* 2012). These Golgi enzymes control the site of *O*-glycosylation. After the first glycan (GalNAc) is added forming the Tn antigen, the action of other glycosyltranferases leads to the synthesis of the various core structures depending on the cell context. In gastric epithelial cells, a Gal-transferase (C1GalT-1) leads to the biosynthesis of the core 1 (T antigen), which can be further branched, extended and terminated by Lewis and ABO blood group antigens. Alternatively, Tn and T antigens can be sialylated by sialyltransferases forming the sialyl-Tn (STn), sialyl-T (ST) and disialyl-T (**Figure 1**) (for a review see Reis *et al.,* 2010).

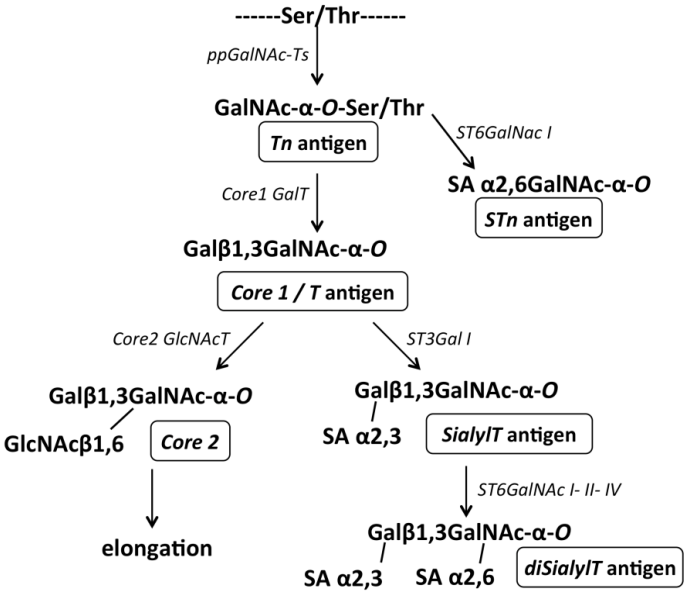

Figure 1: Schematic representation of the biosynthesis of Core *O*-glycan structures and formation of simple type carbohydrate antigens Tn, STn, T and ST.

The alterations in glycosylation observed in pathologic conditions are mostly due to modifications at the glycosylation cell machinery, disorganization of secretory pathway organelles (ER and Golgi) and altered glycosyltransferase expression. The aberrant expression of glycoconjugates bearing these glycans either present on the surface or secreted by cells are a major potential source of biomarkers representing most serological assays used for cancer detection and monitoring. These serological assays detect carbohydrate antigens such as SLe^a (CA19-9) and STn (CA72-4) or mucin glycoproteins such as MUC1 (CA15-3) and MUC16 (CA125) (Reis *et al.,* 2010).

The glycoproteins carrying immature glycans, such as simple mucin type *O*-glycan antigens, can be present in circulating proteins in pathological conditions and are being targeted for early

detection approaches. In this study we assess the immunohistochemical presence of truncated *O*-glycans STn and T antigens in gastric tissues, including normal gastric mucosa, gastritis and IM. We have further screened sera from the same patients searching to identify proteins bearing these truncated glycans. This approach resulted in the identification of fine alterations in the *O*-glycosylation of serum glycoproteins, such as the presence of STn, which is known to be aberrantly expressed in the gastric lesions under study. Specifically, we identified serum proteins carrying abnormal *O*-glycans that can be candidate targets for the non-invasive diagnosis of precursor lesions of gastric cancer.

MATERIALS AND METHODS

Tissue samples and Histology

Stomach biopsies and serum from individuals without gastric lesions and *H. pylori* infection (n=6), gastritis (n=5), complete (n=5) and incomplete (n=3) IM were used. The individuals are part of a cohort from northern Portugal (Viana do Castelo) working in a shipyard that have been study and characterized as previously described for gastric pathologies and *H. pylori* infection (Almeida *et al.,* 2003).

Paraffin sections were used for histochemistry and immunohistochemistry. Tissue sections were stained with hematoxylin and eosin (H&E) for histopathology. Clinical data, including *H. pylori* infection status of every case was considered for selection of the cases and controls.

Immunohistochemistry

Paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min, and then sections were incubated with normal rabbit serum diluted 1:5 in PBS containing 10% BSA. Incubation with the monoclonal antibodies was performed overnight at 4°C. Slides were washed in PBS and incubated for 30 min with secondary biotinylated rabbit anti-mouse antibody (E0354-DakoCytomation, Glostrup, Denmark) diluted 1:200 in PBS containing 5% of BSA. The slides were subsequently washed in PBS and incubated for 30 min with avidin-biotin complex (Vectastain Elite ABC kit, Burlingame, CA, USA) according to manufacturer's recommendations. Staining was performed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) containing 0.02% hydrogen peroxide. Counter staining of the nucleus was done with Mayer's hematoxylin. Monoclonal antibodies used in this study, their specificity and their references are listed in **Table 1**.

Table 1: Specificity of monoclonal antibodies used for immunohistochemistry.

MoAbs	Isotype	Dilution	Antigen and reference
TKH2	IgG1	1:20	STn (Kjeldsen *et al.,* 1988)
3C9	IgM	1:50	T (Bohm *et al.,* 1997)
CLH2	IgG2	1:750	MUC5AC (Reis *et al.,* 1997)
PMH1	IgM	1:40	MUC2 GalNAc (Reis *et al.,* 1998)

Sera sample collection and protein equalization by combinatorial peptide ligand library

Sera sample from the same individuals as the biopsies were pooled according to the clinical data (without gastric lesions and *H. pylori* infection, gastritis, complete and incomplete IM). Equalization of the amount of proteins within each serum sample group was done using a combinatorial peptide ligand library – CPLL (ProteoMiner, BioRad, CA) (Thulasiraman *et al.,* 2005) according to manufacturer's recommendations.

2D gel electrophoresis

Equalized protein samples from CPLL were precipitated (ProteoExtract, Calbiochem), resuspended in rehydration buffer (7M Urea, 2M Thiourea, 4% (v/v) CHAPS and 0.0002% Bromophenol Blue) with 0.2% of ampholyte and quantified (2D Quant Kit from GE Healthcare). Passive rehydration of the strips was performed overnight with 100µg of sample using IPG strips of pH 3-10 NL (ReadyStrip; 0.5 x3 x70 mm, Bio-Rad, Hercules, CA) at room temperature. Isoelectric focusing was performed on Protean IEF cell (Bio-Rad) with an initial voltage of 250 V for 15 min, and then by applying a voltage gradient up to 4000V with limiting current of 50 µA per strip and temperature set at 20°C. The first dimension was concluded at 14-20 kVh.

Following the isoelectric focusing proteins were reduced and alkylated by incubation with 2% DL-dithiothreitol (DTT) followed by 2.5% Iodoacetamide in an equilibration buffer (6M Urea, 2% SDS, 0.002% Bromophenol Blue, 75mM Tris pH 8.8, 29.3% Glycerol) for 10 min each under gentle agitation. The strips were then packed in a 1% low gelling (1% agarose in running buffer - 25 mM Tris, 192 mM Glycine, and 0.1% (w/v) SDS, pH 8.3; Bio-Rad) on top of a 10% acrylamide gel (acrylamide/bisacrylamide 37.5:1, 2.6% from Bio-Rad). Second dimension electrophoresis was performed in a Mini-Protean tetra cell system (Bio-Rad) using 1xTris/Glycine/SDS buffer at constant voltage of 125 V.

Western blot analysis

Gels were transferred to nitrocellulose membranes (Amersham) in a semi-dry system according to manufacturer's recommendations (TE 77 PWR Amersham) and were blocked with 5% BSA in PBS-Tween (PBS-T) 0.05%. Primary antibodies were incubated overnight at 4°C with 5% BSA in PBS-T 0.05%. Membranes were washed three times with PBS-T 0.05% before secondary antibody incubation. Secondary antibodies were isotype specific (Jackson immunoresearch) anti-IgM (dilution 1:100000) and anti-IgG1 (dilution 1:50000) and were incubated for 1 h in 1% BSA in PBS-T 0.05%. Signal detection was obtained by enhanced chemiluminescence (ECL plus, Amersham). The primary antibodies were the same as those used for immunohistochemistry (**Table 1**) and used undiluted.

Protein selection and in-gel tryptic digestion.

2D gels were stained with Coomassie Blue (Bio-Safe Coomassie from Bio-Rad, CA) overnight and images were acquired with a Gel Doc XR system (Bio-Rad, CA).

The spots highlighted in the Western blots were matched in the Coomassie Blue gels and proteins excised with a spotpicker (OneTouch 2D gel spotpicker, 1.5 mm diameter, Gel Company, USA). The selected protein spots were then processed for MALDI MS analysis in agreement with the trypsin manufacturer instructions (Promega, USA): the protein gel plugs were washed with water, destained with methanol:50 mM NH_4HCO_3 and acetonitrile:50 mM NH_4HCO_3 (1:1 v/v each), reduced with 25 mM DTT in 50 mM NH_4HCO_3 for 20 min at 56°C, alkylated with 55 mM IAA in 50 mM NH_4HCO_3 for 20 min in the dark, in gel digested with 10 µL of 2 ng/µL trypsin for 3 h at 37°C in presence of 0.01% surfactant (ProteaseMAX, Promega) and the resulting peptides were extracted with 20 µL of TFA 2.5% for 15 min.

Protein identification by MALDI-TOF/TOF

Protein digests were desalted, concentrated and spotted onto a MALDI plate using ZipTips (Millipore, USA) following the manufacturer's instructions. For the matrix preparation, a solution of 6-8 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA was used. Samples were analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (AB SCIEX, Framingham, MA). Peptide mass fingerprint (PMF) data were collected in positive MS reflector mode in the range of *m/z* 700–4000 and was calibrated with external standards and internally calibrated using trypsin autolysis peaks. If necessary, several of the highest intensity non-tryptic peaks were selected for MS/MS analysis. The MS and MS/MS spectra were processed and analyzed using the software

GPS Explorer (Version 3.6, AB SCIEX, Framingham, MA), and were searched together against the UniProt (release 2012_09) protein sequence database using the Mascot search engine (Version 2.1.04, Matrix Science, UK) limited to *Homo sapiens* taxonomy. The search included 65 peaks with a signal-to-noise ratio greater than 10 and allowed for up to two missed trypsin cleavage sites. The MS tolerance was 50 ppm for PMF analysis and 1.0 Da for MS/MS analysis; fixed modifications, carbamidomethylation of cysteine; variable modifications, oxidation of methionin; keratins were filtered out. To be considered a match, a confidence interval (CI), calculated by the AB SCIEX GPS Explorer/Mascot software, of at least 99% was required.

Data mining for glycosylation sites in glycoproteins

N-Glycosylation in human proteins was predicted by the NetNglyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc), an artificial neural network that examines the sequence context of Asn-Xaa-Ser/Thr (where Xaa is not Pro) sequences. *O*-glycosylation was predicted by NetOglyc 3.1 server (http:// www.cbs.dtu.dk/services/NetOGlyc) that produces neural network predictions of mucin- type GalNAc *O*-glycosylation sites in mammalian proteins (Julenius *et al.,* 2005).

Plasminogen sialoglycopeptides enrichment and characterization

Plasminogen spots from the centre of a 2D gel from each clinical situation were excised and processed as described above. For the enrichment of plasminogen sialoglycopeptides the plasminogen peptide extract was subjected to titanium dioxide chromatography (Larsen *et al.,* 2007) as described by manufacturer (GE Healthcare, USA). The sialoglycopeptides were eluted with the MALDI matrix 2',4',6'-Trihydroxyacetophenone monohydrate (THAP). The mass spectra acquisition was performed in linear positive mode in the instrument MALDI-TOF/TOF 4700 Proteomics Analyzer (AB SCIEX, USA). In order to search for sialoglycopeptides the mass spectra raw data was submitted to the software Glycomod (http://web.expasy.org/glycomod/) and MSBridge, ProteinProspector (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msbridgestandard). The mass error tolerance was 0.4 Da. Mass spectra were internally calibrated by mass plasminogen sialoglycoforms previously described (Hayes *et al.,* 1979a; Hayes *et al.,* 1979b; Hortin 1990; Pirie-Shepherd *et al.,* 1997).

In gel *O*-deglycosylation and permethylation

Enrichment of plasminogen from serum samples of individuals without gastric lesions and *H. pylori* infection, gastritis, complete and incomplete IM cases were performed using Lysine-sepharose affinity chromatography (Deutsch *et al.,* 1970). The enriched plasminogen samples were run in SDS-PAGE gel and stained with Coomassie Blue. The plasminogen bands were removed from SDS-PAGE gels and the protein identity was confirmed by MALDI MS. Equivalent amounts of plasminogen previously digested with neuraminidase from *Clostridium perfringens* (Sigma-Aldrich; Karlsruhe, Germany) were used as a control.

Plasminogen was then in-gel de-*O*-glycosylated by reductive β-elimination upon incubation with 50 mM NaOH and 1 M $NaBH_4$ at 45°C for 16 h. The reaction was stopped with glacial acetic acid until no fizzing was observed and the samples were subsequently filtered using 10-kDa molecular weight cutoff (MWCO; Millipore). The filtrate, containing low molecular weight peptides, *O*-glycans and borate salts, was recovered and incubated several times with methanol containing 5% (v/v) acetic acid under a stream of nitrogen to remove borates as methyl esters.

The *O*-glycans enriched fractions were then permethylated adopting a modification of the method by Ciucanu and Kerek (Ciucanu *et al.,* 1984). Briefly, the samples were dissolved in 100 μL of anhydrous DMSO, and powdered NaOH was added. The mixture was sonicated for 30 min and frozen prior to the addition of 100 μL of CH_3I and then incubated under mild stirring for 1 h. The permethylated samples were recovered from the reaction mixture by extraction with dichloromethane and extensively washed with acidified water (pH 2.0) to avoid base-induced hydrolysis of the carboxymethyl group of permethylated sialic acids. The samples were then de-salted using Dowex ion-exchange resin (Dowex 50W-X8, Dow, USA).

Nano-HPLC-MALDI-TOF/TOF

The permethylated samples were separated in a nano-HPLC Ultimate 3000 system (Dionex, Amsterdam) equipped with a capillary column (Pepmap100 C18; 3 μm particle size, 0.75 μm internal diameter, 15 cm in length). The samples were dissolved in 5% acetonitrile (ACN) aqueous solution containing 0.1% formic acid (phase A). The separation was performed using a linear gradient of 32-50 % B for 45 min, 50-70 % B for 10 min and 70-32% A for 5 min. The eluted glycans were applied directly on a MALDI plate in 10 sec fractions using an automatic fraction collector Probot (Dionex, Amsterdam, Netherlands) under a continuous flow rate of 270 nL of DHB matrix solution (10 mg/mL in 70% acetonitrile/0.1% TFA and internal standard Glu-Fib at

15ftmol). Mass spectra were obtained on a MALDI TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode and obtained in the mass range from 600-4500 Da with 1200 laser shots. For the experiment, Glu-Fib was used for internal calibrations. The MALDI-MS data from each chromatographic run was combined into a three dimensional data array (LC fraction, *m/z*, total ionic current). A survey of plausible analytical signals at 691.36 $[STn+Na]^+$, was performed by determining the most prominent peaks occurring within 0.2 Da of the reference peak. The analysis of the distribution of the STn MS signals allowed the identification of chromatographic profiles. MS^2 were acquired under high-energy collisional ionization dissociation (CID) conditions. Peak assignments MS^2 spectra and database searches were performed using the GlycoWorkBench platform (Ceroni *et al.,* 2008).

RESULTS

The proteomic strategy applied in this study is schematically represented in **Figure 2**.

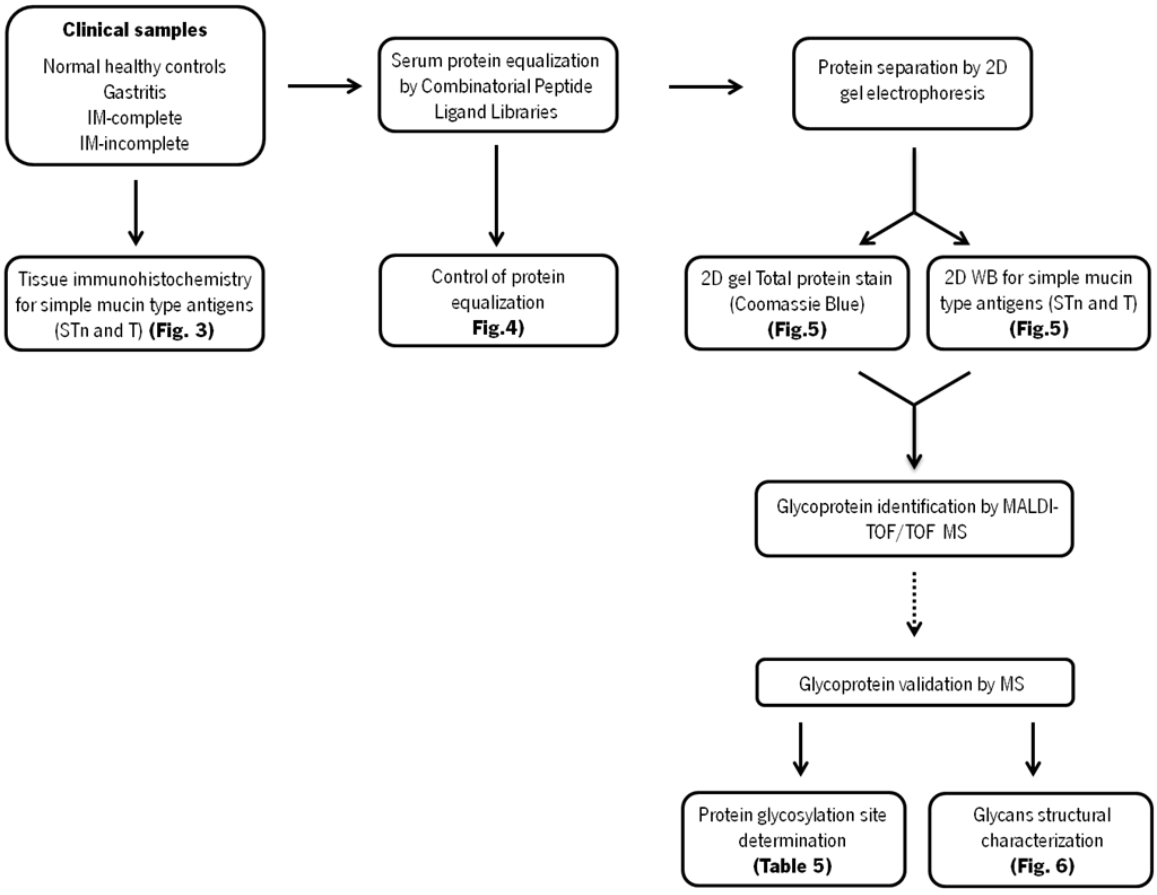

Figure 2: Schematic representation of the proteomic strategy applied in this study.

Expression of aberrant simple mucin-type carbohydrate antigens in gastric tissues

The pattern of expression of STn and T antigens was assessed in normal gastric mucosa, in gastric mucosa with gastritis, and in IM (complete and incomplete types). The pattern of mucin expression (MUC5AC *and* MUC2) allowed the classification of IM in the gastric mucosa as previously described (Reis *et al.,* 1999). **Table 2** show the overall results of STn and T antigen expression and **Figure 3** displays representative cases.

Table 2: In situ analysis of mucins and simple mucin-type carbohydrate antigens by immunohistochemistry in gastric tissues.

Cases	***Immunostaining***			
	MUC2	**MUC5AC**	**STn**	**T**
Control (n=5)	0	5	0	1
Gastritis (n=6)	0	6	0	1
Complete intestinal metaplasia (n=4)	4	0	4	1
Incomplete intestinal metaplasia (n=3)	3	3	3	0

Normal gastric mucosa showed absence of expression of STn and T antigens, with the exception of a single case that displayed a faint staining for T antigen in few cells. As expected, mucin expression in normal mucosa was limited to MUC5AC detection in the foveolar superficial epithelium of the gastric mucosa. No expression of MUC2 was observed in normal gastric mucosa (**Table 2**, **Figure 3**).

Gastric mucosa displaying gastritis showed no expression of STn but staining for the T antigen was observed in one case (**Table 2**, **Figure 3**). Mucosa with gastritis showed expression of MUC5AC with a strong cytoplasmic staining detected in every case. Mucosa with gastritis showed absence of MUC2 expression (**Table 2**, **Figure 3**).

Among the seven cases with IM, three showed a mixed expression of MUC5AC and MUC2 characteristic of the incomplete type of IM. Four cases showed only positive staining for MUC2 consistent with the complete type of IM. Both types of IM showed high levels of STn antigen expression detected mostly in the vacuole of goblet cells (**Table 2**, **Figure 3**) of the metaplastic glands. Absence of expression of T antigen was observed in IM with the exception of one case of the complete type that displayed expression of T antigen in few cells (**Table 2**).

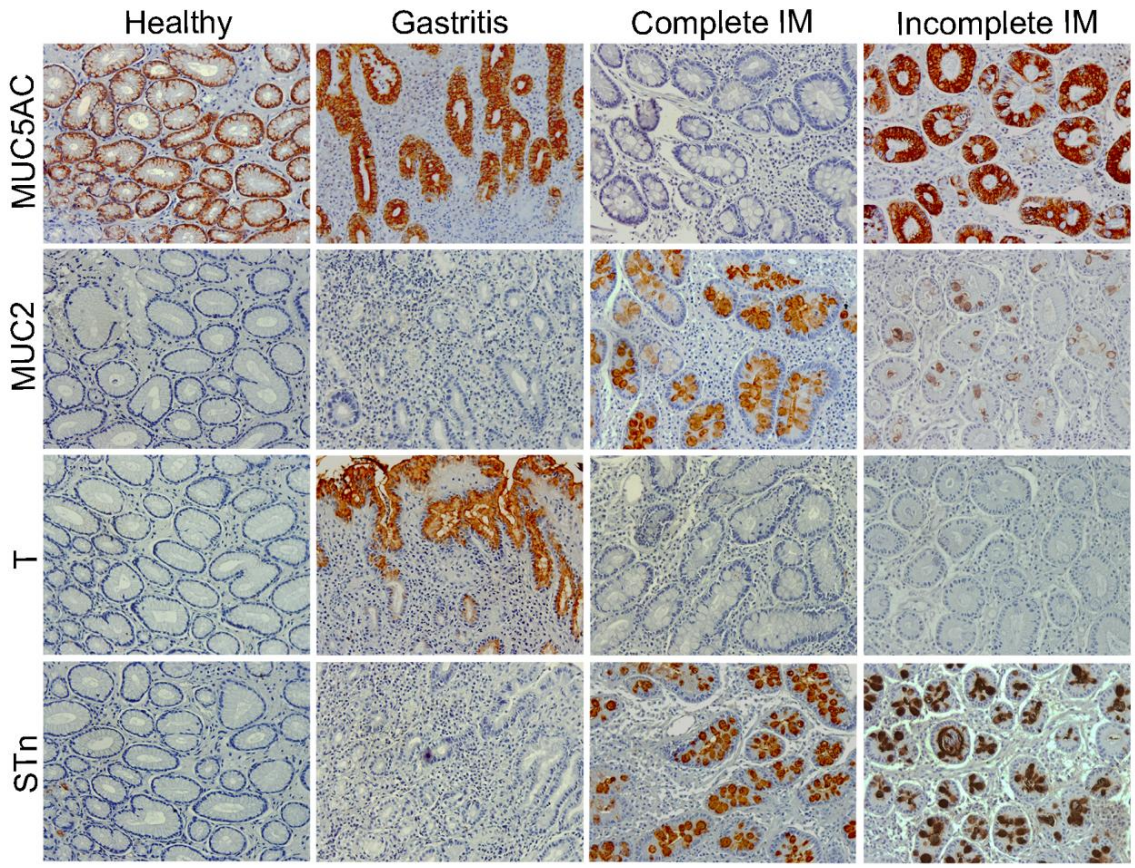

Figure 3: Immunohistochemical staining of normal mucosa and gastric lesions with MUC5AC, MUC2 and the truncated glycoforms T and STn antigens. The figure shows normal expression of MUC5AC and absence of expression of MUC2 in mucosa from healthy individuals, as well as lack of truncated glycoforms (except one normal case that also expressed T antigen – Table 2, not shown in Figure). In gastric lesions we see the presence of truncated glycans (although T antigen was also seen in only one case – Table 2 and Figure 3), and also *de novo* expression of MUC2 in intestinal metaplasia.

Serum protein equalization by combinatory peptide ligand library (CPLL)

Serum is a highly complex biofluid comprehending proteins spanning a wide range of dynamic concentrations (Anderson *et al.,* 2002). In addition, a few proteins account for approximately 70-90% of the overall proteome, which is known to mask subtle alterations associated with pathological events (Chan *et al.,* 2004). To overcome this limitation, the proteome of each clinical group was equalized by CPLL. This proteome equalization resulted in the gel electrophoretic profile presented in **Figure 4**, which displays the bound and unbound fractions of the CPLL. The equalized fractions of the different clinical groups showed a normalized amount of proteins characterized by an increased ratio of low abundant proteins and a decreased ratio of high abundant proteins (**Figure 4**).

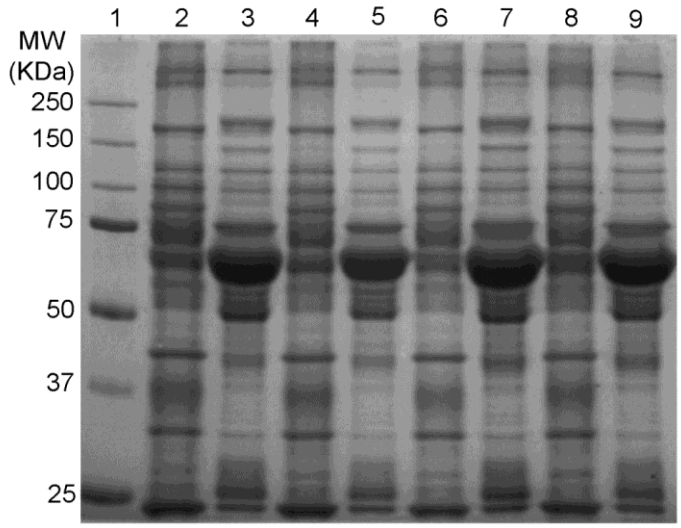

Figure 4: SDS-PAGE of sera before and after CPLL treatment. In the gel are represented serum samples of the four different groups before and after protein equalization using the CPLL technique. Lanes 2, 4, 6 and 8 represent the eluted sample of the ligand library for healthy, gastritis, complete and incomplete intestinal metaplasia sera, respectively. Lanes 3, 5, 7 and 9 represent the unbound fraction of the same samples in the same order. Lane one shows molecular weight standards.

Serum protein separation by two-dimensional gel electrophoresis and simple carbohydrate antigen detection

To identify proteins in the serum displaying simple mucin type carbohydrate antigens a glycoproteomic analysis was performed. This approach included protein separation by two-dimensional (2D) gel electrophoresis combined with a Western blotting directed for the detection of simple mucin type carbohydrate antigens using specific monoclonal antibodies.

The equalized serum protein samples from the different clinical groups were subjected to two-dimensional gel electrophoresis with a first dimension separation according to the protein isoelectric point and a second dimension based on the protein molecular weight. The patterns of protein distribution in 2D gels were similar among the different clinical groups as revealed by Coomassie Blue staining of the gels (**Figure 5**, left panel). These protein maps showed profiles compatible with a good resolution separation of protein isoforms. 2D gels replicates of the same clinical groups that were immunoblotted for simple mucin type carbohydrate antigens showed reactivity for T and STn antigens. This immunoreactivity was restricted to few proteins in the proteomic map (**Figure 5**, center and right panels).

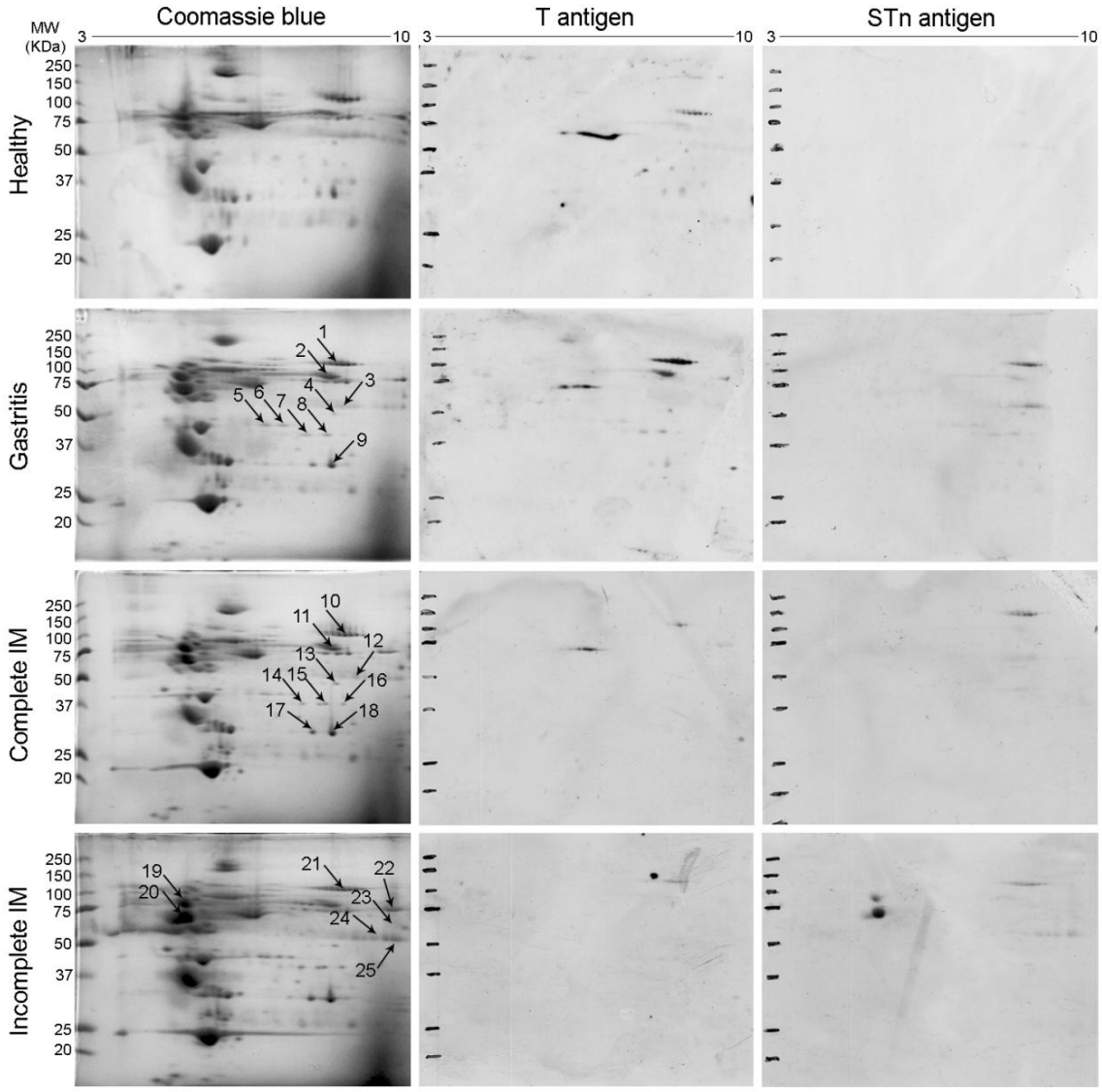

Figure 5: 2D gel electrophoresis and Western blot analysis for *T* and *STn* antigens of serum from healthy individuals, individuals with gastritis and individuals with intestinal metaplasia (complete and incomplete type). In the left side of the figure are represented coomassie blue gels of serum samples equalized with CPLL and in the middle and right sides are represented Western blots against T and STn antigens respectively. The spots that were highlighted in the Western blots were matched on Coomassie blue gels and excised for protein identification by MALDI-TOF TOF analysis.

The 2D immunobloting revealed that the immunoreactivity for the T antigen was higher in the gastritis group with lower detection in IM groups. Furthermore, STn antigen immunoreactivity showed to be higher in proteins from the 2D gel maps of the IM groups and in gastritis when compared with normal control group (**Figure 5**, right panel).

Protein identification by MALDI-TOF/TOF mass spectrometry

Identification of the proteins that were labeled in Western blots for simple mucin type carbohydrate antigens was obtained using the excised spots from the Coomassie Blue stained 2D

gel (**Figure 5**). **Table 3** shows the list of identified proteins by MALDI-TOF/TOF mass spectrometry. Proteins identified due to T antigen detection in samples from gastritis patients were plasminogen (Spot ID 1) and Histidine-rich glycoprotein (Spot ID 2). Plasminogen was also identified based on T antigen immunoreactivity in both types of IM (Spot ID 10 and 21). Proteins identified according to the immunoreactivity with STn antigen included plasminogen which was detected in all disease groups (Spot ID 1, 10, 21). Vitronectin was also identified based on STn immunoreactivity in incomplete IM (Spot ID 19 and 20). Other proteins indentified in the 2D maps showing immunoreactivity with STn are displayed in **Table 3**.

Table 3: Proteins identified in sera of gastritis, complete intestinal metaplasia and incomplete intestinal metaplasia according to the immunoreactivity with antibodies for T and STn antigens.

Spot ID	*Protein description*	*Accession number*	*MASCOT Protein C.I. %*	*Peptide count*	*% cov*	*Peaks matched*	*MOWSE score*
1	Plasminogen	PLMN_HUMAN	100	44	58	49	454
2	Histidine-rich glycoprotein	HRG_HUMAN	100	21	36	21	156
3	IGH protein	Q6GMX6_HUMAN	99.8	9	22	9	79
4	Complement factor H	F8WDX4_HUMAN	100	18	44	18	133
5	Complement factor H-related protein 1	FHR1_HUMAN	100	13	40	14	95
6	Complement factor H-related protein 1	FHR1_HUMAN	99.4	7	21	9	66
7	Complement factor H-related protein 1	FHR1_HUMAN	99.3	10	30	11	74
8	Complement factor H-related protein 1	FHR1_HUMAN	99.4	11	37	12	74
9	Complement C4 gamma chain	B4DDH0_HUMAN	100	17	34	18	105
10	Plasminogen	PLMN_HUMAN	100	43	59	49	442
11	Histidine-rich glycoprotein	HRG_HUMAN	100	22	40	22	169
12	IGH protein	Q6GMX6_HUMAN	99.1	11	27	11	72
13	Complement factor H	CFAH_HUMAN	100	27	65	32	262
14	Complement factor H-related protein 1	FHR1_HUMAN	100	14	43	15	107
15	Complement factor H-related protein 1	FHR1_HUMAN	100	15	42	17	119
16	Complement factor H-related protein 1	FHR1_HUMAN	100	15	37	16	118
17	Complement C4-B gamma chain	B4DDH0_HUMAN	99.8	15	25	15	79
18	Complement C4-B gamma chain	B4DDH0_HUMAN	100	20	31	22	133
19	Vitronectin	VTNC_HUMAN	100	17	36	19	121
20	Vitronectin	VTNC_HUMAN	99.8	13	30	15	79
21	Plasminogen	PLMN_HUMAN	100	42	54	48	424
22	Complement C4-B	B4DIE5_HUMAN	100	25	30	25	170
23	Complement C4-B	B4DIE5_HUMAN	100	10	13	10	86
24	IGH protein	Q6GMX6_HUMAN	99.9	9	24	9	83
25	IGH protein	Q6GMX6_HUMAN	100	10	17	11	106

Glycosylation characterization of the identified proteins

Table 4 depicts the simple mucin type carbohydrate antigens detected by Western blotting in the identified proteins from the sera in the different clinical groups, as well as the bioinformatical prediction of glycosylation in these proteins. Additionally, **Table 4** shows available information on the *N*- and *O*- glycosylation of these proteins previously described, as well as the sites of *O*-glycosylation and *N*-glycosylation predicted bioinformaticaly by NetOGlyc and NetNGlyc, respectively.

We observed expression of T antigen in plasminogen from all clinical groups and a strong STn immunoreactivity in gastritis and IM. Conversely, the expression of T antigens was considerably decreased in the plasminogen from patients with IM. The sites of *O*-glycosylation known to date for plasminogen include Thr346 (Hayes *et al.,* 1979b), Ser248 (Pirie-Shepherd *et al.,* 1997), and Ser339 (Hortin 1990), as well as *N*-glycosylation in Asn289 (Hayes *et al.,* 1979a). NetOGlyc (**Table 4**) has further highlighted Thr290, Thr359, and Thr365 as putative *O*-glycosylation sites.

Table 4: Glycosylation information of the identified proteins.

Protein	Glycosylation*		Predicted glycosylation		T and STn expression in serum by Western blot			
	O-linked	N-linked	Net -O-Glyc	Net -N-Glyc	Healthy	Gastritis	Complete IM	Incomplete IM
Plasminogen	Ser^{248} Ser^{339} Thr^{346}	Asn^{289}	Thr^{171} Thr^{340} Thr^{346}	No	T	T/STn	T/STn	T/STn
Vitronectin	No	Asn^{86} Asn^{169} Asn^{242}	Thr^{113} Ser^{137} Thr^{141}	Asn^{86} Asn^{169}	-	-	-	STn
Complement factor H	No	Asn^{511} Asn^{700} Asn^{784} Asn^{804} Asn^{864} Asn^{893} Asn^{1011} Asn^{1077}	No	Asn^{529} Asn^{882} Asn^{1029} Asn^{1095}	-	STn	STn	STn
Histidine-rich glycoprotein	No	Asn^{45} Asn^{69} Asn^{107} Asn^{184} Asn^{326} Asn^{327}	Ser^{307}	Asn^{63} Asn^{125} Asn^{344}	-	T	-	-

* Glycosylation sites reported in previous studies.

Vitronectin, which has been described until now as an *N*-glycosylated protein (Ogawa *et al.,* 1995), was also found among the proteins showing simple mucin-type *O*-glycans immunoreactivity. Still, its expression was restricted to STn in the context of incomplete IM. In agreement with these observations, NetOGlyc has also predicted possible *O*-glycosylation sites for Vitronectin.

Additionally, we observed STn antigen reactivity in Complement factor H in sera from gastritis and IM. No sites of *O*-glycosylation have been described but at least eight sites of *N*-glycosylation have been shown in Complement factor H (Fenaille *et al.,* 2007). Histidine-rich glycoprotein was identified as displaying T antigen reactivity. No sites of *O*-glycosylation have been described but at least six sites of N-glycosylation have been described in this protein (Jones *et al.,* 2005) (**Table 4**). Noteworthy, despite the absence of reports of *O*-glycosylation in Histidine-rich glycoprotein, theoretically this protein can carry this type of post-translational modification as predicted by NetOGlyc.

In summary, among the proteins found carrying simple mucin type carbohydrate antigens, plasminogen showed the most differentiated pattern of *O*-glycosylation for IM. Namely, it presented decreased levels of T antigen accompanied by the abnormal overexpression of STn.

Structural characterization of STn antigen from Plasminogen and glycosylation site determination

Structural analysis was performed in order to validate immunoblotting assignments regarding the expression of STn in plasminogen. The protein purified by affinity chromatography using Lysine-sepharose, further separated by SDS-PAGE electrophoresis was chemically de-*O*-glycosylated in gel. The released *O*-glycans from plasminogen were then permethylated to avoid desialylation by MALDI in-source and metastable decay and analyzed by MALDI-TOF/TOF. This allowed the distinction of a chromatographic profile for the ion at *m/z* 691.36 corresponding to the sodium adduct of STn antigen (**Figure 6A** and **B**). This assignment was further supported by the absence of the signal upon desialylation of plasminogen with a α-neuraminidase. Moreover, the product ion spectrum exhibited the ions at *m/z* 107.1 resulting from $B_1^{0,2}X_1$ fragmentations, at *m/z* 358.1 from $C_1^{1,3}X_1$, and at *m/z* 416.2 from $^{2,5}X_1$ or C_1 (nomenclature according to Domon and Costello (Domon *et al.,* 1988) (**Figure 6C**), therefore confirming the presence of STn in plasminogen. The prevalence of cross-ring fragmentations are in keeping with previous observations for high CID conditions (Zaia 2004). In agreement with previous reports concerning the *O*-glycosylation of plasminogen (Hayes *et al.,* 1979b; Hortin 1990; Pirie-Shepherd *et al.,* 1997), low abundant ions belonging to mono- (*m/z* 895.5) and di-sialylated T (*m/z* 1256.6) antigens have also been detected (data not shown).

In order to characterize the plasminogen glycosylation sites containing STn, sialoglycopeptides from plasminogen of the different clinical groups were enriched by titanium dioxide chromatography (Larsen *et al.,* 2007) as described in materials and methods. The methodology was optimized for human plasminogen using a commercial plasminogen sample. Using this method we have detected the three *O*-glycosylation sites and one *N*-glycosylation site previously described in human plasminogen (Hayes *et al.,* 1979a; Hayes *et al.,* 1979b; Hortin 1990; Pirie-Shepherd *et al.,* 1997) (**Supplementary Table 1** and **Supplementary Figure 1**). Using this approach we found one STn-containing glycopeptide in healthy controls, five in gastritis, four in complete IM and eight in incomplete IM (**Table 5**).

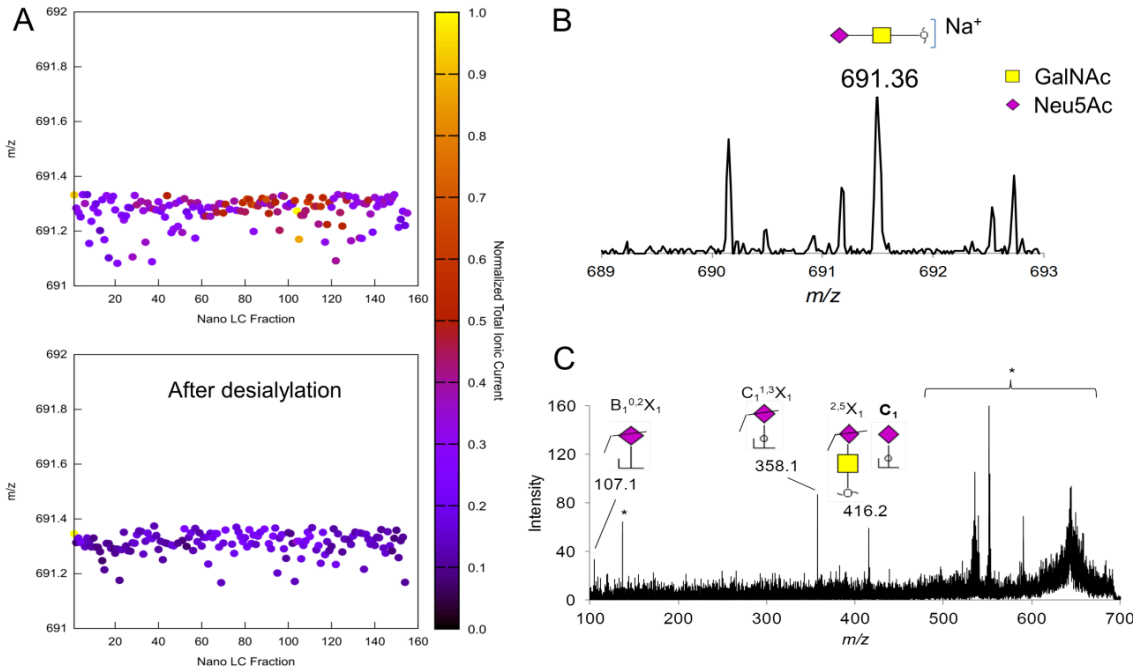

Figure 6: Positive mode MALDI-TOF/TOF identification of permethylated STn in serum plasminogen isolated from patients with intestinal metaplasia. A) Relative Ionic Current of nano-HPLC fractions for the ion at *m/z* 691.36 corresponding to the monoisotopic mass of $[STn+Na]^+$, before and after desialylation. The analytical signals in each plot were normalized to allow the comparison of both sets of data. A chromatographic envelop was observed in the samples for fractions 80-100, that is absent after desialylation. B) MS spectrum showing the ion at *m/z* 691.36 corresponding to the monoisotopic mass of $[STn+Na]^+$. C) MS^2 spectrum of the ion at *m/z* 691.36 highlighting reporter ions resulting from combined sialic acid cross-ring fragmentations (X_1) and glycosidic bound cleavages B1 and C1 fragments (nomenclature according to Domon *et al.,* 1988) and symbology adapted from Glycoworkbench. "*" overlapping signals resulting from cross-ring fragmentations at the sialic moiety, and/or at the reduced GalNAc residue, and/or the loss of methoxy groups (Morelle *et al.,* 2004), induced by high CID conditions (Zaia 2004).

Table 5: Characterization of serum plasminogen[1] sialoglycopeptides.

	Experimental mass (Da)	Theoretical mass (Da)	Number of STn glycoforms	Glycoform Mass	Tryptic missed cleavages	Sialoglycopeptides[2]
Control	2782.59	2782.80	3	1483.36	0	494-504 HSIFTPETNPR
	3189.28	3189.15	4	1977.81	0	108-117 WSSTSPHRPR
Gastritis	4656.01	4656.00	1	494.45	0	330-367IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR
	2382.30	2382.46	2	988.91	0	379-389 C*QSWSSM§TPHR
	2782.40	2782.80	3	1483.36	0	494-504 HSIFTPETNPR
	2319.88	2319.56	1	494.45	2	777-791 VSRFVTWIEGVM§RNN
	2352.49	2352.68	1	494.45	2	71-85 DVVLFEKKVYLSEC*K
Complete IM	2783.07	2782.80	3	1483.36	0	494-504 HSIFTPETNPR
	855.63	855.88	1	494.45	0	777-779 VSR
	2814.02	2814.02	2	988.91	2	777-791 VSRFVTWIEGVM§RNN
	1528.63	1528.54	2	988.91	0	94-98 GTM§SK
	3189.50	3189.15	4	1977.81	0	108-117 WSSTSPHRPR
	4656.33	4656.00	1	494.45	0	330-367IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR
Incomplete IM	2382.46	2382.46	2	988.91	0	379-389 C*QSWSSM§TPHR
	2782.59	2782.80	3	1483.36	0	494-504 HSIFTPETNPR
	4723.11	4722.93	3	1483.36	0	720-750 VQSTELC*AGHLAGGTDSC*QGDSGGPLVC*FEK
	3187.30	3187.62	1	494.45	0	753-776 YILQGVTSWGLGC*ARPNKPGVYVR
	855.49	855.87	1	494.45	0	777-779 VSR

1: Plasminogen aminoacid numbering excludes the 19 amino acid signal peptide.
2: C* - carbamidomethylation of cysteine; M§ = methionine oxidation

DISCUSSION

Alterations of glycosylation are commonly observed in pathological conditions, including gastric cancer (David *et al.,* 1992; Amado *et al.,* 1998), constituting a major source of biomarkers (Reis *et al.,* 2010). These changes in glycosylation are also observed during the process of gastric carcinogenesis, such as in gastritis and in IM, a precursor lesion of gastric carcinoma characterized by the expression of simple mucin type carbohydrate antigens, such as T and STn (David *et al.,* 1992; Ferreira *et al.,* 2006; Conze *et al.,* 2010; Marcos *et al.,* 2011).

Serum remains the ideal biofluid for biomarker identification due to the easy collection and because it frequently displays proteins expressed by pathological tissues. The present study identified serum proteins displaying altered *O*-glycosylation as determined by the expression of the antigens T and STn in patients with gastric pathologies, such as gastritis, IM of the complete and incomplete types, as compared with control individuals without any gastric mucosa lesion.

In order to perform this glycoproteomic analysis we applied an equalizing tool for serum protein content. The CPLL (Thulasiraman *et al.,* 2005) equalized sera from the different clinical groups showed a normalized amount of proteins characterized by an increased ratio of low abundant proteins and a decreased ratio of high abundant proteins. This tool showed to be quite efficient in avoiding the overrepresentation of serum albumin, immunoglobulins, as well as the other 20 most abundant serum proteins (Farrah *et al.,* 2011). The search for serum proteins bearing T and STn antigens was performed using a combination of 2-D gel electrophoresis for protein separation and further detection by Western blotting of T and STn using specific monoclonal antibodies. This strategy directed this serum *O*-glycoproteome search for the protein targets displaying altered simple mucin type carbohydrate antigens T and STn in the different clinical groups. The first dimension of the 2D gel electrophoresis using separation gradients of pH 3-10 and pH 4-7 were applied in the present study, however the pH 3-10 gradient allowed better resolution of proteins localized in the upper limit of the gradient and showing immunoreactivity with the monoclonal antibodies against the STn and T glycan antigens.

The patterns of total protein distribution in 2D gel electrophoresis were similar among the different clinical groups, with protein maps showing good resolution separation of various protein isoforms (**Figure 5**, left panel). The Western blotting analysis showed T and STn antigens detection in few proteins (**Figure 5**, center and right panel) of the different clinical groups. T antigen detection was observed in all groups with higher reactivity observed in gastritis whereas STn antigen detection was observed in gastritis, and in complete and incomplete IM groups.

The detection of serum proteins expressing truncated glycans may reflect the aberrant glycosylation observed in proteins expressed by pathological tissues. This is the case in most serological assays that detect circulating glycoproteins derived from malignant tumors or benign lesions (Reis *et al.,* 2010; Pan *et al.,* 2011). Our results showing the detection of truncated glycans STn and T in the glycoproteomic profiling of patients with gastritis and IM are in agreement with our previous findings that the STn and T antigens are expressed in the gastric mucosa of the patients displaying pre-neoplastic pathological conditions of the stomach (David *et al.,* 1992; Carneiro *et al.,* 1994; Ferreira *et al.,* 2006), particularly in IM, and in contrast to the absence (or almost absence) of expression of these truncated glycans in the normal gastric mucosa of control individuals.

The glycosylation modifications observed in gastric pathologic tissues may stem from altered expression of glycosyltransferases, previously shown in gastric epithelial cells induced by *H. pylori* infection (Marcos *et al.,* 2008), and particular increased expression of specific glycosyltransferases (Marcos *et al.,* 2011). The reasons underlying such glycosylation modifications are still largely unknown but hypothesis such as deregulation of the glycosylation machinery in the Golgi apparatus of the cells (Gill *et al.,* 2011), or as consequence of altered differentiation program observed in IM are being tested (Almeida *et al.,* 2003).

The proteomic mining of the serum for STn and T antigens expression in the different clinical groups tested in the present work has resulted in the identification of relatively abundant circulating proteins, namely plasminogen, vitronectin, complement factor H, and histidine-rich glycoprotein. Even though the amount of these proteins did not vary significantly between groups, the pattern of simple mucin type *O*-glycans immunoreactivity showed considerable alterations.

Plasminogen was identified due to the immunoreactivity for T and STn antigen in all disease groups, but showed no immunoreactivity in healthy controls. Interestingly, among the identified proteins carrying simple mucin type carbohydrate antigens, plasminogen showed the most differentiated pattern of *O*-glycosylation in IM, displaying decreased levels of T antigens accompanied by an increased expression of STn when compared with the other clinical groups. The detailed analysis of the *O*-glycans from purified plasminogen from IM patients was performed in order to validate the STn glycan detection at the molecular level. Plasminogen *O*-glycans released by reductive β-elimination were permethylated and analysed by MALDI mass spectrometry. Our results showed the detection of ions compatible with STn antigens (**Figure 6**). Further MALDI-MS^2 analysis of the ion at *m/z* 691.4 Da exhibited glycosidic bond cleavages B, C

and Z, and cross-ring fragmentation A and X product ions characteristic of STn glycan, therefore demonstrating the presence of STn in plasminogen from serum of incomplete IM patients.

In addition, the MALDI structural analysis of sialoglycopeptides from plasminogen enriched by titanium dioxide chromatography showed the presence of glycopeptides containing STn in all disease groups. The use of the sialoglycopeptides enrichment method by titanium dioxide followed by MALDI showed to be quite efficient with the identification of the three *O*-glycosylation sites and one *N*-glycosylation site previously described in human plasminogen (Hayes *et al.,* 1979a; Hayes *et al.,* 1979b; Hortin 1990; Pirie-Shepherd *et al.,* 1997) (**Supplementary Figure 1** and **Supplementary Table 1**). Based on this approach the analysis of the clinical groups lead to the identification of one STn-containing glycopeptide in healthy control, five STn-containing glycopeptides in gastritis, four in complete IM and eight in incomplete IM (**Table 5**). Some of the sites identified have not been described for plasminogen and may constitute potential novel biomarkers of pre-cancerous gastric lesions.

Further analysis of enriched for sialic acid containing glycoproteins from gastritis, intestinal metaplasia, and carcinoma patients confirmed STn reactivity of plasminogen in an independent set of samples (**Supplementary Figure 2** and **Supplementary Table 2**). These results using alternative glycoprotein enrichment approaches and different sample cohorts further demonstrate the potential application of the altered plasminogen STn glycosylation as a biomarker in these pathologies.

Plasminogen is released as a zymogen from the liver into circulation where it adopts a closed, activation resistant, conformation. Upon binding to blood clots, or to cell surfaces, plasminogen can adopt an open form that can be converted into active plasmin by a variety of enzymes, including tissue plasminogen activator and urokinase plasminogen activator (Miles *et al.,* 2005; Law *et al.,* 2012). Plasmin is a serine protease that acts dissolving fibrin clots and in other proteolysis functions in diverse systems. Two major glycoforms of plasminogen have been described in humans - type I plasminogen containing two glycosylation moieties (*N*-linked to Asn289 and *O*-linked to Thr346), and type II plasminogen containing a single *O*-linked sugar on Thr346 (Hayes *et al.,* 1979a; Hayes *et al.,* 1979b). However, additional sites of *O*-glycosylation of plasminogen have been reported in Ser248 (Pirie-Shepherd *et al.,* 1997), and Thr339 (Hortin 1990). Type II plasminogen has been shown to be preferentially recruited to the cell surface whereas type I plasminogen appears more readily recruited to blood clots (Takada *et al.,* 1985). Nevertheless, only one (Thr346) of these glycosylation sites could be theoretically predicted using

the bioinformatics platform NetOGlyc. On the other hand this approach retrieved other putative glycosylation sites that are still lacking *in vivo* confirmation. The discrepancy between these findings has been previous highlighted for serum proteins (Ferreira *et al.,* 2011) and suggests that *in vivo* processing and pathophysiological states may play a determinant role in the definition of the glycosylation of circulating glycoproteins.

The X-ray crystal structure analysis of closed plasminogen has revealed that *O*-glycosylation alter the position of a Kringle domain, partially explaining the functional differences observed between the type I and type II plasminogen glycoforms (Law *et al.,* 2012). In closed plasminogen, the *O*-linked sugar on Thr346 is one of the requirements for the blocking of the cleavage by tissue plasminogen activator and urokinase plasminogen activator (Law *et al.,* 2012).

Liver is the primary tissue for plasminogen synthesis (Raum *et al.,* 1980) but other tissue sources, including the gut, have been described in animal models (Zhang *et al.,* 2002). The truncated *O*-glycans observed in circulating plasminogen from gastritis and in IM patients may either reflect altered glycosylation in the liver response to inflammatory cytokines or altered glycosylation of locally expressed plasminogen. Pro-inflammatory cytokines produced within the gastric disease context (Goll *et al.,* 2007; Chang *et al.,* 2008; Haghazali *et al.,* 2011), may induce differential expression of glycosyltranferases in hepatocytes leading to alteration of glycosylation of circulating proteins (Gabay *et al.,* 1999). This is in agreement with previous studies that have shown that modification of glycosylation characterized by increased expression of sialylated glycan structures, are observed in hepatocyte derived proteins during acute and chronic inflammatory diseases (De Graaf *et al.,* 1993; Brinkman-van der Linden *et al.,* 1998; Peracaula *et al.,* 2010; Sarrats *et al.,* 2010). In addition, alterations of plasminogen may also be related with the infection by *H. pylori*. Plasminogen-binding proteins in *H. pylori* with subsequent activation to plasmin (Pantzar *et al.,* 1998; Jonsson *et al.,* 2004) may provide proteolytic capacity and may contribute for the virulence of this bacterium (Pantzar *et al.,* 1998; Jonsson *et al.,* 2004). Also, the increased expression of urokinase plasminogen activator has been described in *H. pylori*-associated gastritis (Gotz *et al.,* 1996; Goto *et al.,* 2011). These findings altogether may point towards an important role of plasminogen activation in pathological conditions of the gastric mucosa.

Vitronectin was also identified based on STn immunoreactivity in the incomplete type of IM. Vitronectin is a glycoprotein known to be glycosylated. Human vitronectin has been shown to contain N-glycans but no *O*-glycans have been described, contrary to vitronectin of other mammals that have been shown to contain both types of glycosylation (Kitagaki-Ogawa *et al.,*

1990). Furthermore, it was demonstrated that despite the homology of about 73% between human and rat vitronectin, the sites of glycosylation are highly conserved and have been shown to be important for the protein function (Sano *et al.,* 2007).

Vitronectin is a multifunctional glycoprotein produced mainly by hepatocytes that is present mostly in plasma and extracellular matrix of many tissues (Schvartz *et al.,* 1999). Vitronectin is involved in many functions such as the regulation of coagulation and fibrinolysis, cell adhesion and invasion, as well as in matrix remodeling and humoral defense mechanisms (Preissner 1991). Glycosylation has been reported to be important in the interaction of vitronectin with other molecules and on its functional activities (Yoneda *et al.,* 1998). Additionally, vitronectin oligosaccharide moiety was suggested to be relevant in *H. pylori* binding and in the mechanism of the bacterial immune escape (Ringner *et al.,* 1994; Singh *et al.,* 2010). Furthermore, vitronectin was shown to be important in the adhesion and migration of tumor-infiltrating lymphocytes (Edwards *et al.,* 2006).

Our results showed STn antigen immunoreactivity for vitronectin in the context of incomplete IM. Despite the lack of reports on vitronectin *O*-glycosylation in humans we could predict three possible *O*-glycan sites (Thr113, Ser137, Thr141) using the NetOGlyc tool. Taking into consideration the importance of vitronectin glycosylation on its function and the reported bacterial interactions (Ringner *et al.,* 1994; Singh *et al.,* 2010), our findings may point towards a possible role in the context of gastric lesions development.

The present study also indicated the histidine-rich glycoprotein as a target expressing truncated *O*-glycans, presenting reactivity with T antigen in the gastritis group. Histidine-rich glycoprotein is a plasma glycoprotein produced by the liver and known to bind to a number of ligands in circulation, such as heparin, heparan sulfate, thrombospondin, and plasminogen. Histidine-rich glycoprotein acts as an adapter protein and has been implicated in regulating many processes such as immune complex and pathogen clearance, cell adhesion, angiogenesis, coagulation and fibrinolysis (Ohta *et al.,* 2009). Even though regarded as an *N*-glycosylated protein (six described sites and three predicted) our study has found Western blotting reactivity for the T antigen in gastritis and the NetOGlyc tool identified Ser307 as a putative glycosylation site. This indicates that this type of glycosylation could also be present in Histidine-rich glycoprotein and suggests that further structural insights on the *O*-glycan moiety may be required to complement previous studies focused on the analysis of the *N*-glycans (Ohta *et al.,* 2009).

Similarly, our results showed immunoreactivity to STn antigen in all pathological conditions in proteins from the complement system, namely complement factor H, complement H-related protein and complement C4-B. The human complement pathway is a highly controlled effector mechanism of the immune system. Over 30 plasma proteins and membrane bound molecules are involved in the complement system and the most of these proteins are glycosylated. The complement factor H, complement factor H-related protein and the complement C4-B are proteins that have been described to be *N*-glycosylated (Ritchie *et al.,* 2002). Our results indicating that aberrant *O*-glycosylation can be detected in these members of the complement system warrants further structural characterization of the glycans in these proteins. Most of the protein modules that form the complement system have been crystallized and structural data provides evidence for the role of *N*-glycans in this system. However, the structure and role of glycans in the resistance to proteolysis and functionality activation within pathological conditions is still incomplete. As these data become available, the glycans can be modeled at the appropriate locations and give further insights into the interaction between complement proteins and cofactors.

Our results provide novel glycobiomarkers in serum from patients with gastric pathologies, including gastritis, intestinal metaplasia and gastric carcinoma. The present work opens new avenues for future targeted evaluation of these specific glycobiomarkers in additional immunoassay-based approaches enabling large scale individual analysis.

In summary, this work present a set of proteins displaying altered *O*-glycosylation as detected by antibodies directed to STn and T antigens in the serum from patients with gastritis and IM in opposition to minor or no reactivity in the same proteins of healthy individuals without any gastric disease. We further demonstrated that circulating serum plasminogen from IM patients carry the truncated *O*-glycan STn antigen. These results warrant further studies to address the application of plasminogen STn glycosylation pattern as a serum biomarker of gastric pathologies.

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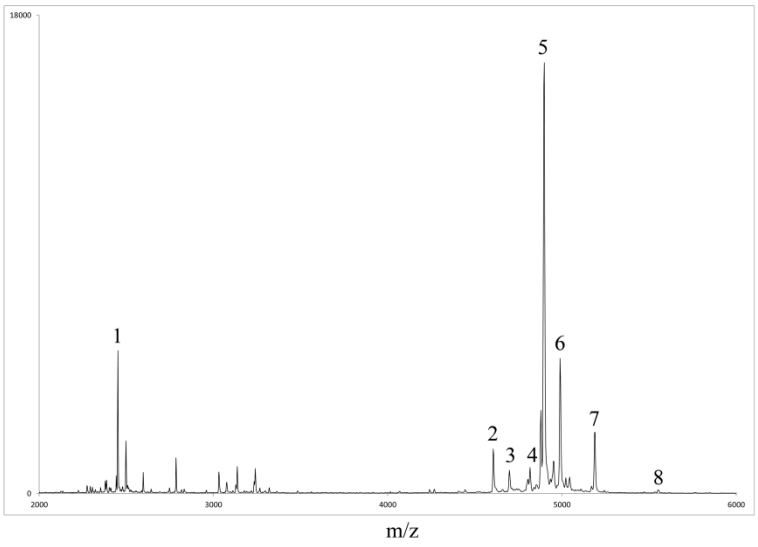
SUPPLEMENTARY DATA

Supplementary data 1 – Characterization of plasminogen glycosylation by MALDI-TOF/TOF mass spectrometry

Supplementary Table 1: List of plasminogen glycopeptides detected by MALDI-MS. Serum plasminogen from a commercial sample was subjected to tryptic digestion followed by titanium dioxide enrichment of sialoglycopeptides (described in material and methods). MALDI mass spectra were acquired in linear positive mode using THAP (2',4',6'-Trihydroxyacetophenone monohydrate) as a matrix with a mass error tolerance of 0.06 %. Different already described plasminogen glycoforms have been detected.

Peak n°	Experimental mass (Da)	Theoretical mass (Da)	Glycosylation type	Glycoform(s)	Sialoglycopeptides #	Reference
1	2451.99	2451.65	O	ST	243-258 C*TTPPPSSGPTYQC*LK(G)	(Pirie-Shepherd *et al.,* 1997)
2	4605.11	4606.87	O	T + Pi	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	
3	4697.11	4698.75	N	Hex2HexNAc2NeuAC1 + Man3GlcNAc2	266-290 GNVAVTVSGHTCQHWSAQTPHTHNR(T)	(Hayes *et al.,* 1979a)
4	4815.91	4818.15	O	ST	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	(Hayes *et al.,* 1979b; Pirie-Shepherd *et al.,* 1997)
5	4896.60	4898.13	O	ST + Pi	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	(Pirie-Shepherd *et al.,* 1997)
6	4989.98	4987.07	N	Hex2HexNAc2NeuAC2 + Man3GlcNAc2	266-290 GNVAVTVSGHTCQHWSAQTPHTHNR(T)	(Hayes *et al.,* 1979a)
7	5188.48	5189.39	O	diST + Pi	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	(Pirie-Shepherd *et al.,* 1997)
8	5554.99	5554.61	O	diST + T + Pi	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	(Hortin 1990)



Supplementary Figure 1: MALDI MS spectrum of human plasminogen after titanium dioxide enrichment. The detected sialoglycopeptides are presented in the Supplementary Table 1.

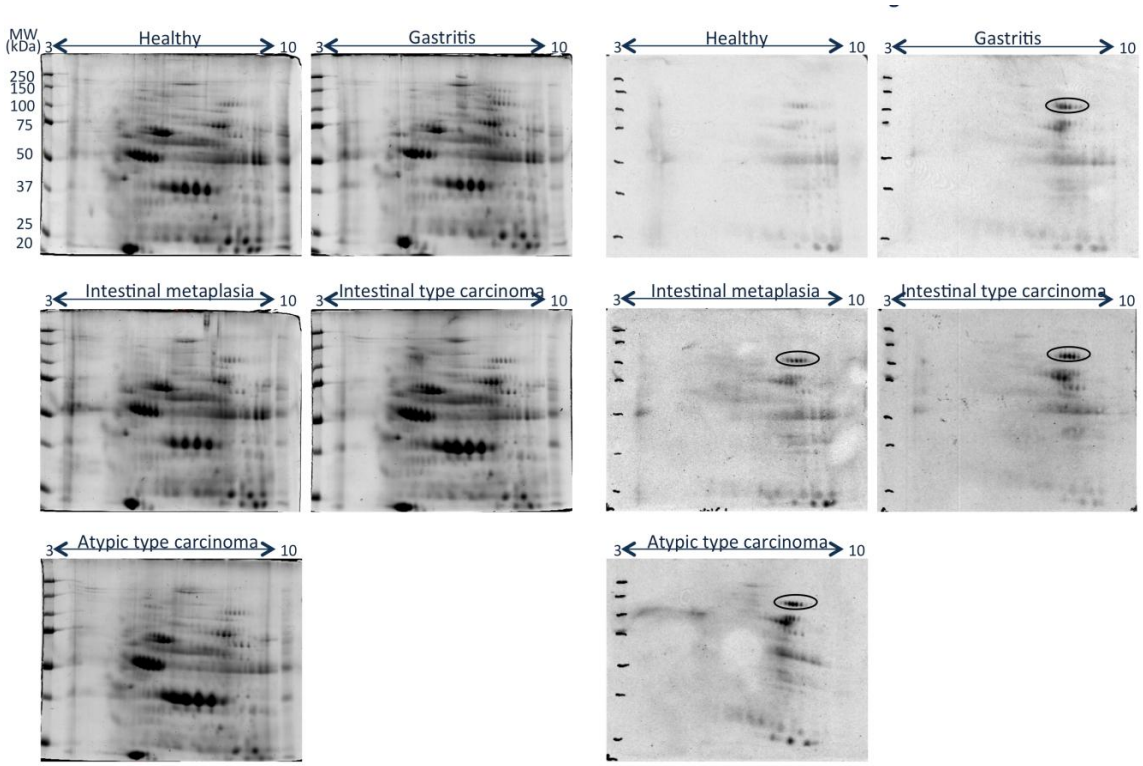
Supplementary data 2 - Identification of STn in Plasminogen from gastritis, intestinal metaplasia and gastric carcinoma of independent set of samples

Supplementary Material and methods

Sera sample collection, glycoprotein enrichment by lectin affinity chromatography and identification of STn containing proteins

A second set of sera sample from Hospital Santo António and Hospital São João were used for the identification of proteins carrying STn structures. Sera sample from ten individuals within each of the following clinical groups were

used: without gastric lesions, gastritis, intestinal metaplasia and from two types of gastric carcinoma (intestinal and atypical). Sera sample were pooled and subjected to albumin and IgG depletion by a commercial kit (ProteoPrep from Sigma Aldrich). For the enrichment of STn containing glycoproteins, depleted sera were subjected to *Sambucus nigra* agglutinin (Vector laboratories) affinity chromatography that specifically recognizes and capture α2,6 sialic acids containing glycoproteins. The affinity chromatography eluted proteins were further subjected to 2D gel electrophoresis, Western blot analysis to detect STn containing proteins, and MALDI-TOF/TOF mass spectrometry protein identification was performed as described in materials and methods of this paper.



Supplementary Figure 2: Glycoproteomic approach to reveal serum proteins carrying STn antigen. Serum samples from healthy, gastric precursor lesions (gastritis and intestinal metaplasia) and two types of gastric carcinoma (intestinal and atypical type) individuals were subjected to albumin and IgG removal and lectin affinity chromatography to capture α2,6 sialic acid containing proteins. Affinity captured proteins were separated by 2D gel electrophoresis and STn highlighted by Western blot. The results show the presence of STn containing proteins in serum from individuals with gastric percursor lesions and with gastric carcinoma, and Plasminogen was identified by MALDI-TOF/TOF as STn protein carrier. Black highlighted areas indicate selected protein dots, reactive for STn antigen, and identified as plasminogen (see Supplementary Table 2).

Supplementary Table 2: Proteins identified in sera of control, gastritis, metaplasia, intestinal type carcinoma and atypic type carcinoma according to the immunoreactivity with antibodies for STn antigen.

Spot ID	*Protein description*	*Accession number*	*MASCOT Protein C.I. %*	*Peptide count*	*% cov*	*Peaks matched*	*MOWSE score*
Control	Plasminogen	PLMN_HUMAN	100	31	32	34	243
Gastritis	Plasminogen	PLMN_HUMAN	100	32	35	32	259
Metaplasia	Plasminogen	PLMN_HUMAN	100	28	32	30	202
Intestinal Type Carcinoma	Plasminogen	PLMN_HUMAN	100	33	37	37	273
Atypic type carcinoma	Plasminogen	PLMN_HUMAN	100	30	33	34	232

3.2

Expression of ST3GAL4 Leads to SLe^x Expression and Induces c-Met Activation and an Invasive Phenotype in Gastric Carcinoma Cells

Content

3.2 Expression of ST3GAL4 leads to SLe^x expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells.

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ABSTRACT

Sialyl-Lewis X (SLe^x) is a sialylated glycan antigen expressed on the cell surface during malignant cell transformation and is associated with cancer progression and poor prognosis. The increased expression of sialylated glycans is associated with alterations in the expression of sialyltransferases.

In this study we determined the capacity of ST3GAL3 and ST3GAL4 sialyltransferases to synthesize the SLe^x antigen in MKN45 gastric carcinoma cells and evaluated the effect of SLe^x overexpression in cancer cell behavior both *in vitro* and *in vivo* using the chicken chorioallantoic membrane (CAM) model. The activation of tyrosine kinase receptors and their downstream molecular targets was also addressed.

Our results showed that the expression of ST3GAL4 in MKN45 gastric cancer cells leads to the synthesis of SLe^x antigens and to an increased invasive phenotype both *in vitro* and in the *in vivo* CAM model. Analysis of phosphorylation of tyrosine kinase receptors showed a specific increase in c-Met activation. The characterization of downstream molecular targets of c-Met activation, involved in the invasive phenotype, revealed increased phosphorylation of FAK and Src proteins and activation of Cdc42, Rac1 and RhoA GTPases. Inhibition of c-Met and Src activation abolished the observed increased cell invasive phenotype.

In conclusion, the expression of ST3GAL4 leads to SLe^x antigen expression in gastric cancer cells which in turn induces an increased invasive phenotype through the activation of c-Met, in association with Src, FAK and Cdc42, Rac1 and RhoA GTPases activation.

INTRODUCTION

Alterations in cell surface glycosylation are considered a hallmark during carcinogenesis. These alterations usually lead to the expression of tumor-associated carbohydrates on glycoproteins or glycolipids that decorate cell surfaces (Reis *et al.,* 2010). One of the most common glycan alterations is the increase of sialylated Lewis-type blood group antigens, such as sialyl Lewis A (SLe^a (NeuAcα2,3Galβ1-3[Fucα1-4]GlcNAc-R) and sialyl Lewis X (SLe^x (NeuAcα2,3Galβ1-4[Fucα1-3]GlcNAc-R)). SLe^a and SLe^x are expressed in cancer cells, mimicking their normal expression on blood cells (monocytes and neutrophils) potentiating cancer cell migration through binding to endothelial cell selectins (Varki 1994; Fuster *et al.,* 2005). Therefore, SLe^a and SLe^x overexpression are a common feature of several carcinomas (e.g., lung, colon, gastric and pancreas) and are associated with increased metastatic capacity (Fukuoka *et al.,* 1998; Kim *et al.,* 1998; Tatsumi *et al.,* 1998; Borsig *et al.,* 2002) and poor overall patients' survival (Nakamori *et al.,* 1997; Amado *et al.,* 1998; Baldus *et al.,* 1998; Nakamori *et al.,* 1999; Grabowski *et al.,* 2000).

The increased expression of sialylated glycans associated to carcinogenesis is the result of altered expression of sialyltransferases (STs) genes which encode for enzymes involved in the biosynthesis of the glycan antigens above described (Harduin-Lepers *et al.,* 2012). Up to 20 different sialyltransferases have been described to catalyse the transfer of sialic acid residues from a donor substrate CMP-sialic acid to the oligosaccharide side chain of the glycoconjugates. This sialic acid generally occupies the terminal non-reducing position on glycan chains (Harduin-Lepers *et al.,* 2005). Different STs show cell and tissue specific expression pattern and differ in substrate specificities and types of linkage formed (Harduin-Lepers *et al.,* 2005). Depending on these characteristics, STs are classified in four families - ST3Gal, ST6Gal, ST6GalNAc and ST8Sia. ST3Gal family are α2,3-STs which catalyze the transfer of sialic acid residues to terminal galactopyranosyl (Gal) residues and include six members from ST3Gal I to ST3Gal VI (Harduin-Lepers *et al.,* 2001).

Among the six ST3Gal sialyltransferases, ST3Gal III, IV and VI have been described to contribute to SLe^x formation (Kono *et al.,* 1997; Okajima *et al.,* 1999), with a substantial role attributed to ST3Gal IV (Ellies *et al.,* 2002; Sperandio *et al.,* 2006).

The sialyl-Lewis antigens are synthesized on type 1 (Gal β1,3 GlcNAc) or type 2 (Gal β1,4 GlcNAc) disaccharide sequences. The sialyltransferase ST3Gal III preferentially acts on type 1 rather than on type 2 disaccharides and is involved in the synthesis of SLe^a (Kitagawa *et al.,*

1993). ST3Gal IV mainly catalyzes the α2,3 sialylation of type 2 disaccharides, leading to the biosynthesis of SLe^x (Ellies *et al.,* 2002; Colomb *et al.,* 2012).

We previously demonstrated the contribution of different ST3Gal sialyltransferases to the synthesis of sialyl Lewis antigens in gastric carcinoma cells, and described that ST3Gal IV is involved in the synthesis of SLe^x antigen (Carvalho *et al.,* 2010). In line with this report, other studies also found that high expression of ST3Gal IV, contributes to the expression of α2,3-linked sialic acid residues, and is associated with the malignant behavior of gastric cancer cells (Jun *et al.,* 2012).

In gastric carcinoma tissues, the increased expression of ST3Gal IV (Petretti *et al.,* 1999) and of sialyl Lewis antigens have been associated with poor prognosis and metastatic capacity (Amado *et al.,* 1998). These reports highlight the role of STs and evidenced that the expression of crucial glycan determinants, such SLe^x, play an important role in tumor progression. However, the molecular mechanisms underlying the aggressive behavior of gastric cancer cells expressing SLe^x are not fully understood. Some studies pointed to the importance of tyrosine kinase receptor activation in ST overexpression models (Cazet *et al.,* 2009; Cazet *et al.,* 2010; Cazet *et al.,* 2012). In the present study we assessed the effect of ST3GAL IV overexpression in the synthesis of SLe^x in gastric carcinoma cells and we evaluated the functional role of SLe^x *in vitro* (proliferation, invasion and adhesion) and *in vivo* (angiogenesis, tumor growth and invasion). We further evaluated the contribution to cell behavior of tyrosine kinase receptors activation and identified the downstream effectors in the context of a ST3Gal IV/SLe^x overexpressing gastric carcinoma cells.

MATERIALS AND METHODS

Cell culture

The gastric cancer cell line MKN45 was obtained from the Japanese Cancer Research Bank (Tsukuba, Japan) and was stably transfected with full length human gene for ST3GAL3 (MST3Gal III), ST3GAL4 (MST3Gal IV) and the empty vector pcDNA3.1 (Mock) as shown previously (Carvalho *et al.,* 2010). The cells were grown in monolayer culture in T75cm^2 flasks and maintained at 37°C in an atmosphere of 5% CO_2, in Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX, HEPES medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin

(P/S) and in the presence of 0.5 mg/mL G418 (all from Invitrogen). Culture medium was replaced every two days.

RNA isolation, cDNA synthesis and real-time PCR analysis

Total RNA was extracted from cell lysates of Mock, MST3Gal III and MST3Gal IV cell lines using TRI Reagent (Sigma) and converted to cDNA using the SuperScript® II Reverse Transcriptase (Invitrogen). Reverse transcription was performed using 3 µg of total RNA, random oligonucleotides primers and SuperScript II RT (Invitrogen) in a total volume of 20 µL as described by the manufacturer. For real-time PCR analysis, cDNA samples were diluted 50-fold with water and PCR amplified in triplicate with 10.0 µL Power SYBRGreen Master Mix (Applied Biosystems), 0.48 µL of each 10 µM primer and 4 µL cDNA using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The primers used were the following: ST3GAL3 for 5'-ggtggcagtcgcaggattt-3'; rev 5'-catgcgaacggtctcatagtagtg-3'; and ST3GAL4 for 5'-cctggtagctttcaaggcaatg-3'; rev 5'-cctttcgcacccgcttct-3'. Expression of 18S (for 5'-cgccgctagaggtgaaattc-3'; rev 5'-cattcttggcaaatgctttcg-3') and GAPDH (for 5'-agtccctgccacactcag-3'; rev 5'-tactttattgatggtacatgacaagg-3') was also measured in triplicate for each sample and used for normalization of target gene abundance. Specificity of amplification was confirmed by melting curve analysis. Standard curves were determined for each gene, and results are presented as ratio between target gene and housekeeping genes, 18S and GAPDH.

Proliferation assays

Cell growth was analyzed using the BrdU reagent (Roche) according to the manufacturer's directions. Cells (1×10^5) were seeded in slides on 24-well plates (Thermo Fisher Scientific) and grown in RPMI containing 10% FBS, 1% P/S in the presence of 0.5 mg/mL G418. When cells reached 50% of confluence, BrdU was incorporated in cell culture medium and incubated for 20 minutes. After incorporation cell culture medium was removed and cells fixed with methanol for 30 min. Cell labeling with anti-BrdU antibody and FITC secondary antibody was performed according to manufacturer's instructions. Three independent assays were performed and each assay was done in quadruplicates for all the cell lines. Percentage of dividing cells was calculated by measuring positive BrdU cells in relation to total cells with the help of ImageJ software. Results are presented as means ± SD for each sample, and proliferation levels obtained were compared with the Mock control cell line.

Invasion assay

Invasion assays were performed in a BD Biocoat Matrigel invasion chamber with an 8-µm diameter pore size membrane and a thin layer of Matrigel, in a 24-well plate. Inserts were rehydrated for at least 1 h in RPMI medium. After detachment of confluent cells with trypsin/EDTA, cells (5×10^4) were seeded in the upper surface of Transwell plates and cultured in RPMI containing 10% FBS, 1% P/S in the presence of 0.5 mg/mL G418 for 6 h, and the same culture medium was added in the lower part of the insert. After incubation, non-invading cells in the upper part of the insert were carefully removed, cells were fixed with methanol and membranes were removed from the inserts and mounted in a slide using Vectashield with DAPI (Vector labs). Three independent assays were performed and cells were seeded in duplicate for each cell line. Invading cells were counted under a fluorescence microscope, and measurement was done by counting cells in three different fields in each sample, with application of ImageJ software. Results are presented as means ± SD for each sample, and invasion levels obtained were compared with the Mock control cell line.

Cell-substrate adhesion assay

Cell adhesion assays were performed in a 96-well plate coated overnight at 4°C with 50 µL of different extracellular matrix (ECM) proteins: collagen IV, fibronectin and vitronectin in the concentration of 20 µg/mL, while bovine serum albumin (BSA) (Sigma-Aldrich) was used as negative control. After coating, the plate was incubated for 1 hour with 0.5% of BSA in phosphate buffer saline (PBS) and viable cells (2×10^4 cells/well) were introduced into the plate and allowed to adhere for 30 min in RPMI serum-free medium at 37°C and 5% CO2. Removal of non-adherent cells was performed by washing the plate with PBS and adherent cells were fixed with methanol for 30 min. Cells were subjected to 0.5% crystal violet dissolved in 20% of methanol for 1 h, and then washed several times with water and allowed to air dry. Crystal violet dye was solubilized with 10% acetic acid and absorbance was measured at λ = 560 nm. Results are presented as means ± SD for each sample, and adhesion levels obtained were compared with the Mock control cell line.

Phospho-RTK array analysis

Cells were cultured until reached confluence on T75cm2 flasks with RPMI medium supplemented with 10% FBS and 100 units/mL penicillin-streptomycin in the presence of 0.5 mg/mL G418. Cells were then lysed in NP40 lysis buffer (1% NP40, 20 mM Tris-HCl (pH 8.0),

137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitor cocktail tablet (Roche)), protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce) and 300 µg of total protein was used for the human Phospho-RTK array kit (R&D Systems). Phospho-RTK array protocol was performed according to manufacturer's instructions. Activated receptors were matched according to the phospho-RTK array coordinates: a1, a2: EphA6; a3, a4: EphA7; a5, a6: EphB1; a7, a8: EphB2; a9, a10: EphB4; a11, a12: EphB6; a13, a14: mouse IgG1 negative control; a15, a16: mouse IgG2A negative control; a17, a18: mouse IgG2B negative control; a19, a20: goat IgG negative control; a21, a22: PBS negative control; b1, b2: Tie-2; b3, b4: TrkA; b5, b6: TrkB; b7, b8: TrkC; b9, b10: VEGFR1; b11, b12: VEGFR2; b13, b14: VEGFR3; b15, b16: MuSK; b17, b18: EphA1; b19, b20: EphA2; b21, b22: EphA3; b23, b24: EphA4; c1, c2: Mer; c3, c4: c-Met; c5, c6: MSPR; c7, c8: PDGFRα; c9, c10: PDGFRβ c11, c12: SCFR; c13, c14: Flt-3; c15, c16: M-CSFR; c17, c18: c-Ret; c19, c20: ROR1; c21, c22: ROR2; c23, c24: Tie-1; d1, d2: EGFR; d3, d4: ErbB2; d5, d6: ErbB3; d7, d8: ErbB4; d9, d10: FGFR1; d11, d12: FGFR2α; d13, d14: FGFR3; d15, d16: FGFR4; d17, d18: insulin R; d19, d20: IGF-IR; d21, d22: Axl; d23, d24: Dtk. Black dots represent phospho-tyrosine positive controls.

c-Met and Src inhibition assay

c-Met and Src inhibitors were used to evaluate the invasive capacity of the cells upon inhibition. c-Met inhibition was performed with 0.1 µM of PHA-665752 (Sigma) and Src inhibition with 20 µM of PP2 (Sigma) both during 10h. Inhibition was assessed by Western blot for the phosphorylation status of c-Met and Src, and invasion capacity of cells was evaluated as described above, after 10h of inhibitors incubation

Immunoblotting

Proteins were obtained from total cell lysates of each cell line. Briefly, confluent T75cm2 flasks were incubated with NP40 lysis buffer and cells were scraped. Total cell lysates were centrifuge at 14000 rpm for 10 min to remove pellet cell debris. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce). Proteins from cell lysates were separated accordingly to protein molecular weight by gel electrophoresis in 7.5% acrylamide/bis acrylamide (Sigma) SDS-PAGE. For c-Met, phospho-Met, phospho-AKT, phospho-STAT3 and phospho-ERK detection, 25 µg of total protein extract were used and for phospho-Src and phospho-FAK detection we used 50 µg of total protein extract. Gels were then transferred onto a nitrocellulose

membrane (Amersham) in a semi-dry system. Membranes were then blocked with 5% non-fat milk, washed three times with Tris buffer saline (TBS), and incubated overnight at 4°C with primary antibodies. After incubation, membranes were washed three times with TBS and incubated 1 h with secondary antibodies. Analysis was done by chemiluminescence using the ECL Western blotting detection reagent and films (both from GE Healthcare).

Antibodies: anti-phosphorylated Akt Ser473, anti-phosphorylated FAK Tyr397, anti-phosphorylated Src Tyr416, anti-phosphorylated ERK Thr202/Tyr204 and anti-phosphorylated MET Tyr1234/1235, anti-phosphorylated STAT3 Tyr705 (all rabbit polyclonal antibody from Cell Signaling Technology) were used at 1:1000 dilution. Mouse monoclonal IgG2a antibody directed against human MET (Invitrogen) was used at 1:2000. Anti-SLe^x clone KM93 (Millipore) was used at 1:500 dilution. Goat anti-actin and rabbit anti-actin (Santa Cruz Biotechnology) were used at 1:8000 dilution. Secondary anti-rabbit and anti-goat antibodies, conjugated with horseradish peroxidase (DAKO), were used at 1:2000, while anti-mouse IgG2a and IgM antibodies, conjugated with horseradish peroxidase (Jackson immunoresearch) were used at 1:25000 and 1:10000, respectively.

Cdc42, Rac1 and RhoA GTPases pull down assay

Cells were cultured in serum free medium for 24 h, and proteins were obtained from total cell lysates. Pull-down assays, using RhoA / Rac1 / Cdc42 Activation Assay Combo Biochem Kit (Cytoskeleton, inc), were performed according to manufacturer's instructions, using 600 µg of total protein lysates. Briefly, rhotekin-RBD effector domain affinity beads were used to bind RhoA active (GTP-bound) protein and PAK-PBD effector domain affinity beads for Cdc42 and Rac1 active proteins. Total proteins were incubated with these beads for 2 h, and pull down proteins were eluted with Laemmli buffer and separated on 12% acrilamide/bis acrilamide gels. Pull down negative and positive controls were performed according to manufacturer's instructions; briefly total cell lysates were incubated with GTPases inhibitors or activators prior to pull down. To confirm the presence of GTPases in the cell protein extract, a 5% input control were also runned in the gels. Gels were transferred to nitrocellulose membranes and antibodies against Cdc42, RAC1 and RhoA (included in the kit) were incubated overnight with gentle agitation. Proteins were analyzed by chemiluminescence using the ECL Western blotting detection reagent and films (both from GE Healthcare).

Chicken embryo *in* vivo tumorigenesis and angiogenic assay

The chicken embryo chorioallantoic membrane (CAM) model was used to evaluate the angiogenic response and growth capability of Mock and MST3Gal IV cells (n=13 for each group). According to the European Directive 2010/63/EU, ethical approval is not required for experiments using embryonic chicken. Correspondingly, the Portuguese law on animal welfare does not restrict the use of chicken eggs. Briefly, fertilized chick (*Gallus gallus*) eggs obtained from commercial sources were incubated horizontally at 37.8°C in a humidified atmosphere and referred to embryonic day (E). On E3 a square window was opened in the shell after removal of 1.5-2 mL of albumin to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and the eggs returned to the incubator. The window in the egg shell does not interfere in any way with the normal development of the chick embryo. Cells, re-suspended in 10 µL of complete medium ($1x10^6$ cells per embryo), were placed on top of E10 growing CAM into a 3 mm nylon ring under sterile conditions. The eggs were re-sealed and returned to the incubator for an additional 3 days. At this point the embryos are at embryonic development day 13, thus still in the first 2/3 of development. The embryos were euthanized by adding 2 mL of fixative in the top of the CAM which is a very efficient and fast method. After removing the ring, the CAM was excised from the embryos, photographed *ex ovo* under a stereoscope, at 20x magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (less than 15 µm diameter) growing radial towards the ring area was counted in a blind fashion manner. The area of CAM tumors was determined using the Cell A (Olympus) software.

Immunohistochemistry analysis and tumor invasive phenotype

Excided CAMs were fixed in 10% neutral-buffered formalin, paraffin-embedded for slide sections and stained with hematoxilin-eosin for histological examination. Slides with clear view of the CAM tumors were also processed for cytokeratin, SLe^x and p-Met immunohistochemical detection in order to characterize the phenotype of CAM tumors. Briefly, sections were dewaxed, rehydrate and the endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min. Then, sections were incubated with normal rabbit or swine serum diluted 1:5 in PBS containing 10% BSA for 30 min followed by incubation with the monoclonal antibodies overnight at 4°C. Incubation with both biotinylated rabbit anti-mouse and swine anti-rabbit secondary antibodies (DAKO) was done during 30 min at room temperature followed by avidin/biotin complex detection (Vectastain). Staining was performed with 3,3′-diaminobenzidine

tetrahydrochloride (Sigma) containing 0.02% hydrogen peroxide and counter staining of the nucleus was done with Mayer's hematoxylin. Monoclonal antibodies used were KM93 1:60, p-Met 1:100 and cytokeratins AE1/AE3 1:300, and for both antigen retrieval was achieved with citrate buffer pH 6.0. Evaluation of tumor invasion was performed in a blind fashion way by two independent observers. The semi-quantitative evaluation took into consideration the quantity of human AE1/AE3 labeled cells present in the CAM mesenchyme.

Statistical analysis

Statistical analysis was performed using Graph Pad program. ANOVA tests were used to calculate significance in an interval of 95% confidence level. All statistics were compared with Mock group and values of $p<0.05$ were considered to be statistically significant.

RESULTS

Induction of SLe^x by overexpression of ST3GAL4 in gastric carcinoma cells

To evaluate the role of ST3GAL3 and ST3GAL4 sialyltransferases in the synthesis of SLe^x structures, the previously established MKN45 cell line model stably transfected with full length of either ST3GAL3 (MST3Gal III), ST3GAL4 (MST3Gal IV) genes, or an empty vector as control (Mock) were used (Carvalho *et al.,* 2010). The evaluation of the expression levels of ST3GAL3 and ST3GAL4 genes by Real Time-PCR (**Figure 1A**), showed approximately 4 fold increase of ST3GAL3 gene in MST3Gal III cells in comparison with Mock and MST3Gal IV cells, and a 160 fold increase of ST3GAL4 gene expression in MST3Gal IV cells in comparison with Mock and MST3Gal III cells.

The biosynthesis of SLe^x antigen was further assessed by immunofluorescence and by Western blot analysis of total cell lysates and secreted proteins (secretome). Immunofluorescence results showed expression of SLe^x in MST3Gal IV cells when compared with Mock and MST3Gal III cell lines (**Figure 1B**). Consistently, Western blot results demonstrated the expression of SLe^x in MST3Gal IV cells, both in total cell lysates as previously described (Carvalho *et al.,* 2010) and secreted proteins (**Figure 1C**). No expression of SLe^x was detected in total protein extracts or secreted proteins from Mock and MST3Gal III cells.

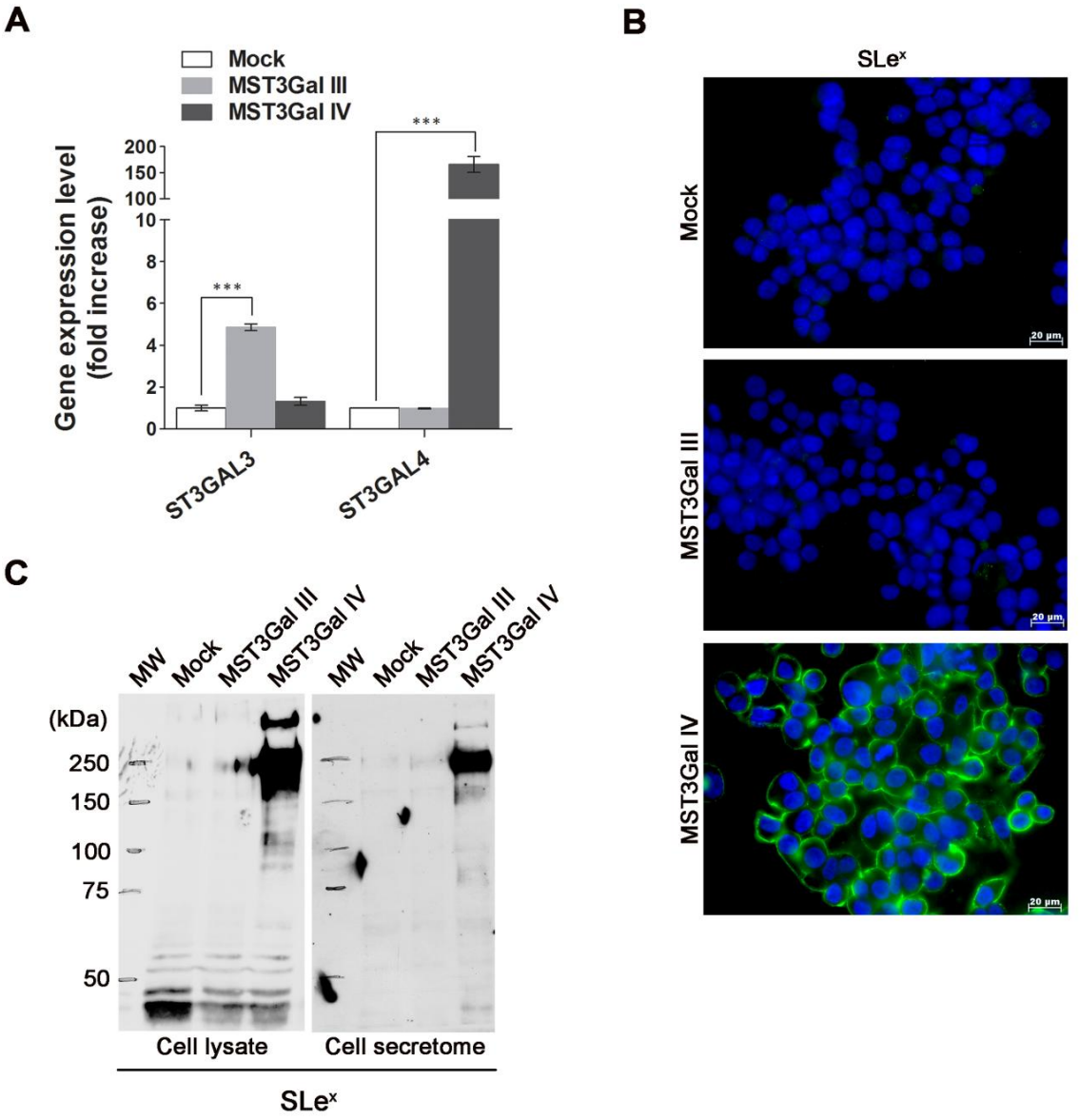

Figure 1: Induction of SLe^x expression by ST3Gal IV transfection in gastric carcinoma cells. A – Relative quantification of ST3GAL3 and ST3GAL4 mRNA expression in MOCK, MST3Gal III and MST3Gal IV transfected cells showing a significant overexpression of ST3GAL3 gene in MST3Gal III cells and ST3GAL4 in MST3Gal IV cells when comparing gene expression levels in Mock cells; ***$p < 0.001$ Mock versus MST3Gal III for ST3GAL3 gene and ***$p < 0.001$ MST3Gal IV versus Mock for ST3GAL4 gene. Results are presented as means ± SD. **B –** Immunofluorescence detection of SLe^x expression in Mock, MST3Gal III and MST3Gal IV cells evidencing the presence of SLe^x in MST3Gal IV (magnification 200x); **C –** Western blot detection of SLe^x in proteins from total cell lysate and secreted proteins from Mock, MST3Gal III and MST3Gal IV cells. SLe^x expression was observed on cell lysates and secretome from ST3GAL4 transfected cells (MST3Gal IV).

Glycan structural characterization of glycolipids were also performed with mass spectrometry technology and no differences were observed in the pattern of glycosylation in the different cell lines, and no SLe^x structures were found in all the cell lines (**Supplemented Figure 1**).

Since only MST3Gal IV transfected cells were able to produce SLe^x antigen, further experiments were performed using the MST3Gal IV and Mock cells.

In vitro biological behavior of SLe^x expressing cells - MST3Gal IV

Cell growth, invasive capacity and adhesion properties of cells transfected with the ST3Gal IV gene were evaluated in order to characterize these cellular phenotypes, and also to address the biological role of SLe^x expression. MST3Gal IV cells showed no statistical differences when compared to Mock cells in terms of BrdU incorporation (**Figure 2A**), suggesting that the expression of ST3Gal IV sialyltransferase and of SLe^x do not affect the proliferation rate of these cells.

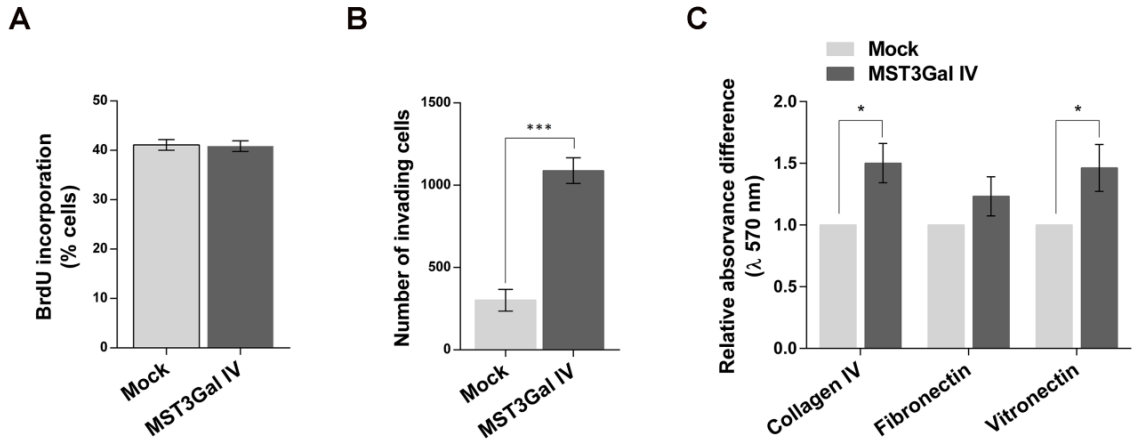

Figure 2: SLe^x overexpression induces cell invasion and increases matrix-cell adhesion *in vitro*. A – BrdU proliferation assay in transfected cells. After 50% confluence, cells were incubated for 30 min with BrdU reagent and fluorescent labeled for proliferative index measurement. No differences were found between the different cell lines. **B –** Cell invasion assay on Matrigel chambers. Cells were seeded on Matrigel-coated filters inserted into two-compartment chambers and invading capacity was measured by counting the number of cells that invade, through the Matrigel-coated filter, 6 h after incubation. MST3Gal IV cells demonstrated an invasive phenotype presenting a significant increased number of invasive cells when compared with MOCK cells (***$p < 0.001$). **C –** Cell adhesion to ECM proteins. Adhesion potential of cells was assessed by incubating cells 30 min in pre-coated plates with collagen IV, fibronectin and vitronectin. Results demonstrate an increased adhesion of SLe^x expressing cells to collagen IV (*$p < 0.05$ MST3Gal IV versus Mock) and vitronectin (*$p < 0.05$ MST3Gal IV versus Mock) matrix proteins. No statistical difference was found in the adhesion capacity of cells to fibronectin. Results are presented as means ± SD.

Cell invasion was analyzed by counting the number of invasive cells on Transwell Matrigel invasion chambers. This analysis revealed that cells overexpressing ST3Gal IV sialyltransferase presented 3 fold increased ability to invade *in vitro,* when compared with Mock control cells (**Figure 2B**). This result evidence the importance of SLe^x expression for the invasive phenotype of MST3Gal IV cells.

In addition, the adhesion to extracellular matrix proteins was also evaluated by seeding cells in plates pre-coated with collagen type IV, fibronectin or vitronectin. Interestingly, SLe^x expressing cells (MST3Gal IV) present an increase capacity to adhere to collagen IV and to vitronectin when

compared with Mock control cells (**Figure 2C**). In contrast, no statistical differences were found in the adhesion capacity of these cells to fibronectin.

In vivo evaluation of angiogenesis, tumor growth and invasion capacity of MST3Gal IV cell line using chicken embryo chorioallantoic membrane model

Transfected cells were inoculated into the chicken embryo chorioallantoic membrane (CAM) and different parameters were evaluated after 3 days of inoculation, specifically, the angiogenic response, tumor size and tumor cell invasive capacity (**Table 1**).

Table 1: Parameters evaluated in the CAM model: Angiogenesis, tumor growth and invasion potential of cells.

	MOCK	MST3Gal IV	p-value
Angiogenesis *(vessel number)*	20.15 ± 4.06	19.31±4.05	n.s.
Tumour growth *(total area mm²)*	4.13± 1.45	4.29± 1.24	n.s.
Cell invasion on CAM *(% cases)*	1/7 (14.3%)	5/7 (71.43%)	* (0.0308)

n.s.(non-significant) $p \geq 0.05$
* $p < 0.05$

The angiogenic potential was assessed by counting the number of vessels with less than 20 µm diameter growing radially towards the inoculation area. The results show no statistical differences in vessel number between Mock control cells and MST3Gal IV indicating that ST3Gal IV and SLe^x expression do not influence the angiogenic response. Tumor size was assessed by measuring the area (mm^2) of the tumor in the different groups. The results show no statistical differences in tumor size arising from the different cell lines, indicating no influence of ST3Gal IV and SLe^x expression in tumor growth potential.

For the evaluation of tumor cell invasive capacity, CAMs were excised from the embryos, fixed with formalin and paraffin-embedded. Invasion of inoculated cells was evaluated in sections of CAM tumors immunostained for human citokeratins. The results show an increased invasive capacity of MST3Gal IV cells inoculated in CAM in comparison to Mock cells (**Table 1**). To assess if cells invading the CAM expressed SLe^x antigens, CAM sections were immunostained for SLe^x. The results show that MST3Gal IV invasive cells expressed SLe^x antigens, contrary to the observed in Mock control cells (**Figure 3**).

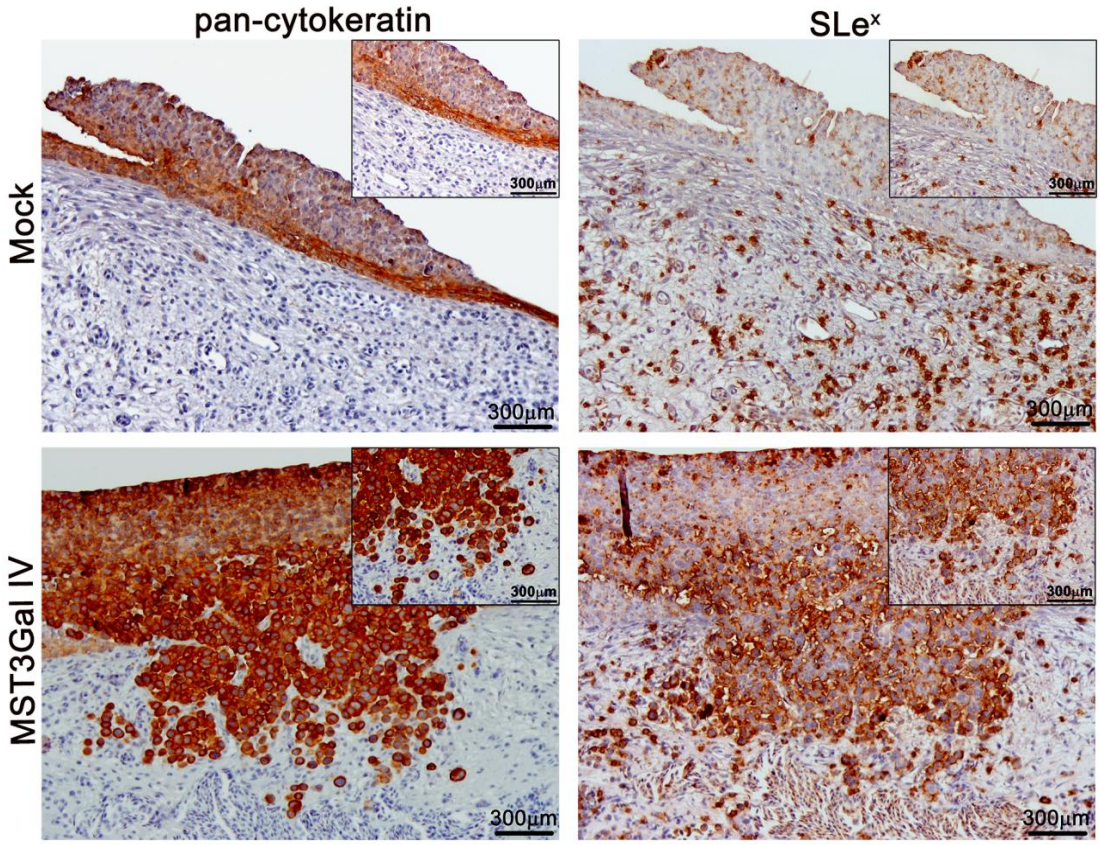

Figure 3: SLe^x expressing cells present an invasive phenotype *in vivo* chicken embryo chorioallantoic membrane (CAM) model. Transfected cells were inoculated in CAM and different parameters were evaluated after 3 days of inoculation. Invasive capacity of inoculated cells was evaluated by immunolabeling CAM tumors with human cytokeratin and SLe^x to assess the presence of human epithelial cells expressing SLe^x. Human cytokeratins immunostaining was used to prove the presence of inoculated human gastric carcinoma cells. SLe^x expression and the invasive capacity of cells were match up to cytokeratins expression. Immunostained tissues evidence the presence of SLe^x structures in CAM tumors from MST3Gal IV cells that, in contrast with Mock cells, invaded the CAM tissue.

Increased activation of c-Met receptor in SLe^x expressing cells - MST3GalIV

To evaluate the possible effects of SLe^x expression on the activation of cell surface receptors and on the induction of the cancer cell invasive phenotype, a receptor tyrosine kinase array was performed using total cell lysates from Mock and MST3Gal IV cells. The results show consistently that MST3Gal IV cells induce increased activation of hepatocyte growth factor receptor (HGFR/c-Met) (**Figure 4A**).

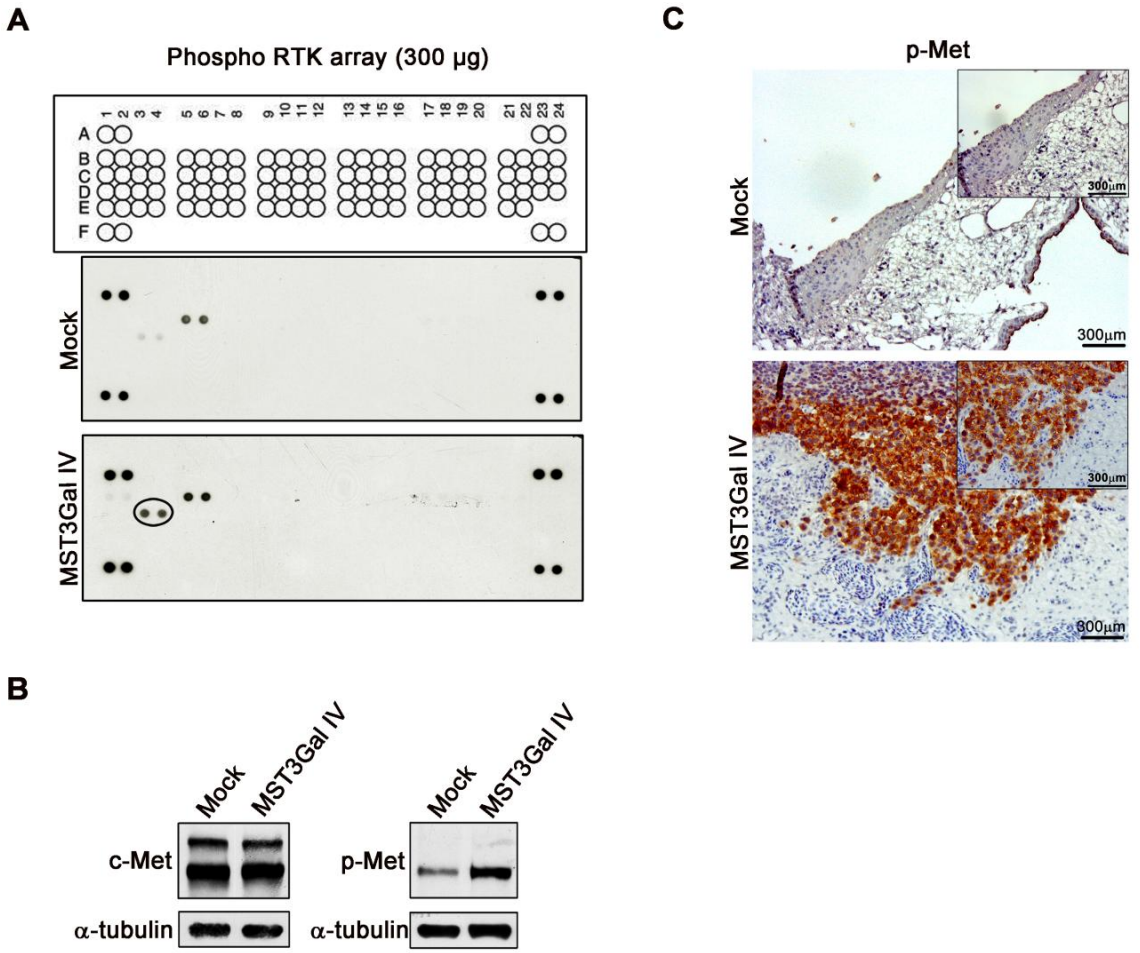

Figure 4: Tyrosine kinase receptors activation evaluation in gastric carcinoma cells; increased c-Met in MST3GAL IV cells. A – phospho-RTK array of transfected Mock and MST3Gal IV cells. Total cell lysates were collected and 300 µg of total protein were incubated into a phospho-RTK membrane array. The array shows an increased activation of c-Met (HGFR) in MST3Gal IV cells. Activated receptors were matched according to the phospho-RTK array coordinates indicated in the material and methods section. c3, c4 correspond to c-Met ; **B –** Analysis of c-Met activation in MOCK and MST3Gal IV cells by Western blot; Cell lysates were analyzed by Western blot with antibodies directed against human c-Met and the phosphorylated tyrosine residues 1234/1235 of the kinase domain to confirm the activation of c-Met in MST3GAL IV cells. The results show an increase expression of phosphorylated c-Met (p-Met) in MST3Gal IV cell line with no differences in c-Met total protein levels in both cell lines. Anti-tubulin antibody was used to assess protein loading. **C –** Expression of phosphorylated c-Met was assessed in CAM tissues. The evaluation of c-Met activation in CAM tumors show that MST3Gal IV/SLe^x expressing cells present positive staining for phospho c-Met and that the resulting invading cells are also positive for the phosphorylated form of this receptor.

The increased level of c-Met receptor tyrosine phosphorylation (p-Met) was further evaluated by Western blot, and the results confirmed that phosphorylation of c-Met is increased in MST3Gal IV cells, with no differences in total c-Met protein expression levels (**Figure 4B**). To assess if the CAM invading cells are expressing the activated c-Met, CAM sections were immunostained for phospho c-Met, demonstrating that MST3Gal IV invasive cells are indeed expressing activated c-Met (**Figure 4C**).

Evaluation of downstream effectors of c-Met activation

c-Met activation relies on stereotypical signaling modulators common to many RTKs (Liu *et al.,* 2010; Organ *et al.,* 2011). To evaluate possible downstream effectors of c-Met activation, we analyzed the activation of Src, FAK, STAT3, AKT and ERK, proteins involved in different c-Met downstream pathways. Our results show that MST3Gal IV cells present increased activation of Src and FAK proteins which are known to be involved in cell motility and invasion (**Figure 5A**, **B**).

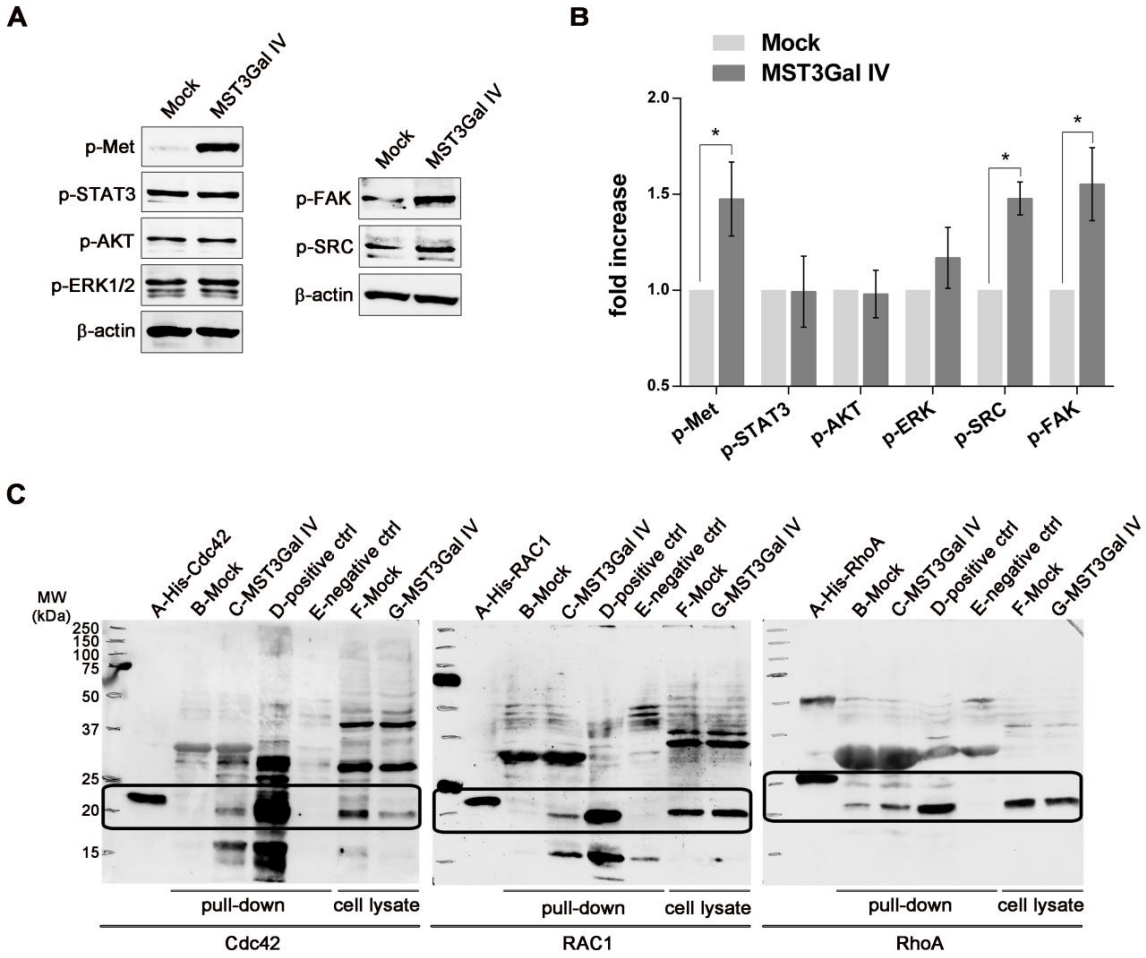

Figure 5: Evaluation of downstream effectors of c-Met activation. A – Increased levels of p-FAK and p-Src proteins in MST3Gal IV cells. The contribution of other effector proteins, such as AKT, ERK, FAK and Src was evaluated by Western blot for their phosphorylated forms in Mock and MST3Gal IV cell lines, and expression of β-actin protein was used as protein loading control. Results show increased levels of phophorylated FAK and Src supporting their involvement as downstream effectors of phosphorylated c-Met (p-Met). **B –** Analysis of 5 independent Western blot of c-Met, STAT3, AKT, ERK, FAK and Src phosphorylated forms in MOCK and MST3 Gal IV cells showing statistically significant increased levels of p-FAK and p-Src, concomitantly with increased phosphorylated c-Met. Results are presented as means ± SD. **C –** Evaluation of Cdc42, Rac1 and RhoA GTPases as potential modulators of c-Met activation by pull-down assay of their activated forms. Western blot analysis of pull-down proteins evidence an increased activation of Cdc42, Rac1 and RhoA in MST3Gal IV cell line. A-GTPase WB protein positive control (His-Cdc42, His-Rac1 and His-RhoA); B-Mock total cell protein pull down; C-MST3Gal IV total cell protein pull down; D-Mock total cell protein pull down with previous GTPases activation (pull down positive control); E-Mock total cell protein pull down with previous GTPases inhibitors (pull down negative control); F-Mock total cell protein input; G-

MST3Gal IV total cell protein input. Highlighted areas represent regions of interest regarding the specific protein migration.

The small GTPases of the Rho family, such as Rac1, Cdc42, and RhoA were also evaluated as possible downstream modulators of c-Met activation by pull-down of activated GTPases. Our results demonstrate that in MST3Gal IV cells, the expression of ST3Gal IV and SLe^x induce activation of Rac1, Cdc42 and RhoA (**Figure 5C**).

Inhibition of invasion in SLe^x expressing cells using c-Met and Src activation inhibitors

In order to confirm the biological role of c-Met and Src activation in the invasive capacity of SLe^x expressing cells, inhibition of phosphorylation of c-Met, Src and both in combination were performed. The inhibition was tested using different concentration of each inhibitor, and different time-points of incubation (data not shown). Longer incubations with 0.1 μM of PHA-665752 c-Met inhibitor (24h and 48h) led to decrease in cell proliferation and cell death (data not shown), therefore a 10h incubation time-point, showing no alteration in cell proliferation, was used for the evaluation of cell invasion. Src inhibition occurred after 10h of incubation with 20 μM of PP2 and no differences in cell death and proliferation was observed after longer incubation periods (data not shown). The activation status of c-Met and Src was assessed by Western blot analysis, and results confirmed the decreased in activation of both proteins after 10 h incubation with the inhibitors (**Figure 6A**). Given the observation that SLe^x expressing cells present increased cell invasive capacity resulting from the activation of c-Met and Src, invasion of cells was evaluated after c-Met and Src inhibition. The results confirmed the increased invasion of SLe^x expressing cells in DMSO control treatment, and demonstrated the abolishment of this invasion capacity upon inhibition of c-Met, Src and both proteins in combination (**Figure 6B**). Moreover, the results showed that inhibition of Src or both Src and c-Met in combination were more effective in precluding cell invasion (**Figure 6B**).

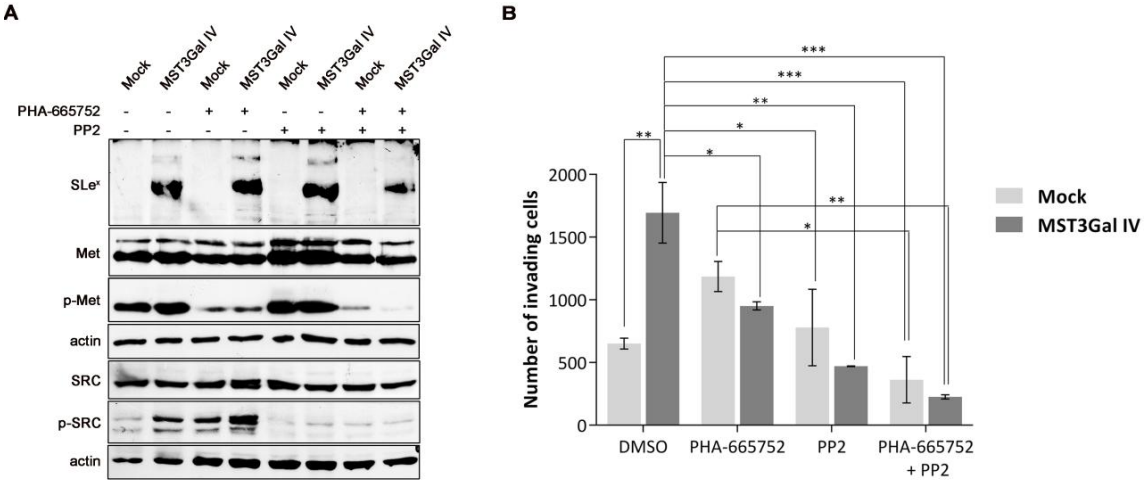

Figure 6: Inhibition of invasion in SLe^x expressing cells by targeting c-Met and Src activation. A – Evaluation by Western blot of the activation of c-Met and Src in the MST3Gal IV and Mock cells with or without the presence of inhibitors of c-Met (PHA-665752) and Src (PP2). **B –** Cell invasion assay on Matrigel chambers after inhibition of c-Met and Src activation. Cells were seeded on Matrigel-coated filters after incubation with inhibitors for 10 h. Invading capacity was measured by counting the number of cells that invade, through the Matrigel-coated filter, after 6 h. MST3Gal IV cells demonstrated an invasive phenotype presenting a significant increased number of invasive cells when compared with MOCK cells. Inhibition of c-Met, Src, and both in combination led to abolishment of cell invasion capability of the MST3Gal IV cells (*p<0.05; **p<0.01; ***p < 0.001).

DISCUSSION

Aberrant glycosylation has been described for many years as a hallmark of cancer, and many of the resulting altered glycosyl epitopes are tumor associated antigens (Hakomori 2002; Drake *et al.,* 2010). These cancer-related antigens are caused by disease-specific alterations in the glycan synthesis pathway such as changes in the Golgi and Endoplasmic Reticulum compartments, mutations in enzymes or chaperons, altered expression of enzymes and biochemical competition, and even variations in sugar donor availability (for a review see Reis *et al.,* 2010). A common alteration is the abnormal expression of sialyltransferases, responsible for adding sialic acids residues to cell surface molecules and to secreted proteins, and which have been involved in the oncogenic transformation, as well as in invasion and metastasis (Hakomori 2002; Dube *et al.,* 2005; Drake *et al.,* 2010). Sialic acids are typically attached to the outermost ends of glycoproteins and glycolipids that can mediate and modulate a wide variety of physiological and pathological processes (Varki 2008).

The SLe^x antigen is a sialylated glycan structure which expression has been associated with cancer progression and aggressiveness as well as poor overall patient survival (Nakamori *et al.,* 1997; Amado *et al.,* 1998; Baldus *et al.,* 1998; Fukuoka *et al.,* 1998; Kim *et al.,* 1998; Tatsumi *et al.,* 1998; Nakamori *et al.,* 1999; Grabowski *et al.,* 2000; Borsig *et al.,* 2002). The expression

of SLe^x in cancer results from the altered expression of sialyltransferases, that adds the sialic acid in a α2,3 linkage to Galactose residues on type-II chains (Harduin-Lepers *et al.,* 2012).

In this study, we have characterized the role ST3Gal IV sialyltransferase in the synthesis of SLe^x antigen. Expression analyzes of SLe^x in stably transfected gastric carcinoma cells by immunofluorescence and Western blot confirmed that ST3Gal IV leads to the biosynthesis of SLe^x. Moreover, our results indicate that SLe^x antigen is expressed on proteins from total cell lysates as well as on secreted proteins from MST3Gal IV cells. These results confirm previous observations that described the importance of ST3Gal IV in the synthesis of SLe^x, the glycan ligand of selectins (Ellies *et al.,* 2002; Sperandio *et al.,* 2006). In addition, our results are in agreement with recent reports showing an increased mRNA level of ST3Gal IV and α2,3 sialic acid residues expression in gastric cancer tissues (Jun *et al.,* 2012).

The carbohydrate SLe^x functions as a ligand for cell adhesion molecules of the selectin family, usually expressed on vascular endothelial cells. The expression of SLe^x on cancer cells is known to facilitate tumor cell spreading by mediating tumor-endothelial cell interactions (Takada *et al.,* 1993; Kannagi 1997). The SLe^x antigen is known to be important in selectin interactions participating in the adhesion of cancer cells to vascular endothelium and contributing to hematogenous metastasis (Kannagi *et al.,* 2004). These previous observations further support that SLe^x antigen plays a functional role in malignant cancer cell behavior. Noteworthy, the crosstalk between cancer cells and host mechanisms like cell-cell adhesion and cell-matrix adhesion interactions, tumor cell growth and motility are known to be important in modulating the process of cancer cell invasion. In the present study we performed a comprehensive evaluation of the biological role of SLe^x in gastric cancer cells using *in vitro* and *in vivo* models. The *in vitro* analysis showed that SLe^x expressing cells display a similar proliferative rate when compared with Mock transfected cells. However, SLe^x expressing cells demonstrated a higher capacity to invade *in vitro* in Matrigel chambers, demonstrating the active role of this sialylated glycan structure in tumor cell motility and invasion. Concomitant to this invasive capacity, SLe^x expressing cells evidenced higher capacity to adhere to collagen IV and vitronectin extracellular matrix proteins. These findings highlight the importance of this sialylated glycan in the malignant invasive phenotype. Furthermore, this invasive phenotype was also confirmed *in vivo* where cells transfected with ST3Gal IV and expressing SLe^x antigen presented increased capacity to invade the chorioallantoic membrane of the chicken embryo. Our results are in keeping with studies that associate SLe^x expressing tumors with more aggressive phenotypes (Nakamori *et al.,* 1993;

Amado *et al.,* 1998; Ichikawa *et al.,* 2000). In the gastric carcinoma context it has also been described that SLe^x antigen expression correlates with liver metastasis (Tatsumi *et al.,* 1998). The modulation of cancer cell biological behavior by sialylated glycans has been previously described in human pancreatic cells. In this pancreatic model the restoration of α1,2 fucosyltransferase activity, a enzymatic competitor of ST3Gal transferases, reduces the expression of Sialyl Lewis antigens and decreases the adhesive and metastatic properties of these cells (Aubert *et al.,* 2000).

In addition, previous reports have shown that increased cellular sialylation leads to receptor and signaling pathways activation and that the hypersialylation contributes to cancer progression and increased cell motility (Seales *et al.,* 2005a; Seales *et al.,* 2005b). Moreover, it has been described that TNF-α can induce SLe^x and 6-sulfo-SLe^x expression in human cancer cells, by increasing the expression of ST3GAL4 (Colomb *et al.,* 2012). This mechanism has also been shown to be mediated by neutrophils expressing TNF-α leading to cancer cells invasiveness and metastasis (St Hill *et al.,* 2011).

In order to clarify the potential implication of ST3Gal IV and of its product SLe^x in the biological behavior of gastric cancer cells, we evaluated the expression of activated tyrosine kinase receptors and downstream modulators involved in cancer cell invasion. The tyrosine kinase receptor array allowed the identification of a constitutive activation of c-Met in SLe^x expressing cells. The activation of tyrosine receptors, directly or indirectly by glycan antigens has previously been observed in other cancer cell models. Singh and colleagues described that the Thomsen-Friedenreich antigen (T antigen) present in CD44v6 promotes the activation of c-Met and mitogen-activated protein kinase (MAPK) signaling leading to cancer cell proliferation (Singh *et al.,* 2006). Furthermore, activation of c-Met receptor has been described in a breast cancer cell model that overexpress glycosyltransferases and this activation has been implicated in proliferation and invasion of cancer cells (Cazet *et al.,* 2009; Cazet *et al.,* 2010; Cazet *et al.,* 2012). The MKN45 cell line model has been reported to have a high level of expression and dependence on c-Met (Smolen *et al.,* 2006) and therefore modulation of cellular glycosylation can have implications in this c-Met dependent cells.

c-Met overexpression has been considered a hallmark of cancer, playing a role in many tumors and in metastatic progression (Sierra *et al.,* 2011). In gastric cancer, c-Met expression alterations have been reported, such as the Tpr/Met rearrangement (Soman *et al.,* 1991; Yu *et al.,* 2000) and c-Met copy number amplification (Lee *et al.,* 2011), as well as increased c-Met activation

(Inoue *et al.,* 2004; Dua *et al.,* 2011). We evaluated the c-Met activation in a series of gastric carcinoma tissues (data not shown). The variability in tissue sample processing is known to lead to loss of protein phosphorylation which precluded the detection of phosphorylated c-Met in these samples. However, sections analyzed from the chicken chorioallantoic membrane tumors, derived from either Mock or SLe^x expressing gastric carcinoma cells, confirmed phosphorylated c-Met positive staining in SLe^x cancer invading cells. This result further supports the hypothesis that SLe^x expressing cells exhibit invasive capacity through the activation of c-Met.

The activation of c-Met is well known to induce docking sites for proteins that mediate downstream signaling leading to the activation of the MAPK, phosphatidylinositol 3-kinase (PI3K)-AKT, v-src oncogene homolog (Src), signal transducer and activator of transcription (STAT), which are signaling pathways that are involved in increased cell growth, scattering, motility, invasion, protection from apoptosis, branching morphogenesis, and angiogenesis (Liu *et al.,* 2010; Organ *et al.,* 2011). Taking that into consideration, we evaluated the downstream effectors of c-Met activation and found that FAK and Src proteins showed increased activation in cells expressing ST3Gal IV. In combination with our invasion assays results (*in vitro* and *in vivo*), these results strongly suggest that c-Met activation mediates tumor cell motility and invasion, also in gastric cancer cells. These results are in agreement with previous studies that associate Src-FAK signaling pathway with the metastization process (Peng *et al.,* 2009; Lim *et al.,* 2012; Sanchez-Bailon *et al.,* 2012; Shen *et al.,* 2012). Furthermore, our results show that inhibition of c-Met and Src could preclude the increased invasion observed in SLe^x expressing cells supporting the importance of this glycosylation alteration in the activation of this invasive related pathways.

Oncogenic transformation is often associated with changes in organization of the cytoskeleton, which can influence cell migration, adhesion and invasion. The c-Met activation can cause changes in gene expression of cell-cycle regulators (Cdk6 and p27), extracellular matrix proteinases (such as matrix metalloproteinases and urokinase plasminogen activator), and in alterations of cytoskeleton functions that control migration, invasion and proliferation (Birchmeier *et al.,* 2003). The cytoskeleton is composed of a complex and organized network of various fibrous proteins within the cytoplasm, playing an essential structural and regulatory role in the maintenance of cell structure and strength, in cell division, proliferation, motility, invasion and also in signaling functions (Tapon *et al.,* 1997; Machesky *et al.,* 1999; de Curtis *et al.,* 2012). The activation of tyrosine kinase receptors can modify the phosphorylation status of cytoskeleton regulatory and structural proteins. Signaling pathways initiated by the activation of cell surface

receptors can promote distinct membrane protrusions by converging onto the Rho family of GTPases (Hall 1999; Kjoller *et al.,* 1999). Rho proteins are small (21-25 kDa) molecules that share structural homology and become activated only when bound to GTP. One of the best characterized Rho GTPase family members is RhoA regulating the formation of stress fibers and focal adhesion assembly, while Rac1 and Cdc 42 are mainly involved in membrane ruffling and formation of filopodia, respectively (Pertz 2010). Estimation of GTPases activation is frequently a molecular marker in the evaluation of cytoskeleton alterations during cell migration (Evers *et al.,* 2000; Parri *et al.,* 2010; Wessler *et al.,* 2011). Here we showed the activation of Rho GTPases, specifically RhoA, Rac1 and Cdc42. These results further supports the evidence that SLe^x expression leads to cytoskeleton protein alterations in cancer cells, underlying the observed increased cell motility and invasion of these cells. Our findings are in keeping with previous reports showing the importance of RhoA, Rac1 and Cdc42 in cancer progression (Kamai *et al.,* 2004), and also the crosstalk between these GTPases and other signaling pathways like Src-FAK in the migratory phenotype of cancer cells (Leve *et al.,* 2011). Our present findings support the hypothesis that increased expression of SLe^x on the surface of malignant cells plays an important role in tumor invasion and metastasis. Overall, our study showed that tumor cell invasion is induced by SLe^x expression on gastric cancer cells through the activation of c-Met in association with downstream signaling effectors Src, FAK and RhoA GTPases activation (**Figure 7**).

These results open new avenues for the designing of intervention strategies that target ST3Gal IV/SLe^x in cancer cells as well as the inhibition of c-Met and Src in order to improve gastric cancer treatment by targeting invasion and metastasis.

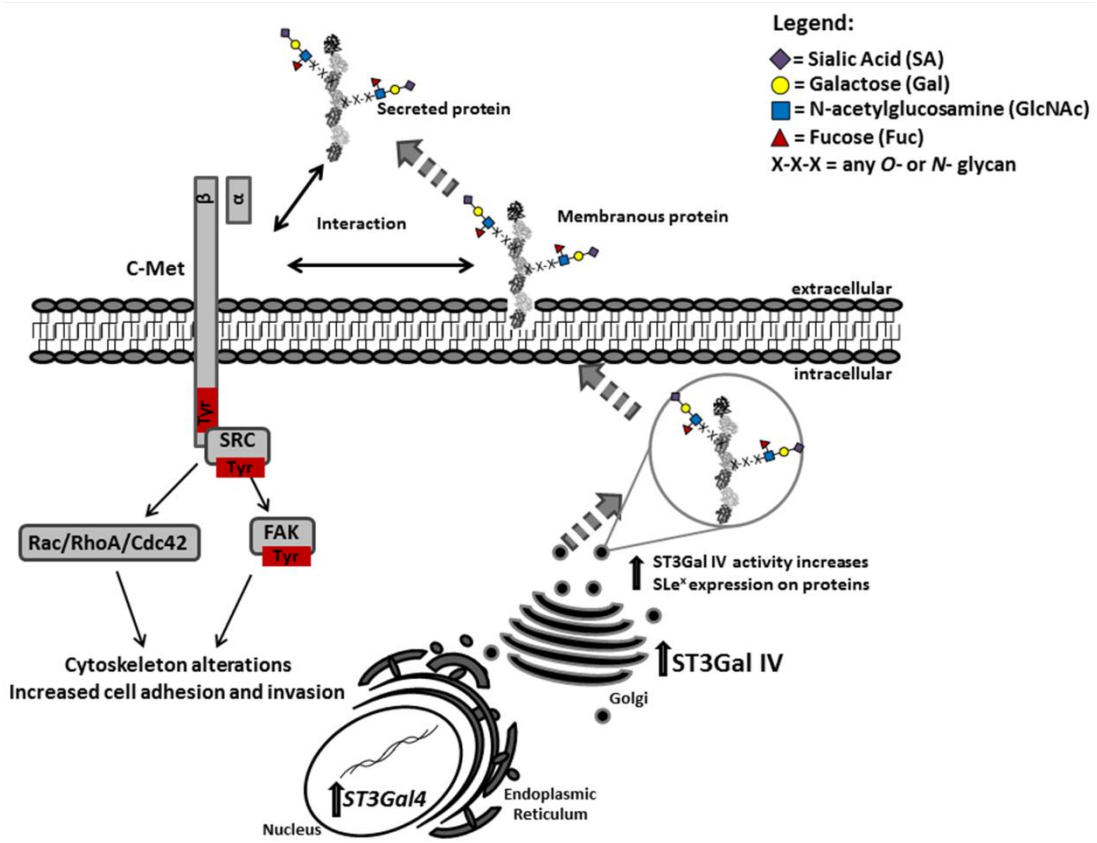

Figure 7: Schematic representation of the alterations induced by increased expression of SLe^x and activation of c-Met. Increased transcription of ST3GAL 4 leads to increased expression of the ST3Gal IV enzyme in the Golgi apparatus of the cells. This enzyme will glycosylate type-2 terminal oligosaccharide chains leading to the presence of SLe^x in glycoproteins targeted for the membrane or to be secreted by the cells. The expression of SLe^x in membrane-associated and secreted proteins can promote the interactions between these proteins and c-Met leading to its activation. c-Met activation leads to downstream signaling activation target proteins Src, FAK and Rho GTPAses leading to a modified cell-matrix adhesion and an increased cell invasion.

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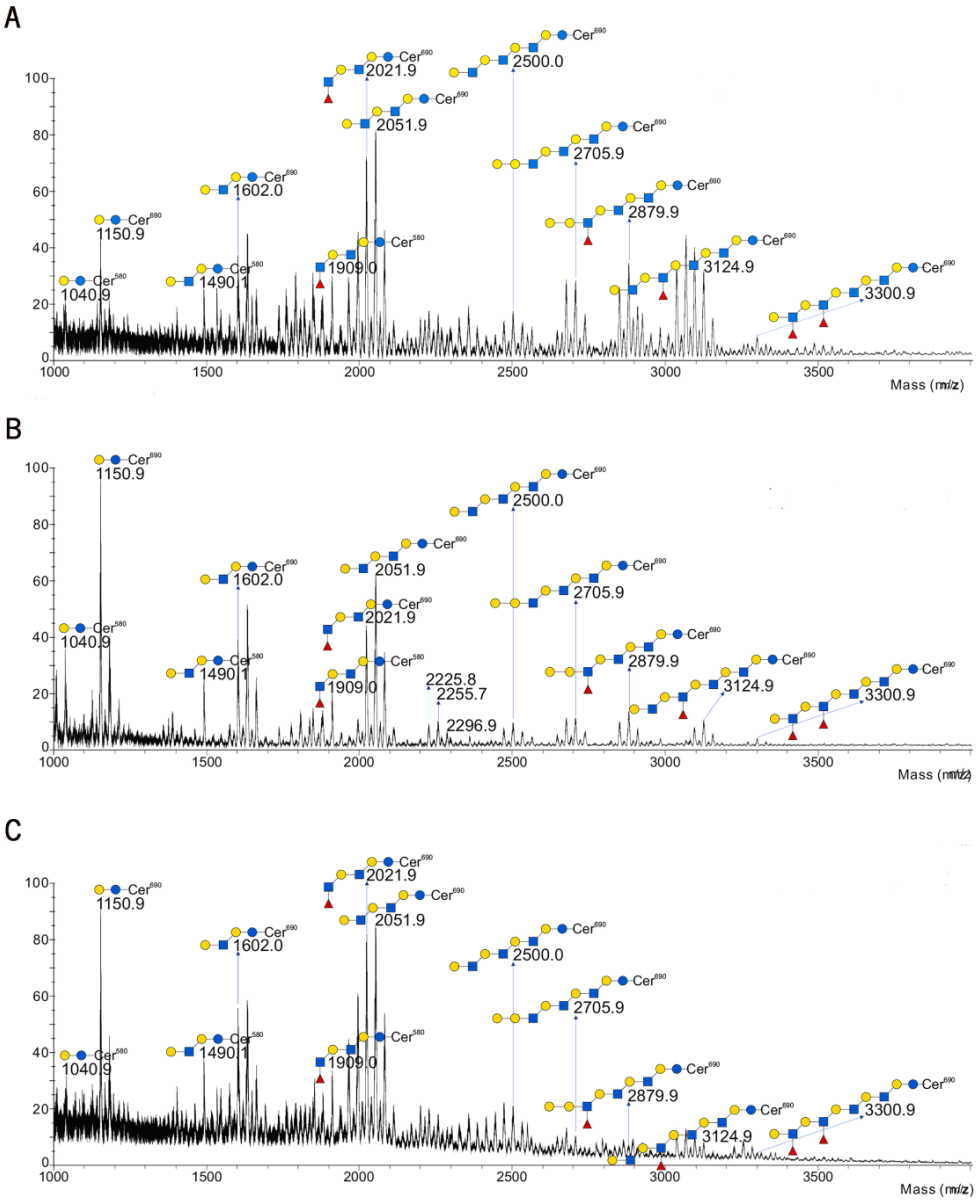
SUPPLEMENTARY DATA

Mass spectrometry analysis of glycosphingolipids

Prior to mass spectrometry analysis, glycosphingolipids were permethylated according to Ciucanu and Kerek (Ciucanu *et al.,* 1984). Briefly, compounds were incubated 2 h in a suspension of 200 mg/mL of NaOH in dry dimethyl sulfoxide (300 µL) and ICH_3 (200 µL). The methylated derivatives were extracted in $CHCl_3$ and washed several times with water. The reagents were evaporated and the sample was dissolved in CHCl 3 in the appropriate dilution. Mass spectrometry analysis of permethylated GSLs was performed by matrix-assisted laser desorption-ionization (MALDI) time-of-flight (TOF) on a Voyager Elite reflectron mass spectrometer (PerSeptive Biosystems, Framingham, MA), equipped with a 337-nm UV laser. Samples were prepared by mixing on a tube 5 µL of diluted permethylated derivatives solution in $CHCl_3$ and 5 µL of 2,5-dihydroxybenzoic acid matrix solution [10 mg/mL dissolved in $CHCl_3/CH_3OH$(1:1, v/v)]. The mixtures (2 µL) were then spotted on the target plate and air-dried.



Supplementary Figure 1: MALDI-TOF analysis of permethylated glycosphingolipids isolated from MKN45 Mock and transfected cells. Permethylated GSLs isolated from (A) MKN45 transfected with empty vector - Mock, (B) MKN45 transfected with ST3Gal III, (C) and MKN45 transfected with ST3Gal IV. No differences were observed in the type of gangliosides expressed in the three different cell lines. Blue circle, Glc; yellow circle, Gal; blue square, GalNAc; red triangle, Fuc.

3.3

CEACAM5 Carcinoembryonic Antigen Carries SLe^x in Gastric Carcinoma Cells - Implications for Diagnosis Improvement

Content

3.3 CEACAM5 carcinoembryonic antigen carries SLe^x in gastric carcinoma cells - implications for diagnosis improvement.

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ABSTRACT

Glycosylation alterations are common features of cancer cells, constituting a main source of cancer biomarkers. These alterations in cancer are consequence of the deregulated expression of glycosyltransferases such as α2,3 sialyltransferases that lead to expression of the SLe^x antigen.

In this work, we demonstrated in a gastric carcinoma cell line that overexpression of ST3Gal IV leads to the biosynthesis of SLe^x and identified by mass spectrometry carcinoembryonic antigen glycoprotein as a major carrier of SLe^x. We validated this result by immunoprecipitation and proximity ligation assay. The analysis of CEA and SLe^x expression in a series of gastric carcinoma cases demonstrated that 83.9% of the total cases co-expressed both CEA and SLe^x. This association was further validated by demonstrating CEA/SLe^x molecular *in situ* expression in 80.6% of the cases as determined by PLA. The presence of CEA/SLe^x molecular complexes in the tumor tissues was correlated with the pattern of tumor growth and venous invasion.

In conclusion we identified CEA as a glycoprotein carrier of SLe^x antigen in gastric carcinoma cells, and demonstrated a co-expression and presence of molecular complexes of SLe^x and CEA in gastric carcinoma tissues. The co-expression of CEA/SLe^x may contribute to improve gastric cancer specific diagnosis and monitoring.

INTRODUCTION

Multiple molecular alterations are involved in the transition of healthy to tumor tissues, and these include changes in the pattern of protein glycosylation. Most membrane-associated and secreted cellular proteins are glycosylated, containing *N*- or *O*-linked glycans that decorate the cell

surfaces. The glycan composition of glycoproteins play various functions in the cell, such as protein conformation and trafficking, cell-cell and cell-matrix interactions and recognition (Varki 1993). Aberrant glycosylation is a common feature associated with cancer, constituting an important source of cancer biomarkers (Drake *et al.,* 2010; Reis *et al.,* 2010).

Deregulated expression or localization of glycosyltransferases and associated proteins within the tumor cell, are underlying causes of glycosylation changes observed in cancer (Hakomori 2002; Gill *et al.,* 2011; Meany *et al.,* 2011; Harduin-Lepers *et al.,* 2012). Altered expression of sialyltransferases and their associated products have been widely studied in cancer (Harduin-Lepers *et al.,* 2012). The most frequently reported tumor-associated glycosylation alterations are the increased sialylation and polysialic acid synthesis, the appearance of sialylated Lewis antigens in glycolipids and glycoproteins, the formation of truncated *O*-glycan chains and the increase in branching of *N*-glycans, (David *et al.,* 1992; Reis *et al.,* 2010; Kang *et al.,* 2011; Pinho *et al.,* 2011; Dall'Olio *et al.,* 2012). The expression of Sialyl Lewis X (SLe^x (NeuAcα2,3Galβ1-4[Fucα1-3]GlcNAc-R)) is significantly upregulated in cancer and has been shown to correlate with more aggressive phenotype and poor prognosis (Nakamori *et al.,* 1993; Nakamori *et al.,* 1997; Fukuoka *et al.,* 1998; Kim *et al.,* 1998; Julien *et al.,* 2011). Several glycosyltransferases have been described to be involved in the synthesis of SLe^x structures in cancer cells, such as the α1,3 fucosyltransferases (FucT-VI and FucT-VII) (Britten *et al.,* 1998; Liu *et al.,* 2008; Trinchera *et al.,* 2011) and the α2,3 sialyltransferase IV (ST3Gal IV) (Ellies *et al.,* 2002; Carvalho *et al.,* 2010; Colomb *et al.,* 2012). In gastric carcinomas increased expression of SLe^x (Amado *et al.,* 1998) and ST3Gal IV (Jun *et al.,* 2012) have been described and associated with an aggressive cancer cell behavior.

Although association between glycan expression alterations in tumor tissues and clinical prognosis has been documented, its application in the clinical setting has been limited to serological assays that detect glycoconjugates shed by the tumors into circulation. Among these, various serological assays have been approved such as carcinoembryonic antigen (CEA) for colorectal cancer, cancer antigen 125 (CA125) for ovarian cancer, CA19.9 for pancreatic and gastric cancer, and prostate-specific antigen (PSA) for prostate cancer (Drake *et al.,* 2010; Reis *et al.,* 2010; Pan *et al.,* 2011).

Currently the clinical application of these makers is mostly for monitoring treatment and relapses with no consensual application in early diagnosis. This limited diagnosis application is mostly due to the low tumor specificity of these biomarkers.

In the present work our main goal was the identification of proteins in gastric carcinoma cells that carry the SLe^x glycan antigen. For this intend, we used a gastric carcinoma cell line expressing SLe^x due to the overexpression of the ST3Gal IV enzyme, and indentified by mass spectrometry analysis carcinoembryonic antigen (CECAM5; CEA) as a SLe^x protein carrier. This result was further validated by CEA immunoprecipitation and Proximity Ligation Assays, and the presence of this molecular complex was also verified in gastric carcinoma tissues and associated with venous invasion.

MATERIALS AND METHODS

Cell culture

MKN45 gastric carcinoma cell line (Japanese Cancer Research Bank, Tsukuba, Japan) was stably transfected with a full length human gene for ST3GAL4 (MST3Gal IV) and the empty vector (Mock) (Carvalho *et al.,* 2010). The cells were routinely grown in monolayer in T75 cm^2 flasks using RPMI 1640 GlutaMAX, HEPES medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) in the presence of 0.5 mg/mL G418 (all from Invitrogen. Cell culture was done at 37°C in a 5% CO_2 atmosphere and culture medium was replaced every two days.

SDS-PAGE and Western blot analysis

Transfected cells were collected at high confluence, incubated and scrapped with NP40 lysis buffer (1% NP40, 20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail tablet (Roche)) to obtain total protein cell lysates. For secretome protein collection, cells in approximately 80% of confluence were carefully washed with phosphate buffer saline (PBS) and RPMI followed by a 24 h cell culture in serum free RPMI medium. The culture medium was collected and concentrated in 10 MW cutoff filters according to manufacturer's instructions (Millipore). Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce), and 50 µg of total protein were loaded on 7.5% acrylamide/bisacrylamide gel (Bio-Rad). After electrophoresis gels were stained or transferred to nitrocellulose membranes (GE Healthcare). Gels were stained with Coomassie blue (Imperial Protein Stain from Thermo Scientific) or with periodic acid-Schiff (PAS) method (Glycoprotein Staining Kit from Thermo Scientific Pierce) that specifically detects glycosylated proteins having sialic acid and other oxidizable carbohydrate groups according to

manufacturer's instructions (Pierce) and images were acquired with a GS800 scanner (Bio-Rad). Membranes were blocked with 5% BSA in PBS with 0.05% Tween 20 (PBST), incubated with primary antibody followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgM and goat anti-mouse IgG1 (Jackson ImmunoResearch) secondary antibodies and ECL detection (GE Healthcare). For lectin blots, after membrane blocking with 5% BSA PBST, biotinylated SNA lectin was incubated for 1 h at room temperature followed by avidin/biotin detection reagents (Vectastain) incubation and ECL detection.

Antibodies and Lectin: Anti- SLe^x clone KM93 was purchased from Millipore and used at 1:500 dilution, rabbit monoclonal IgG1 antibody directed against human CEA was purchased from Cell Signaling Technology and used in 1:3000 dilution and biotinylated SNA from Vector labs was used in 1:600 dilution.

Protein selection and identification by MALDI-TOF/TOF mass spectrometry

The spots highlighted in the Western blots were matched in the stained gel and proteins excised with a spotpicker (OneTouch 2D gel spotpicker, 1.5 mm diameter, Gel Company, USA). After reduction and alkylation, the selected protein bands were *in-gel* digested with trypsin and the respective peptidic mass spectra were acquired by MALDI-TOF/TOF (4700 Proteomics Analyzer MALDI-TOF/TOF, AB SCIEX, CA) as previously described (Gomes *et al.,* 2013a). Proteins were identified using the combined information of Peptide Mass Fingerprint (PMF) and MS/MS peptide sequencing approaches by the Mascot protein search software (Matrix Science, UK), integrated in the GPS Explorer software (AB SCIEX, CA).

CEA Immunoprecipitation

CEA immunoprecipitation was performed in Mock and MST3Gal IV total cell proteins. Briefly, 2 µL of mouse anti-CEA antibody (Cell Signaling) was coupled to 60 µL of ProteinG Sepharose (Sigma) for 2 h at 4°C, followed by BS3 (Sigma) crosslink of the antibody and total cell proteins (600 µg) were incubated overnight at 4°C. Bead's washes were performed with PBS and after protein incubation 1% of Triton X-100 (Sigma) was added. The immunoprecipitated proteins were eluted in 60 µL of SDS Laemmeli buffer and 10 µL was used for CEA Western blot and 25 µL for both SLe^x and SNA Western blot.

Immunofluorescence staining

MKN45 transfected cells were seeded and cultured on glass coverslips in 24-well plates (Orange Scientific). Cells were washed and fixed 30 min with methanol. After fixation, cells were washed and blocked with swine or rabbit serum in 10% BSA PBS for 30 min at room temperature followed by primary antibody incubation overnight at 4°C. Then, cells were washed and incubated for 30 min with both rabbit anti-mouse 1:100 and swine anti-rabbit 1:70 FITC-conjugated secondary antibodies (DAKO). Nuclear counter staining was done with DAPI and coverslips were mounted in a microscope slide with vectashield (Vector Labs). Fluorescence was examined in a fluorescence microscopy and images were acquired using a Zeiss Axio cam MRm and the AxioVision Rel. 4.8 software (Carl ZEISS) and in some cases immersion oil was used. Monoclonal antibodies used: mouse monoclonal KM93 1:60, mouse monoclonal antibody anti-CEA 1:600.

Tissue samples and Immunohistochemistry analysis

Gastric carcinomas and gastric mucosa adjacent to carcinomas were obtained from individuals undergoing surgery at Centro Hospitalar São João (CHSJ), University of Porto Medical Faculty (Porto, Portugal). Study was performed with the approval of the local CHSJ ethical committee. Gastric carcinoma tissues (n=31) formalin-fixed parafin-embedded were used for slide sections and stained with hematoxilin-eosin for histological examination. Hematoxylin and eosin-stained sections were used to classify gastric carcinomas according to the classifications of Carneiro *et al.* (1995), Laurén (1965), Ming (1977), and the World Health Organization (WHO). Clinicopathological characteristics of the cases (lymphatic invasion, venous invasion, pTNM staging) were also recorded in every case.

Paraffin sections were dewaxed, rehydrated and blocked for endogenous peroxidase activity with 3% H_2O_2 in methanol for 30 min. Sections were then incubated with normal rabbit or swine serum diluted 1:5 in PBS containing 10% BSA for 30 min followed by incubation with the monoclonal antibodies overnight at 4°C. Incubation with biotinylated rabbit anti-mouse (DAKO) was done during 30 min at room temperature followed by avidin/biotin complex detection (Vectastain). Staining was performed with 3,3′- diaminobenzidine tetrahydrochloride from Sigma containing 0.02% hydrogen peroxide and counter staining of the nucleus was done with Mayer's hematoxylin. Mouse monoclonal antibodies used KM93 1:60, anti-CEA (CB30) 1:300 with antigen retrieval with citrate buffer pH 6.0.

Proximity ligation assay (PLA)

PLA assay was performed in cultured cells on glass coverslips and on gastric carcinoma tissue sections for detection of ligation dependent SLe^x on CEA glycoproteins, according to previous studies showing that PLA is an appropriate approach for glycoproteins identification in tissue sections (Pinto *et al.,* 2012). Briefly, paraffin tissue sections were dewaxed and rehydrated followed by antigen retrieval with citrate buffer pH6.0. Tissue sections and cells on glass coverslips were then incubated with normal goat serum diluted 1:5 in PBS containing 10% BSA for 30 min followed by incubation with the two monoclonal antibodies in the same solution overnight at 4°C. Proximity ligation assays (PLA) were performed using the DuoLink® II Fluorescence Kit (Olink® Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. PLA probes anti-IgG plus and anti-IgM minus were incubated 1 h at 37°C, followed by ligation step 30 min at 37°C and amplification step 100 min at 37°C. Nuclei were stained with DAPI and slides were mounted in an appropriated medium (Duolink II). PLA products are seen as fluorescent red dots. Fluorescence was examined in a fluorescence microscopy and images were acquired using a Zeiss Axio cam MRm and the AxioVision Rel. 4.8 software (Carl ZEISS) and in some cases immersion oil was used.

RESULTS

Identification of SLe^x protein carriers in MKN45 ST3Gal IV gastric carcinoma cell line

The expression of SLe^x on proteins from Mock and ST3Gal IV overexpressing cells were evaluated by Western blot. We observed expression of SLe^x antigen exclusively on total cell lysates and secretome from MST3Gal IV cells (**Figure 1**).

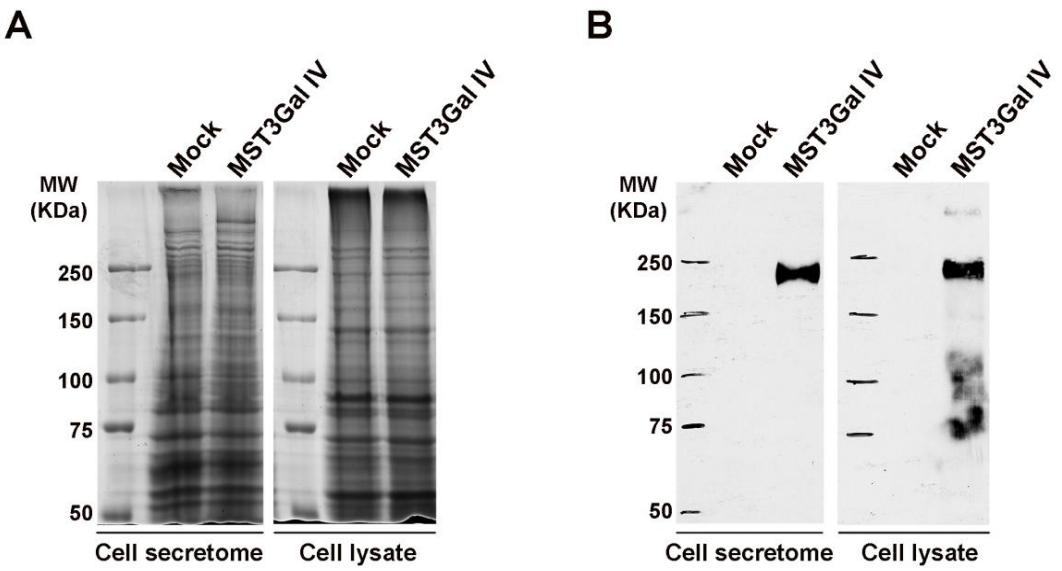

Figure 1: ST3Gal IV induces expression of SLe^x in proteins of MKN45 gastric cancer cells. Gastric carcinoma cells stably transfected with ST3Gal IV show SLe^x expression. **A –** Coomassie blue gel of proteins from total cell lysates and secretome from Mock and MST3Gal IV cells; **B –** Western blot analysis of SLe^x expression in Mock and MST3Gal IV. This evaluation shows expression of SLe^x antigen in proteins from MST3Gal IV cell line.

In order to detect high molecular weight proteins labeled in the SLe^x western blot, we separated total cell lysates in SDS-PAGE and stained glycoproteins using the PAS method that specifically stains glycan residues (**Figure 2A**). The SLe^x bands in the Western blot (**Figure 2B**) were matched with the glycan stained gel and the proteins identified by MALDI-TOF/TOF mass spectrometry analysis.

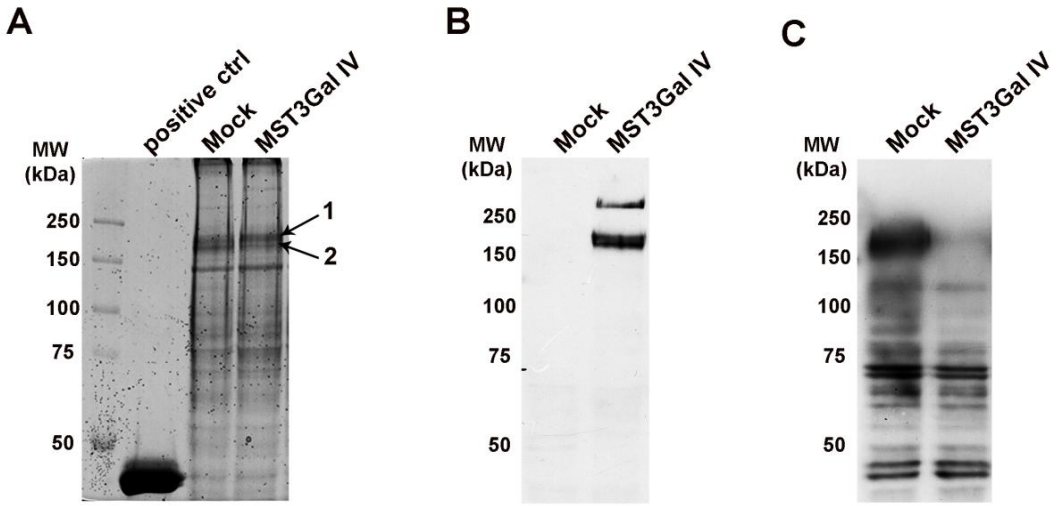

Figure 2: Glycoprotein analysis of Mock and MST3Gal IV cells. A – Glycan staining using the periodic acid-Schiff (PAS) method of SDS-PAGE glycoproteins present in Mock and MST3Gal IV; Horseradish peroxidase was used as positive staining control; **B –** SLe^x expression on glycoproteins from Mock and MST3Gal IV cells; **C –** α2-6 sialic acid expression detected using the SNA lectin in glycoproteins from Mock and MST3Gal IV cells. The results show SLe^x expression (**B**) and reduced α2-6 sialic acids expression (**C**) in MST3Gal IV cells. The PAS staining method (**A**) allowed the detection of glycoproteins in Mock and MST3Gal IV cells. Arrows 1 and 2 indicate the selected proteins for MALDI-TOF/TOF identification.

Two proteins were identified (**Table 1**), and among them the Carcinoembryonic antigen (CEACAM5; CEA) was the only previously described to be glycosylated. In addition, the detection of α2,6 sialic acids in total cell lysate proteins shows a loss of α2,6 sialic acids expression (**Figure 2C**) in SLe^x positive proteins from MST3Gal IV cells (**Figure 2B**).

Table 1: Proteins identified in MST3Gal IV cells according to SLe^x antigen detection by western blot

Spot ID	*Protein description*	*Accession number*	*MASCOT Protein C.I. %*	*Peptide count*	*% cov*	*Peaks matched*	*MOWSE score*
1	Carcinoembryonic antigen-related cell adhesion molecule 5	CEAM5_HUMAN	99	4	8	5	92
2	Ras GTPase-activating-like protein IQGAP1	IQGA1_HUMAN	100	39	21	40	268
	Carcinoembryonic antigen-related cell adhesion molecule 5	CEAM5_HUMAN	100	5	10	7	234

Attempts to obtain a protein separation in 2D gel electrophoresis were performed, but Coomassie blue and SLe^x staining (**Supplementary Figure 1**) did not allow the visualization of high molecular weight proteins that were observed in 1D SDS-PAGE.

Characterization of SLe^x glycan antigen in CEA

In order to validate that CEA is a carrier of SLe^x we performed CEA immunoprecipitation of total cell lysates from Mock and MST3Gal IV cells, followed by Western blot analysis for SLe^x antigen. The results confirmed that gastric carcinoma cells express CEA (**Figure 3A**), and that CEA glycan structure from ST3Gal IV transfected cells contains the SLe^x antigen (**Figure 3B**). Furthermore, it was observed a shift in the type of sialylation with a reduced α2,6 sialic acids and an increase in α2,3 sialylation present in CEA from ST3Gal IV cells (**Figure 3C**). Our results also show that CEA glycoprotein molecules from MST3Gal IV cells display a slightly higher molecular weight when compared with CEA protein molecules from Mock cells. These differences can be due to the alteration of the glycan structures in CEA protein molecules from MST3Gal IV cells.

To further confirm the expression of SLe^x in CEA molecules, Mock and MST3Gal IV gastric cells were evaluated by PLA, which indicates close proximity of the two molecules. The PLA results show positive expression signals in MST3GAl IV cells confirming that SLe^x and CEA are in molecular proximity (**Figure 3D**).

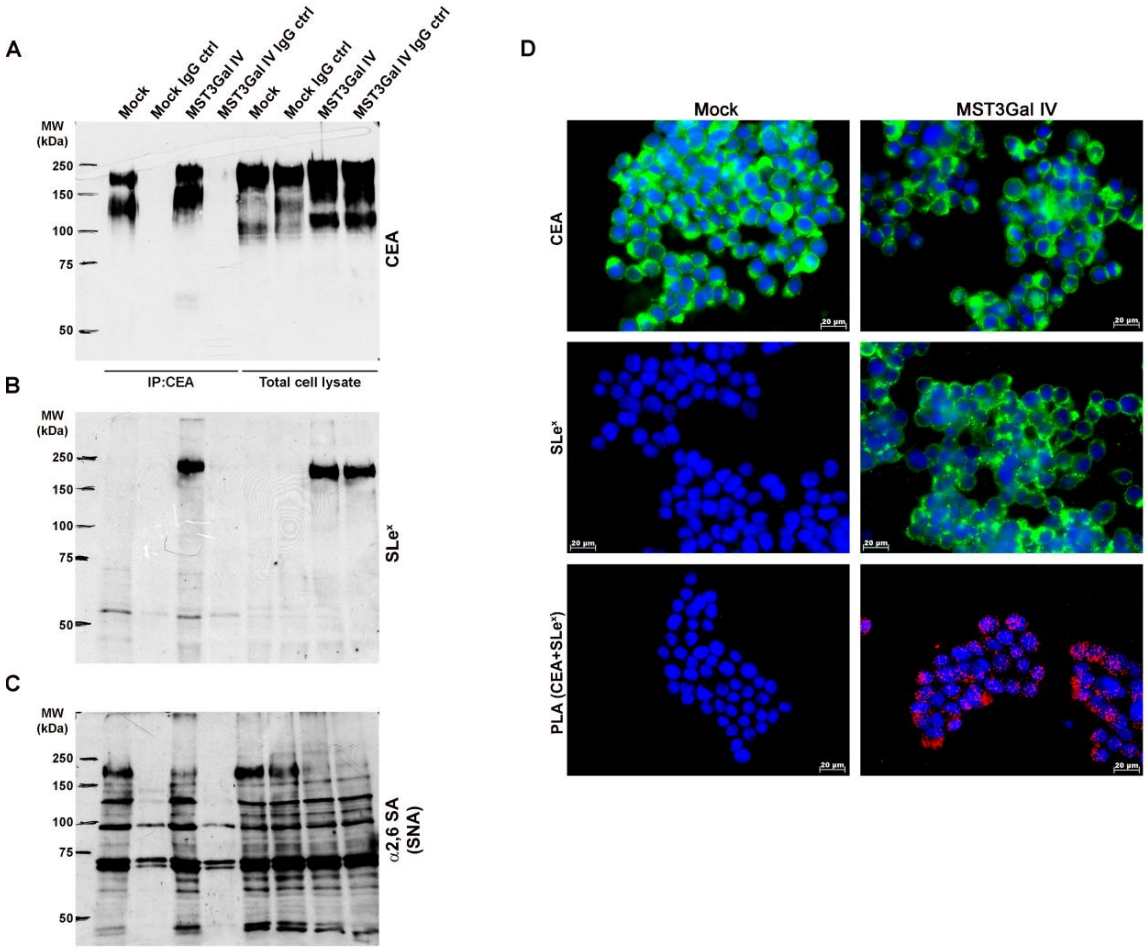

Figure 3: SLe^x expression in CEA molecules from MST3Gal IV. To assess expression of SLe^x in CEA molecules immunoprecipitation of proteins and PLA was performed in Mock and MST3Gal IV cells. **A –** CEA Western blot analysis to confirm CEA expression in Mock and MST3Gal IV cells. The results show that CEA is expressed in both cell lines; **B –** SLe^x expression analysis in CEA immunoprecipitated proteins from Mock and MST3Gal IV cells confirming the expression of SLe^x in MST3Gal IV CEA protein; **C –** α2-6 sialic acids expression analysis in CEA immunoprecipitated proteins from Mock and MST3Gal IV showing reduced expression of α2-6 sialic acids in CEA. Lanes: 1-molecular weight; 2-Mock CEA immunoprecipitated proteins; 3- Mock IgG immunoprecipitated proteins (immunoprecipitation control); 4- MST3Gal IV CEA immunoprecipitated proteins; 5- MST3Gal IV IgG immunoprecipitated proteins (immunoprecipitation control); 6- Mock CEA protein input; 7- Mock IgG protein input; 8- MST3Gal IV CEA protein input; 9- MST3Gal IV IgG protein input. **D –**Immunofluorescence analysis was performed to evaluate the expression of CEA and SLe^x in Mock and MST3Gal IV gastric carcinoma cells. Both Mock and MST3Gal IV cells expressed CEA whereas only MST3Gal IV cells expressed SLe^x. To assess if CEA carries SLe^x, PLA analyses was performed and showed that only MST3Gal IV cells present CEA with SLe^x antigen.

SLe^x and CEA expression in gastric carcinoma tissues

We next evaluated the expression of SLe^x and CEA in gastric carcinoma. A series of 31 gastric carcinoma tissues were screened for the expression of SLe^x, CEA and the presence of the CEA/SLe^x molecular complex. **Figure 4** shows representative images of the immunohistochemical expression of CEA, SLe^x and PLA in two gastric carcinoma cases as well as in normal gastric mucosa.

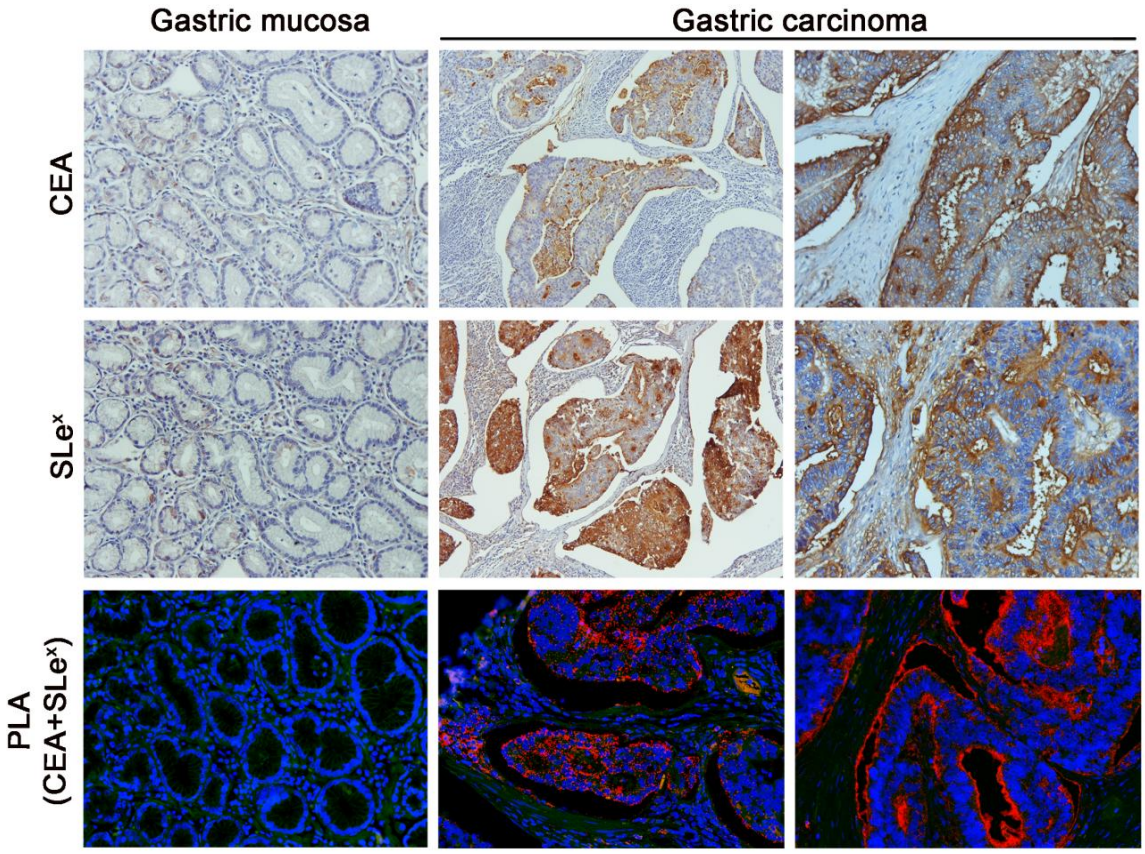

Figure 4: CEA is a SLe^x carrier in gastric carcinoma tissues. Immunohistochemical evaluation of the expression of SLe^x and CEA in gastric carcinoma tissues. The figure illustrates a normal gastric mucosa (left column) and two gastric carcinomas. The result shows CEA and SLe^x expression in gastric carcinoma tissues and no expression in normal gastric mucosa. PLA signal was only observed in gastric carcinoma (200x magnification).

Normal and adjacent mucosa to gastric carcinomas showed no CEA expression whereas few cells with a weak immunostaining were observed for SLe^x in gastric mucosa. Every gastric carcinoma showed CEA expression (100%) with staining varying from less than 25% to more than 75% of the carcinoma cells. Expression of CEA were associated with lymphatic invasion and venous invasion (**Table 2**). There was also a trend for association between CEA expression and more advanced stages of gastric carcinoma (pTNM staging).

Expression of SLe^x was observed in 26 (83.9%) out of 31 gastric carcinoma cases. The expression of SLe^x was heterogeneous among the cases, with 9 (29%) cases showing more than 75% positive cells, 17 (54.9%) cases showing 25-75% positive cells, and 5 (16.1%) cases being negative or showing few positive cells (**Table 2**). The levels of SLe^x expression were associated with Ming´s classification (**Table 2**).

Table 2: Expression of CEA and SLe^x in gastric carcinoma tissues, and expression of CEA/SLe^x by PLA

	CEA				SLe^x				PLA signal		
	0-25%	**25-75%**	**>75%**	***P****	**0-25%**	**25-75%**	**>75%**	***P****	**Neg.**	**Pos.**	***P****
Laurén classification											
Intestinal (n=13)	1 (7.7%)	2 (15.4%)	10 (76.9%)		1 (7.7%)	9 (69.2%)	3 (23.1%)		1 (7.7%)	12 (92.3%)	
Diffuse (n=2)	0 (0%)	1 (50%)	1 (50%)	ns	0 (0%)	2 (100%)	0 (0%)	ns	1 (50%)	1 (50%)	ns
Unclassified (n=16)	1 (6.2%)	5 (31.3%)	10 (62.5%)		5 (25%)	6 (37.5%)	6 (37.5%)		4 (25%)	12 (75%)	
Carneiro classification											
Glandular (n=13)	1 (7.7%)	2 (15.4%)	10 (76.9%)		1 (7.7%)	9 (69.2%)	3 (23.1%)		1 (7.7%)	12 (92.3%)	
Mixed (n=15)	1 (6.7%)	5 (33.3%)	9 (60 %)	ns	3 (20%)	6 (40%)	6 (40%)	ns	3 (20%)	12 (80%)	0.069
Others (Isolated cells + solid) (n=3)	0 (0 %)	1 (33.3%)	2 (66.7%)		1 (33.3%)	2 (66.7%)	0 (0%)		2 (66.7%)	1 (33.3%)	
Ming classification											
Expansive (n=8)	1 (12.5%)	2 (25%)	5 (62.5%)		4 (40%)	3 (37.5%)	1 (12.5%)		4 (50%)	4 (50%)	
Infiltrative (n=22)	1 (4.5%)	5 (22.8%)	16 (72.7%)	ns	1 (4.5%)	13 (59.1%)	8 (36.4%)	0.04	2 (9.1%)	20 (90.9%)	0.038
Unclassified (n=1)	0 (0%)	1 (100%)	0 (0%)		0 (0%)	1 (100%)	0 (0%)		0 (0%)	1 (100%)	
OMS classification											
Well-differentiated (n=15)	2 (13.3%)	2 (13.4%)	11 (73.3%)	ns	2 (13.3%)	9 (60%)	4 (26.7%)	ns	2 (13.3%)	13 (86.7%)	ns
Others (n=16)	0 (0%)	6 (37.5%)	10 (62.5%)		3 (18.75%)	8 (50%)	5 (31.25%)		4 (25%)	12 (75%)	
Lymphatic invasion											
Present (n=24)	1 (4.2%)	4 (16.6%)	19 (79.2%)	0.042	4 (16.7%)	13 (54.1%)	7 (29.2%)	ns	4 (16.7%)	20 (83.3%)	ns
Absent (n=7)	1 (14.3%)	4 (57.1%)	2 (28.6%)		1 (14.3%)	4 (57.1%)	2 (28.6%)		2 (28.6%)	5 (71.4%)	
Venous invasion											
Present (n=19)	0 (0%)	3 (15.8%)	16 (84.2%)	0.029	1 (5.3%)	12 (63.1%)	6 (31.6%)	ns	1 (5.3%)	18 (94.7%)	0.012
Absent (n=12)	2 (16.7%)	5 (41.7%)	5 (41.7%)		4 (33.3%)	5 (41.7%)	3 (25%)		5 (41.7%)	7 (58.3%)	
pTNM											
I+II (n=16)	1 (6.25%)	7 (43.75%)	8 (50%)	0.058	4 (25%)	8 (50%)	4 (25 %)	ns	5 (31.25)	11 (68.75%)	0.08
III+IV (n=15)	1 (6.7%)	1 (6.7%)	13 (86.6%)		1 (6.7%)	9 (60%)	5 (33.3%)		1 (6.7%)	14 (93.3%)	
Total	**2 (6.5%)**	**8 (25.8%)**	**21 (67.7%)**		**5 (16.1%)**	**17 (54.9%)**	**9 (29%)**		**6 (19.4%)**	**25 (80.6%)**	

The evaluation of PLA for CEA/SLe^x showed no signal in normal or adjacent gastric mucosa. In gastric carcinomas, PLA results showed that 25 out of 31 cases (80.6%) were positive. Furthermore, 25 out of the 26 cases that were positive for SLe^x (displaying from 25-100% of positive cells) were also positive in PLA. The evaluation of the association between PLA signal and clinicopathological variables of the cases showed that PLA signal was correlated with Ming´s classification and venous invasion (**Table 2**). In addition there was a trend for association between PLA signals and Carneiro´s classification and pTNM staging.

DISCUSSION

The identification and characterization of molecular markers specific of cells undergoing malignant transformation are key steps for the selection of biomarkers that can be used for improvement of cancer diagnosis and patient's monitoring. The cancer cell proteome has been a target for many studies in the last years, and post translational modifications of proteins, such as glycosylation, can provide key insights and added information to the proteome alterations in cancer (Hakomori 2002; Drake *et al.,* 2010). Glycosylation is deregulated in human cancers resulting in the expression of distinct carbohydrate profiles in malignant tissues (Hakomori 2002; Drake *et al.,* 2010; Reis *et al.,* 2010) and therefore, protein aberrant glycosylation represents an important target for novel cancer biomarker discovery. One of the most typical glycosylation alterations in cancer is characterized by increased cellular sialylation. Many cancers specifically express sialylated structures such as the SLe^x antigen (Hakomori 2002), which is known to be associated with an increase of ST3Gal IV sialyltransferase activity (Ellies *et al.,* 2002; Sperandio *et al.,* 2006; Carvalho *et al.,* 2010; Jun *et al.,* 2012).

In this study we have confirmed that ST3Gal IV overexpression leads to SLe^x biosynthesis in membrane-associated proteins (Carvalho *et al.,* 2010), and also demonstrated expression of SLe^x in secreted proteins from gastric carcinoma cells. The SLe^x expression analysis of MST3Gal IV cells revealed a restricted expression limited to a set of glycoproteins, demonstrating specificity of the SLe^x protein carriers. In addition, analysis of α2,6 sialic acid expression in MST3Gal IV cells, determined by SNA staining, showed that α2,6-sialylation was reduced in the protein bands that were positive for SLe^x. This concurrent expression of SLe^x (α2,3 sialic acid) and α 2,6 sialic acid in MST3Gal IV and in Mock control cells, respectively, suggests an enzymatic competition in the addition of sialic acids to glycoproteins. This can result from the increased expression of ST3Gal

IV in the cell and its location in the Golgi compartment that may limit the CMP-sialic acid donor and the acceptor substrate availability in the cell due to previous α2,3 sialylation.

Previous studies have shown that SLe^x expression is implicated in the malignant behavior of cancer cells and is associated with more aggressive tumors (Nakamori *et al.,* 1993; Nakamori *et al.,* 1997; Amado *et al.,* 1998; Fukuoka *et al.,* 1998; Kim *et al.,* 1998). Recently, we evaluated the biological role of SLe^x in a gastric carcinoma cell model, showing that these cells display increased adhesion capacity to extracellular matrix proteins as well as an increased invasive phenotype both *in vitro* and *in vivo* (Gomes *et al.,* 2013b). This phenotype was also associated with an increased activation of c-Met signaling pathways controlling cellular motility and invasion (Gomes *et al.,* 2013b). These previous observations highlight the importance of identifying the glycoproteins carrying SLe^x and determining the biological contribution of such specific protein glycosylation to the malignant phenotype of cancer cells. In this regard, and in order to disclose new targets for diagnostic and therapeutic applications, in this work we identify the proteins carriers of SLe^x by MALDI-TOF/TOF mass spectrometry analysis. The protein identification revealed CEA as a major SLe^x glycoprotein carrier in the ST3Gal IV expressing cells. The SLe^x expression in CEA was confirmed by immunoprecipitation and PLA assays. The immunoprecipitation results showed that MKN45 cells expresses CEA and the SLe^x western blot analysis confirmed the presence of this glycan structure in CEA molecules in ST3Gal IV expressing cells. These observations were further supported with the PLA results showing positive signal for CEA/SLe^x molecular complexes in ST3Gal IV expressing cells when compared with no PLA signal in the Mock cells.

CEA is a heavily glycosylated protein (Paxton *et al.,* 1987) with 28 possible *N*-glycosylation sites described (Oikawa *et al.,* 1987). CEA shows limited expression in normal adult tissues, most restricted to epithelial cells (Frangsmyr *et al.,* 1999; Hammarstrom 1999). The cellular expression of CEA is limited to the glycocalyx commonly in the apical membrane domain of the cell as a glycophosphatidylinositol-linked surface glycoprotein (Hefta *et al.,* 1988; Takami *et al.,* 1988; Frangsmyr *et al.,* 1999; Hammarstrom 1999). However, the loss of cell polarity and tissue architecture observed in cancer leads to the shedding of CEA into the blood of cancer patients which can be detected by the CEA serological assay (Gold *et al.,* 1965; Benchimol *et al.,* 1989). Additionally, the increased invasive and metastasis capacity of cancer cells also result in release of CEA molecules into the lymph and blood vessels further contributing for the increase CEA serum detection.

Currently, CEA serological detection in the cancer clinical setting is mostly limited to monitoring the disease with limited application in diagnosis. The differential diagnostic application of benign and malignant lesions remains a hurdle which can be eventually overcome by taking into consideration the type and level of glycosylation of the CEA protein (Garcia *et al.,* 1991; Saeland *et al.,* 2012). In this work, we also observed that SDS-PAGE analysis of the CEA immunoprecipitated from MST3Gal IV cells displayed a slightly higher molecular weight than CEA from Mock cells. These molecular weight variations in CEA may be attributed to its different glycosylation pattern in ST3Gal IV cells. This feature has been previously observed in colonic tissues, where normal colonic mucosa and preneoplastic lesions display CEA molecules with different molecular weights when compared to colon cancer cells (Garcia *et al.,* 1991).

In gastric cancer the overexpression of CEA has been well documented (Chevinsky 1991; Berinstein 2002) with association between the increased serum levels and poor outcome of the patients (Park *et al.,* 2008; Chen *et al.,* 2012). Nevertheless, at the moment there is no information on the glycosylation modifications of CEA in gastric tissues. Therefore in order to clarify the potential application of CEA glycosylation in gastric carcinoma tissues we evaluated possible associations with clinicopathological characteristics of the cases. Our results showed that all gastric carcinoma cases evaluated expressed CEA, in contrast with an absence of detection in adjacent normal gastric tissue. Furthermore, analysis of SLe^x expression in gastric carcinomas showed that 83.9% of the cases displayed SLe^x antigen. The evaluation of expression of both SLe^x antigen and CEA molecules in gastric carcinoma tissues by PLA revealed that 80.6% of the cases showed CEA/SLe^x PLA signal, strongly suggesting that CEA carries SLe^x antigens in gastric carcinoma. The expression of CEA/SLe^x, detected by PLA, was significantly associated with clinicopathological features of the cases, including the pattern of tumor growth (Ming's classification) and the presence of venous invasion.

The importance of glycosylation changes during tumorigenesis has been described in other cancer biomarkers used in the clinical practice. Some reports have demonstrated altered PSA glycosylation in benign and malignant prostate lesions, highlighting the importance in considering the glycan composition of this biomarker in the clinical diagnosis of the disease (Peracaula *et al.,* 2003; Tajiri *et al.,* 2008; Meany *et al.,* 2009; White *et al.,* 2009; Dwek *et al.,* 2010; Li *et al.,* 2011; Gilgunn *et al.,* 2012; Vermassen *et al.,* 2012). Furthermore, CEA has been also reported as displaying abnormal glycosylation in colon cancer (Saeland *et al.,* 2012) and has been demonstrated to contribute to the tumor biological behavior and immune evasion due to

interactions of CEA glycans with lectins of the immune system (van Gisbergen *et al.,* 2005; Nonaka *et al.,* 2008; Saeland *et al.,* 2012). As shown for PSA, the evaluation of CEA glycoforms also offers interesting diagnostic perspectives to improve specificity to this serum biomarker.

In conclusion, we report the identification of CEA as a SLe^x protein carrier in the ST3Gal IV expressing gastric carcinoma cells, and we validate this association in a series of gastric carcinoma tissues. The expression of CEA/SLe^x in gastric carcinoma detected by PLA was associated with the presence of venous invasion, supporting the role of SLe^x for an aggressive behavior of the tumor cells. Our results open new avenues for addressing CEA altered glycosylation with SLe^x in order to improve diagnosis and potential therapeutic decisions in gastric cancer.

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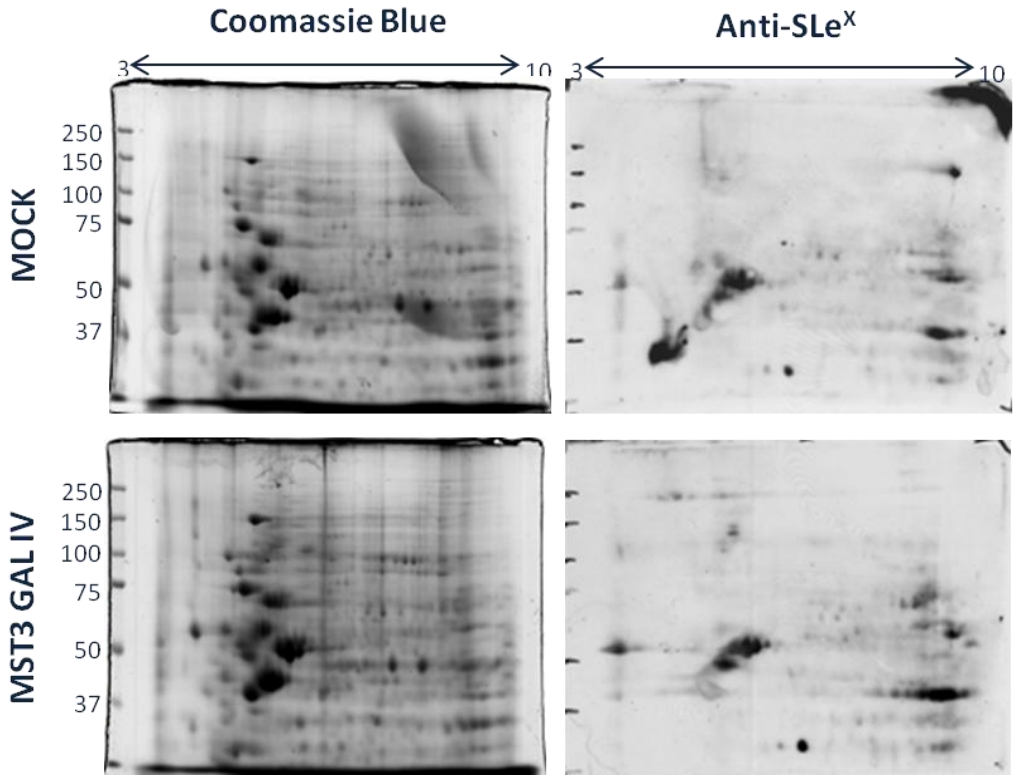
SUPPLEMENTARY DATA

1) Detection of SLe^x protein carriers by 2D gel electrophoresis separation

Material and Methods: 2D gel electrophoresis

Total cell proteins were precipitated using a ProteoExtract Kit (Calbiochem), resuspended in rehydration buffer (7M Urea, 2M Thiourea, 4% (v/v) CHAPS and 0.0002% Bromophenol Blue) with 0.2% of ampholyte and quantified using a 2D Quant Kit (GE Healthcare). Passive rehydration of the strips was performed overnight with 200 µg of total protein using IPG strips of pH 3-10 NL (ReadyStrip; 0.5 x3 x70 mm, Bio-Rad) at room temperature. Isoelectric focusing was performed on Protean IEF cell (Bio-Rad) with an initial voltage of 250 V for 15 min, and then by applying a voltage gradient up to 4000 V with limiting current of 50 µA per strip and temperature set at 20°C. The first dimension was concluded at 14-20 kVh. Following the isoelectric focusing proteins were reduced and alkylated by incubation with 2% DL-dithiothreitol (DTT)(Sigma) followed by incubation with 2.5% Iodoacetamide (Sigma) in an equilibration buffer (6M Urea, 2% SDS, 0.002% Bromophenol Blue, 75 mM Tris pH8.8, 29.3% Glycerol) for 15 min each under gentle agitation. The strips were then packed in a 1% low gelling (1% agarose in running buffer - 25 mM Tris, 192 mM Glycine, and 0.1% (w/v) SDS, pH 8.3; Bio-Rad) on top of a 9% acrylamide gel (acrylamide/bisacrylamide 37.5:1, 2.6% from Bio-Rad). Second dimension electrophoresis was performed in a Mini-Protean tetra cell system (Bio-Rad) using 1xTris/Glycine/SDS buffer (Bio-Rad) at constant voltage of 125 V.



Supplementary Figure 1: 2D gel electrophoresis and Western blot analysis of SLe^x antigen from Mock and MST3Gal IV proteins. Coomassie blue gels from Mock and MST3Gal IV proteins are represented in the left side of the figure and Western blot analysis of SLe^x antigen is represented in the right side. Proteins from total cell lysates of Mock and MST3Gal IV cells were separated in 2D gels and proteins highlighted in the Western blot were matched and excised for protein identification by MALDI-TOF/TOF analysis. The 2D methodology difficult the separation and identification of high molecular weight proteins that were positive for SLe^x antigen. The proteins identified were not confirmed to carry SLe^x antigen.

Chapter 4

General Discussion Summary and Conclusions

Content

GENERAL DISCUSSION

Cancer is a leading cause of death worldwide and a remarkable effort has been made to improve cancer diagnosis and cancer patients treatment, in order to reduce cancer related deaths.

Many of the studies on cancer research aim at the discovery of new and appropriate biomarkers for cancer detection. Nowadays, various serological assays have been approved in the clinics such as carcinoembryonic antigen (CEA) for colorectal cancer, cancer antigen 125 (CA125) for ovarian cancer, carbohydrate antigen 19-9 (CA19.9) for pancreatic and gastric cancer, and prostate-specific antigen (PSA) for prostate cancer (Drake *et al.,* 2010; Reis *et al.,* 2010; Pan *et al.,* 2011). However, the clinical application of these biomarkers is mostly for monitoring treatment and relapses with no consensual application in diagnosis. The limited diagnosis application of these biomarkers is mostly due to the low tumor specificity and sensitivity, being an imperative the discovery of new cancer biomarkers to improve cancer diagnosis.

Gastric cancer remains one of the most common cancers and is the second leading cause of cancer-related death in the world, although the trends in gastric cancer incidence have been declining along the last decades (Ferlay *et al.,* 2010). In clinical practice, CA19-9 (SLe^a antigen) is one of the serological markers used in gastric cancer. Nevertheless, this biomarker lacks the level of sensitivity and specificity required for the early detection of stomach carcinoma and is mainly employed for post resection monitoring rather than diagnostic purposes (Carpelan-Holmstrom *et al.,* 2002; Qiu *et al.,* 2009).

Alterations of glycosylation are commonly observed in pathological conditions, including gastric cancer (David *et al.,* 1992; Amado *et al.,* 1998). During carcinogenesis, glycans are recognized to be involved in different stages of tumor progression, being key players in the proliferation, invasion, metastasis and angiogenesis processes (Fuster *et al.,* 2005). Deregulation of glycosylation observed in cancer leads to the expression of distinct carbohydrate profiles in malignant tissues (Hakomori 2002; Drake *et al.,* 2010; Reis *et al.,* 2010) and therefore, protein aberrant glycosylation represents an important target for novel cancer biomarker discovery.

GLYCOPROTEOMICS FOR DISCOVERY OF NEW CANCER BIOMARKERS

Glycan structures that decorate cell surfaces present a large structural complexity and are crucial for a wide variety of cellular processes. The high complexity of glycan structures combined with the challenge in the glycan analysis have limited the glycomics, especially when compared to genomics or proteomics. In the last years, advantages in glycomic analysis assist to attenuate the differences in the different "Omic" fields. The recent progress in understanding the importance of glycosylation in both normal and disease conditions have emphasized the value of glycan analysis, specially for finding new biomarkers of disease.

Serum glycoproteomics for biomarker discovery in patients with gastric lesions

Serum remains the ideal biofluid for biomarker identification due to the easy collection and because it frequently displays proteins expressed by pathological tissues. Protein glycosylation alterations are a hallmark in cancer progression, and glycoproteins produced during the carcinogenesis pathway can be released from cells into circulation acting as biosensors of the disease. In the particular case of gastric carcinoma, these changes in glycosylation are also observed in gastric cancer precursor lesions, for instance in gastritis and in intestinal metaplasia (IM), where we found expression of simple mucin type carbohydrate antigens, such as T and STn (David *et al.,* 1992; Ferreira *et al.,* 2006; Conze *et al.,* 2010; Marcos *et al.,* 2011). The expression of these truncated glycans modulates protein function and consequently the cell behavior and immune recognition (Liotta *et al.,* 2001; Pinho *et al.,* 2007), being important as cancer biomarkers (Reis *et al.,* 2010) and presenting potential for immunotherapy (Julien *et al.,* 2009). In light of this, many efforts have been made to develop new glycoproteomic strategies to improve the identification of these glycans and the associated protein carriers in disease.

In the first part of this work (Chapter 3.1) we have focused our attention in the identification of proteins carriers of truncated *O*-glycans that are known to be expressed in the different steps of the gastric carcinogenesis pathway that might be shed into circulation as source for biomarker discovery. To achieve this goal we used two different glycoproteomic strategies: 1) serum protein equalization using a combinatorial peptide ligand library (CPLL) (Thulasiraman *et al.,* 2005); and 2) enrichment of serum glycoproteins, after albumin and immunoglobulins depletion, using lectin affinity chromatography (Abbott *et al.,* 2010). Followed this two procedures proteins were separated according to both their isoelectric point and molecular mass using 2D gel electrophoresis, and truncated *O*-glycans were highlighted using Western blot analysis. The

highlighted proteins were matched to Coomassie blue gels and proteins were identified by MALDI-TOF/TOF analysis.

The detection of serum proteins expressing truncated glycans may reflect the aberrant glycosylation observed in proteins in the pathological tissues. Therefore, the expression of these truncated glycans were firstly evaluated in gastric biopsies, confirming the presence of T and STn antigens in gastritis and in the two types of IM (Figure 3 and Table 2, Chapter 3.1) in agreement with our previous findings (David *et al.,* 1992; Carneiro *et al.,* 1994; Ferreira *et al.,* 2006). Serum from the same individuals were used for the serum proteomic screening. Using the different glycoproteomic approaches described above, we were able to identify proteins displaying T and STn antigens in serum of patients with gastritis, and with both types of IM, comparing with control individuals without any gastric mucosa lesion. The Western blot analysis showed T and STn antigens detection in few proteins of the different clinical groups (Figure 5, chapter 3.1), with T immunoreactivity observed in all groups with higher expression in gastritis whereas STn antigen detection was observed in gastritis, and in complete and incomplete IM groups. The mass spectrometry analysis of the immunoreactive proteins resulted in the identification of four main proteins, namely plasminogen, vitronectin, complement factor H, and histidine-rich glycoprotein. Moreover, the amount of these proteins did not vary significantly between groups, except the pattern of simple mucin type *O*-glycans immunoreactivity that showed considerable alterations.

All of the identified proteins have been described to be glycosylated, however only plasminogen has been reported to carry *O*-glycans. Nevertheless, and according to the NetOGlyc search performed in this study, vitronectin and histidine-rich glycoprotein display putative *O*-glycan sites.

Vitronectin was identified based on STn immunoreactivity in the incomplete type of IM. Despite the lack of reports on vitronectin *O*-glycosylation in humans, contrary to vitronectin of other mammals that have been shown to contain both types of glycosylation (Kitagaki-Ogawa *et al.,* 1990), we could predict three possible *O*-glycan sites (Thr113, Ser137, Thr141) using the NetOGlyc tool. Furthermore, it was demonstrated that despite the homology of about 73% between human and rat vitronectin, the sites of glycosylation are highly conserved and have been shown to be important for the protein function (Sano *et al.,* 2007). Glycosylation in vitronectin has been reported to be important in the interaction of vitronectin with other molecules and on its functional activities (Yoneda *et al.,* 1998). Additionally, vitronectin oligosaccharide moiety was suggested to be relevant in *H. pylori* binding and in the mechanism of the bacterial immune

escape (Ringner *et al.,* 1994; Singh *et al.,* 2010). Taking into consideration the importance of vitronectin glycosylation on its function and the reported bacterial interactions (Ringner *et al.,* 1994; Singh *et al.,* 2010), our findings may point towards a possible role in the context of gastric lesions development. In addition, and taking in consideration the fact that patients with incomplete IM present higher risk of developing gastric cancer when compared with complete IM (Silva *et al.,* 1990; Rokkas *et al.,* 1991; Tosi *et al.,* 1993), it would be worthy the deeply study of the role of altered vitronectin glycosylation in incomplete intestinal metaplasia context.

Regarding histidine-rich glycoprotein, our study identified it as expressing truncated *O*-glycans, presenting reactivity with T antigen in the gastritis group. Even though regarded as an *N*-glycosylated protein (6 described sites and 3 predicted), the NetOGlyc tool identified Ser307 as a putative glycosylation site. Histidine-rich glycoprotein is a plasma glycoprotein produced by the liver and known to bind to a number of ligands in circulation, such as heparin, heparan sulfate, thrombospondin, and plasminogen. Histidine-rich glycoprotein acts as an adapter protein and has been implicated in regulating many processes such as immune complex and pathogen clearance, cell adhesion, angiogenesis, coagulation and fibrinolysis (Ohta *et al.,* 2009). The predicted *O*-glycosylation site indicates that this type of glycosylation could also be present in histidine-rich glycoprotein and suggests that further structural insights on the *O*-glycan moiety may be required to complement previous studies focused on the analysis of the *N*-glycans (Ohta *et al.,* 2009).

Besides vitronectin and histidine-rich glycoprotein, our results showed immunoreactivity to STn antigen in all pathological conditions in proteins from the complement system, namely complement factor H, complement H-related protein and complement C4-B. The human complement pathway is a highly controlled effector mechanism of the immune system. Over 30 plasma proteins and membrane bound molecules are involved in the complement system and most of these proteins are glycosylated. The complement factor H, complement factor H-related protein and the complement C4-B are proteins that have been described to be *N*-glycosylated (Ritchie *et al.,* 2002). Most of the protein modules that form the complement system have been crystallized and structural data provides evidence for the role of *N*-glycans in this system. However, the structure and role of glycans in the resistance to proteolysis and functional activation within pathological conditions is still incomplete. As these data become available, the glycans can be modeled at the appropriate locations and give further insights into the interaction between complement proteins and cofactors.

Overall, our results indicate that aberrant *O*-glycosylation can be detected in these three main serum glycoproteins (vitronectin, histidine-rich glycoprotein and complement factor H), however warrants further structural characterization of the glycans to confirm the presence of these aberrant glycosylation in gastric lesions. Nevertheless, in this study from the four main proteins identified, plasminogen has been described as being *O*-glycosylated, and the respective glycan composition has been demonstrated to be important for its function (Santos *et al.,* 2010). Taking this fact in consideration, we decided to deepen with the structural characterization of glycans in plasminogen from serum of individuals with IM.

Serum plasminogen glycan characterization in gastric lesions

The identification of glycan alterations in gastric cancer has been limited to alterations on mucin glycoproteins (David *et al.,* 1992; Carneiro *et al.,* 1994; Amado *et al.,* 1998; Reis *et al.,* 1998; Conze *et al.,* 2010) or in glycosyltransferases and their associated glycans that show increased expression in cancer (Gretschel *et al.,* 2003; Kim *et al.,* 2004; Marcos *et al.,* 2011; Jun *et al.,* 2012). In addition, most of the proteomic studies in serum of gastric cancer patients are based in the differential expression of proteins compared with serum from normal individuals (Aburatani 2005; Miki *et al.,* 2007; Lam *et al.,* 2008; Liu *et al.,* 2010a; Liu *et al.,* 2012) with few reports about serum protein altered glycosylation (Adamczyk *et al.,* 2012; Lin *et al.,* 2012). To our knowledge, very few studies have succeeded in the detection of alterations in serum proteins glycosylation and identification of new biomarkers in stomach cancer. One of the first studies was performed by Goodarzi and Turner in 1998, which detected differences in the *N*-glycosylation pattern of a specific protein, haptoglobin, in gastric cancer claiming a potentially clinically application of haptoglobin glycosylation changes in patient samples (Goodarzi *et al.,* 1998). In 2010, Bones *et al.* were able to identify cancer associated alterations in serum of patients bearing stomach adenocarcinoma (Bones *et al.,* 2010). In this study the authors evaluated the contribution of *N*-glycosylation present on four highly abundant serum glycoproteins as they found an increase in sialylation of haptoglobin, transferrin, and α1-acid glycoprotein and increased levels of core fucosylated biantennary glycans and decreased levels of monogalactosylated core fucosylated biantennary glycans on IgG (Bones *et al.,* 2010). More recently, Bones *et al.* showed an increase in the levels of SLe^x present on triantennary glycans of clusterin, leucine-rich-R2-glycoprotein, and kininogen-1 and also an increased levels of core fucosylated agalactosyl

biantennary glycans present on IgG which were associated with increasing disease pathogenesis (Bones *et al.,* 2011).

In our study, plasminogen was identified due to the immunoreactivity for T and STn antigen in patients with preneoplastic lesions in the gastric mucosa as well as in gastric carcinoma, with no immunoreactivity in healthy controls. Interestingly, among the identified proteins carrying simple mucin type carbohydrate antigens, plasminogen showed the most differentiated pattern of *O*-glycosylation in IM and gastric cancer, displaying decreased levels of T antigens accompanied by an increased expression of STn when compared with the other clinical groups.

Liver is the primary tissue for plasminogen synthesis (Raum *et al.,* 1980) but other tissue sources, including the gut, have been described in animal models (Zhang *et al.,* 2002). The truncated *O*-glycans observed in circulating plasminogen from gastritis, IM and gastric cancer patients may either reflect altered glycosylation in the liver response to inflammatory cytokines or altered glycosylation of locally expressed plasminogen. Pro-inflammatory cytokines produced within the gastric disease context (Goll *et al.,* 2007; Chang *et al.,* 2008; Haghazali *et al.,* 2011), may induce differential expression of glycosyltranferases in hepatocytes leading to alteration of glycosylation of circulating proteins (Gabay *et al.,* 1999). This is in agreement with previous studies that have shown that modification of glycosylation characterized by increased expression of sialylated glycan structures, are observed in hepatocyte derived proteins during acute and chronic inflammatory diseases (De Graaf *et al.,* 1993; Brinkman-van der Linden *et al.,* 1998; Peracaula *et al.,* 2010; Sarrats *et al.,* 2010). Moreover, modification of glycosylation in gastric cancer has been observed in hepatocyte derived proteins and in immune system glycoproteins such as immunoglobulins (Bones *et al.,* 2010; Bones *et al.,* 2011). In addition, alterations of plasminogen may also be related with the gastric mucosa infection by *H. pylori*. Plasminogen-binding proteins expressed in *H. pylori* leads to subsequent activation of plasminogen in plasmin (Pantzar *et al.,* 1998; Jonsson *et al.,* 2004) that may provide proteolytic capacity and may contribute for the pathogenesis of this bacterium (Pantzar *et al.,* 1998; Jonsson *et al.,* 2004). Also, the increased expression of urokinase plasminogen activator has been described in *H. pylori*-associated gastritis (Gotz *et al.,* 1996; Goto *et al.,* 2011). These findings may point towards an important role of plasminogen activation in pathological conditions of the gastric mucosa.

In this study, and in order to obtain new insights about plasminogen glycosylation in the serum of individuals with gastric lesions, we performed a detailed analysis of the *O*-glycans from purified plasminogen from IM patients to validate the STn glycan detection at the molecular level.

Plasminogen *O*-glycans released by reductive β-elimination were permethylated and analyzed by MALDI mass spectrometry. Our results showed the detection of ions compatible with STn antigens (Figure 6, Chapter 3.1). Further MALDI-MS/MS analysis of the ion at *m/z* 691.4 Da exhibited glycosidic bond cleavages B, C and Z, and cross-ring fragmentation A and X product ions characteristic of STn glycan, therefore demonstrating the presence of STn in plasminogen from serum of incomplete IM patients.

Two major glycoforms of plasminogen have been described in humans: type I plasminogen containing two glycosylation moieties (*N*-linked to Asn289 and *O*-linked to Thr346), and type II plasminogen containing a single *O*-linked sugar on Thr346 (Hayes *et al.,* 1979a; Hayes *et al.,* 1979b). However, additional sites of *O*-glycosylation of plasminogen have been reported in Ser248 (Pirie-Shepherd *et al.,* 1997), and Thr339 (Hortin 1990). In this study we further performed a MALDI structural analysis of sialoglycopeptides from plasminogen enriched by titanium dioxide chromatography demonstrating the presence of STn containing glycopeptides in all disease groups. The use of the sialoglycopeptides enrichment method by titanium dioxide followed by MALDI showed to be quite efficient with the identification of the three *O*-glycosylation sites and one *N*-glycosylation site previously described in human plasminogen (Hayes *et al.,* 1979a; Hayes *et al.,* 1979b; Hortin 1990; Pirie-Shepherd *et al.,* 1997) (Supplementary Figure 1 and Supplementary Table 1, Chapter 3.1). Based on this approach the analysis of the clinical groups lead to the identification of one STn-containing glycopeptide in healthy control, five STn-containing glycopeptides in gastritis, four in complete IM and eight in incomplete IM (Table 5, Chapter 3.1). Some of the sites identified have not been described for plasminogen and may constitute potential novel biomarkers of pre-cancerous gastric lesions and gastric cancer. Nevertheless, only one (Thr346) of these glycosylation sites could be theoretically predicted using the bioinformatics platform NetOGlyc. On the other hand this approach retrieved other putative glycosylation sites that are still lacking *in vivo* confirmation. The discrepancy between these findings has been previous highlighted for serum proteins (Ferreira *et al.,* 2011) and suggests that *in vivo* processing and pathophysiological states may play a determinant role in the definition of the glycosylation of circulating glycoproteins.

Furthermore, in our study we were also able to identify STn reactivity in plasminogen from an independent set of serum samples (Supplementary Figure 2 and Supplementary Table 2, Chapter 3.1), enriched for sialic acid containing glycoproteins with SNA lectin from gastritis, intestinal metaplasia, and carcinoma patients. These results using alternative glycoprotein enrichment

approaches and different sample cohorts further demonstrate the potential application of the altered plasminogen STn glycosylation as a biomarker in these pathologies.

In summary, this work present a set of proteins displaying altered *O*-glycosylation as detected by antibodies directed to STn and T antigens in the serum from patients with gastritis, IM and gastric cancer in opposition to minor or no reactivity in the same proteins of healthy individuals without any gastric disease. We further demonstrated that circulating serum plasminogen from IM patients carry the truncated *O*-glycan STn antigen. Altogether these data provide putative novel glycobiomarkers in serum from patients with gastric pathologies, including gastritis, intestinal metaplasia and gastric carcinoma. These results warrant further studies to address the application of plasminogen STn glycosylation pattern as a serum biomarker of gastric pathologies.

MODULATION OF GASTRIC CELLULAR GLYCOPHENOTYPE BY SIALYLTRANSFERASES OVEREXPRESSION: BIOLOGICAL BEHAVIOR AND BIOMARKER IDENTIFICATION

Aberrant glycosylation has been described for many years as a hallmark of cancer, and many of the resulting altered glycosyl epitopes are tumor associated antigens (Hakomori 2002; Drake *et al.,* 2010). These cancer-related antigens are caused by disease-specific alterations in the glycan synthesis pathway such as changes in the Golgi and Endoplasmic Reticulum compartments, mutations in enzymes or chaperons, altered expression of enzymes and biochemical competition, and even variations in sugar donor availability (Reis *et al.,* 2010; Wang *et al.,* 2010; Gill *et al.,* 2013).

A common alteration observed in cancer is the abnormal expression of sialyltransferases, responsible for adding sialic acids residues to cell surface molecules and to secreted proteins, which have been involved in the oncogenic transformation, as well as in invasion and metastasis (Hakomori 2002; Dube *et al.,* 2005; Drake *et al.,* 2010). The identification and characterization of molecular markers specific of cells undergoing malignant transformation are key steps for the selection of biomarkers that can be used for improvement of cancer diagnosis and patient's monitoring.

Biological role of SLe^x expression, due to overexpression of ST3Gal IV, in gastric cancer cells

Sialic acids are typically attached to the outermost ends of glycoproteins and glycolipids that can mediate and modulate a wide variety of physiological and pathological processes (Varki 2008). The SLe^x antigen is a sialylated glycan structure which expression has been associated with cancer progression and aggressiveness (Nakamori *et al.,* 1997; Baldus *et al.,* 1998; Fukuoka *et al.,* 1998; Kim *et al.,* 1998; Nakamori *et al.,* 1999; Grabowski *et al.,* 2000; Borsig *et al.,* 2002). In the gastric carcinoma context, it has also been described that increased expression of SLe^x is associated with an aggressive cancer cell behavior as well as poor overall patient survival (Amado *et al.,* 1998; Tatsumi *et al.,* 1998). The expression of SLe^x in cancer results from the altered expression of sialyltransferases, that adds the sialic acid in a α2,3 linkage to Galactose residues on type-II chains (Harduin-Lepers *et al.,* 2012).

In the second part of this study (Chapter 3.2), we evaluate the capacity of two sialyltransferases, ST3Gal III and ST3Gal IV, to produce SLe^x both in glycoproteins and in glycolipids in gastric carcinoma cells. Expression analyzes of SLe^x, in both ST3Gal III and ST3Gal IV stably transfected gastric carcinoma cells, by immunofluorescence and Western blot confirmed that ST3Gal IV leads to the biosynthesis of SLe^x antigens (Figure 1, Chapter 3.2). Moreover, our results indicate that SLe^x antigen is expressed on proteins from total cell lysates as well as on secreted proteins from cells expressing ST3Gal IV. Furthermore, glycolipidic component analysis by mass spectrometry demonstrated that the pattern of glycosylation did not change in both ST3Gal III and ST3GalIV transfected cells when compared with Mock cells (Supplementary results, Chapter 3.2). These results demonstrated that ST3Gal IV participate in SLe^x biosynthesis, preferentially in glycoproteins. Overall, these results confirm previous observations that described the importance of ST3Gal IV in the synthesis of SLe^x (Ellies *et al.,* 2002; Sperandio *et al.,* 2006), and are in agreement with recent reports showing an increased mRNA level of ST3Gal IV and α2,3 sialic acid residues expression in gastric cancer tissues (Jun *et al.,* 2012).

The carbohydrate SLe^x functions as a ligand for cell adhesion molecules of the selectin family, usually expressed on vascular endothelial cells, and the expression of SLe^x on cancer cells is known to facilitate tumor cell spreading by mediating tumor-endothelial cell interactions contributing to hematogenous metastasis (Takada *et al.,* 1993; Kannagi 1997; Kannagi *et al.,* 2004). These previous observations further support the hypothesis that SLe^x antigen plays a functional role in malignant cancer cell behavior. Noteworthily, the crosstalk between cancer cells

and host mechanisms like cell-cell adhesion and cell-matrix adhesion interactions, tumor cell growth and motility are known to be important in modulating the process of cancer cell invasion. On light of this, and on the clinical pathological evidence that SLe^x expression in gastric cancer are associated with invasive carcinomas (Amado *et al.,* 1998), we analyzed the *in vitro* and *in vivo* biological behavior of SLe^x expressing cells. Thus, we performed a comprehensive evaluation of the biological role of SLe^x in gastric cancer cells using classical *in vitro* models and the *in vivo* chorioallantoic membrane of the chicken embryo (CAM) model. The *in vitro* analysis showed that SLe^x expressing cells display a similar proliferative rate when compared with Mock transfected cells. However, SLe^x expressing cells demonstrated a higher capacity to invade *in vitro* in Matrigel chambers, demonstrating the active role of this sialylated glycan structure in tumor cell motility and invasion. Concomitant to this invasive capacity, SLe^x expressing cells evidenced higher capacity to adhere to collagen IV and vitronectin extracellular matrix proteins (Figure 2, Chapter 3.2). Furthermore, this invasive phenotype was also confirmed *in vivo* where cells transfected with ST3Gal IV and expressing SLe^x antigen presented increased capacity to invade the chorioallantoic membrane of the chicken embryo (Figure 3 and Table 1, Chapter 3.2). These findings, altogether highlight the importance of this sialylated glycan in the malignant invasive phenotype, and are in keeping with studies that associate SLe^x expressing tumors with more aggressive phenotypes (Nakamori *et al.,* 1993; Amado *et al.,* 1998; Tatsumi *et al.,* 1998; Ichikawa *et al.,* 2000). In conclusion, these results support the role of ST3Gal IV in the biosynthesis of SLe^x glycan antigen and evidenced an important role in gastric tumor invasion. However, the molecular mechanisms underlying this altered enzyme expression and aggressive behavior of gastric cancer cells expressing these glycan determinants are not fully understood.

Role of signaling pathways in gastric cancer cells expressing SLe^x structures

Previous reports have shown that increased cellular sialylation leads to receptor and signaling pathways activation and that the hypersialylation contributes to cancer progression and increased cell motility (Seales *et al.,* 2005a; Seales *et al.,* 2005b). Moreover, it has been described that TNF-α can induce SLe^x and 6-sulfo-SLe^x expression in human cancer cells, by increasing the expression of ST3GAL4 (Colomb *et al.,* 2012). This mechanism has also been shown to be mediated by neutrophils expressing TNF-α leading to cancer cells invasiveness and metastasis (St Hill *et al.,* 2011).

Having as starting point the hypothesis that expression of SLe^x can induce activation of signaling pathways involved in cancer cell invasion, we intended to evaluate the possible role of tyrosine kinase receptors activation and downstream signaling effectors in ST3Gal IV expressing cells. To assess receptors activation we used a tyrosine kinase receptor array that allowed the identification of increased activation of c-Met in SLe^x expressing cells (Figure 4, Chapter 3.2). In MKN45 cell line model it has been reported a high level of expression and dependence on c-Met (Smolen *et al.,* 2006) and therefore modulation of cellular glycosylation can have implications in this c-Met dependent cells. In addition, we evaluated the *in vivo* c-Met activation in gastric cancer cells in the CAM model and observed the expression of phosphorylated c-Met on cancer invading cells expressing SLe^x (Figure 4, Chapter 3.2). This result further supports the hypothesis that SLe^x expressing cells exhibit invasive capacity through the activation of c-Met.

In general, c-Met overexpression has been considered a hallmark of cancer, playing a role in many tumors and in metastatic progression (Sierra *et al.,* 2011). In gastric cancer, alterations in c-Met expression have been reported, such as the Tpr/Met rearrangement (Soman *et al.,* 1991; Yu *et al.,* 2000), c-Met copy number amplification (Lee *et al.,* 2011), as well as increased c-Met activation (Inoue *et al.,* 2004; Dua *et al.,* 2011).

The activation of tyrosine receptors, directly or indirectly, by glycan antigens has previously been observed in other cancer cell models. Singh and colleagues described that the Thomsen-Friedenreich antigen (T antigen) present in CD44v6 promotes the activation of c-Met and mitogen-activated protein kinase (MAPK) signaling leading to cancer cell proliferation (Singh *et al.,* 2006). Furthermore, activation of c-Met receptor has been described in a breast cancer cell model that overexpress glycosyltransferases and this activation has been implicated in proliferation and invasion of cancer cells (Cazet *et al.,* 2009; Cazet *et al.,* 2010; Cazet *et al.,* 2012).

The activation of c-Met is well known to induce docking sites for proteins that mediate downstream signaling leading to the activation of the MAPK, phosphatidylinositol 3-kinase (PI3K)-AKT, v-src oncogene homolog (Src), signal transducer and activator of transcription (STAT), which are signaling pathways that are involved in increased cell growth, scattering, motility, invasion, protection from apoptosis, branching morphogenesis, and angiogenesis (Liu *et al.,* 2010b; Organ *et al.,* 2011). Taking that into consideration, we evaluated the downstream effectors of c-Met activation and found that FAK and Src proteins showed increased activation in cells expressing ST3Gal IV (Figure 5, Chapter 3.2). In combination with our invasion assays results (*in vitro* and *in vivo*), these data strongly suggest that c-Met and downstream FAK and Src activation mediates

tumor cell motility and invasion, also in gastric cancer cells. These results are in agreement with previous studies that associate Src-FAK signaling pathway with the metastization process (Peng *et al.,* 2009; Lim *et al.,* 2012; Sanchez-Bailon *et al.,* 2012; Shen *et al.,* 2012). Furthermore, our results show that inhibition of c-Met and Src could preclude the increased invasion observed in SLe^x expressing cells supporting the role of this glycosylation alteration in the activation of this invasive related pathway (Figure 6, Chapter 3.2).

Oncogenic transformation is often associated with changes in organization of the cytoskeleton, which can influence cell migration, adhesion and invasion. The c-Met activation can cause changes in gene expression of cell-cycle regulators (Cdk6 and p27), extracellular matrix proteinases (such as matrix metalloproteinases and urokinase plasminogen activator), and in alterations of cytoskeleton functions that control migration, invasion and proliferation (Birchmeier *et al.,* 2003). The cytoskeleton is composed of a complex and organized network of various fibrous proteins within the cytoplasm, playing an essential structural and regulatory role in the maintenance of cell structure and strength, in cell division, proliferation, motility, invasion and also in signaling functions (Tapon *et al.,* 1997; Machesky *et al.,* 1999; de Curtis *et al.,* 2012). The activation of tyrosine kinase receptors can modify the phosphorylation status of cytoskeleton regulatory and structural proteins. Signaling pathways initiated by the activation of cell surface receptors can promote distinct membrane protrusions by converging onto the Rho family of GTPases (Hall 1999; Kjoller *et al.,* 1999). Rho proteins are small (21-25 kDa) molecules that share structural homology and become activated only when bound to GTP. One of the best characterized Rho GTPase family members is RhoA that regulates the formation of stress fibers and focal adhesion assembly, while Rac1 and Cdc42 are mainly involved in membrane ruffling and formation of filopodia, respectively (Pertz 2010). Estimation of GTPases activation is frequently a molecular marker in the evaluation of cytoskeleton alterations during cell migration (Evers *et al.,* 2000; Parri *et al.,* 2010; Wessler *et al.,* 2011). In this study we showed the activation of Rho GTPases, specifically RhoA, Rac1 and Cdc42 (Figure 5, Chapter 3.2). These results further supports the evidence that SLe^x expression leads to cytoskeleton protein alterations in cancer cells, underlying the observed increased cell motility and invasion of these cells. Our findings are in keeping with previous reports showing the importance of RhoA, Rac1 and Cdc42 in cancer progression (Kamai *et al.,* 2004), and also the crosstalk between these GTPases and other signaling pathways like Src-FAK in the migratory phenotype of cancer cells (Leve *et al.,* 2011).

Our present findings support the hypothesis that increased expression of SLe^x on the surface of malignant cells plays an important role in tumor invasion and metastasis. Overall, our study showed that tumor cell invasion is induced by SLe^x expression on gastric cancer cells through the activation of c-Met in association with downstream signaling effectors Src, FAK and RhoA GTPases activation (Figure 7, Chapter 3.2). These results open new avenues for the designing of intervention strategies that target glycosyltransferases leading to the biosynthesis of SLe^x in cancer cells as well as the inhibition of c-Met and Src in order to improve gastric cancer treatment by targeting invasion and metastasis.

Gastric cancer cell model expressing SLe^x as a source for cancer biomarkers discovery

One of the most typical glycosylation alterations in cancer is characterized by increased cellular sialylation. Many cancers specifically express sialylated structures such as the SLe^x antigen (Hakomori 2002), which is known to be associated with an increase of ST3Gal IV sialyltransferase activity (Ellies *et al.,* 2002; Sperandio *et al.,* 2006; Carvalho *et al.,* 2010; Jun *et al.,* 2012).

In Chapter 3.2 we show expression of SLe^x antigens in proteins from ST3Gal IV overexpressing cells, and this expression revealed to be restricted to a set of glycoproteins, demonstrating specificity of the SLe^x protein carriers. In addition, SLe^x expression was associated with increased invasive capacity of cancer cells, and this result was in accordance to previous studies that demonstrated an association between SLe^x expression with more aggressive tumors (Nakamori *et al.,* 1993; Nakamori *et al.,* 1997; Amado *et al.,* 1998; Fukuoka *et al.,* 1998; Kim *et al.,* 1998). All of these previous observations highlight the importance of identifying the glycoproteins carrying SLe^x and determining the biological contribution of such specific protein glycosylation to the malignant phenotype of cancer cells. In this regard, and in order to disclose new targets for diagnostic and therapeutic applications, in this work (Chapter 3.3) we identify the proteins carriers of SLe^x by MALDI-TOF/TOF mass spectrometry analysis. The protein identification revealed carcinoembryonic antigen (CEA) as a major SLe^x glycoprotein carrier in the ST3Gal IV expressing cells (Figure 2 and Table 1, Chapter 3.3). The SLe^x expression in CEA was confirmed by immunoprecipitation and proximity ligantion assays (PLA). The immunoprecipitation results showed that MKN45 cells expresses CEA and the SLe^x Western blot analysis confirmed the presence of this glycan structure in CEA molecules in ST3Gal IV expressing cells (Figure 3,

Chapter 3.3). These observations were further supported by the PLA results showing positive signal for CEA/SLe^x molecular complexes in ST3Gal IV expressing cells when compared with no PLA signal in the Mock cells (Figure 3, Chapter 3.3).

CEA is a heavily glycosylated protein (Paxton *et al.,* 1987) with 28 possible *N*-glycosylation sites described (Oikawa *et al.,* 1987). CEA shows limited expression in normal adult tissues, most restricted to epithelial cells (Hammarstrom 1999). The cellular expression of CEA is limited to the glycocalyx commonly in the apical membrane domain of the cell as a glycophosphatidylinositol-linked surface glycoprotein (Hefta *et al.,* 1988; Takami *et al.,* 1988; Hammarstrom 1999). However, the loss of cell polarity and tissue architecture observed in cancer leads to the shedding of CEA into the blood of cancer patients which can be detected by the CEA serological assay (Gold *et al.,* 1965; Benchimol *et al.,* 1989). Additionally, the increased invasive and metastasis capacity of cancer cells also result in release of CEA molecules into the lymph and blood vessels further contributing for the increase CEA serum detection.

The use of CEA serological test was first described as a circulating tumor-specific antigen in colon and rectum cancers (Thomson *et al.,* 1969). Currently, CEA serological detection in the cancer clinical setting is mostly limited to monitoring the disease with limited application in diagnosis. In gastric carcinoma, CEA serological test is also used, however its application is mainly to the detection of cancer recurrence (Tamada *et al.,* 1982; Chen *et al.,* 2012) and CEA increased expression has been associated with poor patients' prognosis (Park *et al.,* 2008). The identification of the different CEA glycoforms can contribute to the improvement of the diagnostic application of this biomarker (Garcia *et al.,* 1991; Saeland *et al.,* 2012). In this work, we also observed that SDS-PAGE analysis of the CEA immunoprecipitated from MST3Gal IV cells displayed a slightly higher molecular weight than CEA from Mock cells. These molecular weight variations in CEA may be attributed to its different glycosylation pattern in ST3Gal IV cells. This feature has been previously observed in colonic tissues, where normal colonic mucosa and preneoplastic lesions display CEA molecules with different molecular weights when compared to colon cancer cells (Garcia *et al.,* 1991).

In gastric cancer the overexpression of CEA has been well documented (Chevinsky 1991; Berinstein 2002) with association between the increased serum levels and poor outcome of the patients (Park *et al.,* 2008; Chen *et al.,* 2012). Nevertheless, at the moment there is no information on the glycosylation modifications of CEA in gastric tissues. Therefore in order to clarify the potential application of CEA glycosylation in gastric carcinoma tissues we evaluated

possible associations with clinicopathological characteristics in gastric carcinoma cases. Our results showed that all gastric carcinoma cases evaluated expressed CEA, in contrast with an absence of detection in adjacent normal gastric tissue. Furthermore, analysis of SLe^x expression in gastric carcinomas showed that 83.9% of the cases displayed SLe^x antigen. The evaluation of expression of both SLe^x antigen and CEA molecules in gastric carcinoma tissues by PLA revealed that 80.6% of the cases showed CEA/SLe^x PLA signal, strongly suggesting that CEA carries SLe^x antigens in gastric carcinoma. The expression of CEA/SLe^x, detected by PLA, was significantly associated with clinicopathological features of the cases, including the pattern of tumour growth (Ming's classification) and the presence of venous invasion (Figure 4 and Table 2, Chapter 3.3).

The importance of glycosylation changes during tumorigenesis has been described in other cancer biomarkers used in the clinical practice. Some reports have demonstrated altered PSA glycosylation in benign and malignant prostate lesions, highlighting the importance in considering the glycan composition of this biomarker in the clinical diagnosis of the disease (Peracaula *et al.,* 2003; Tajiri *et al.,* 2008; Meany *et al.,* 2009; White *et al.,* 2009; Dwek *et al.,* 2010; Li *et al.,* 2011; Gilgunn *et al.,* 2012; Vermassen *et al.,* 2012). Furthermore, CEA has been also reported as displaying abnormal glycosylation in colon cancer when compared with normal colonic mucosa and preneoplastic lesions (Saeland *et al.,* 2012) and has been demonstrated to contribute to the tumor biological behavior and immune evasion due to interactions of CEA glycans with lectins of the immune system (van Gisbergen *et al.,* 2005; Nonaka *et al.,* 2008; Saeland *et al.,* 2012). Overall, and as shown for PSA, the evaluation of CEA glycoforms offers interesting diagnostic perspectives to improve specificity of this serum biomarker.

In conclusion, we report the identification of CEA as a SLe^x protein carrier in the ST3Gal IV expressing gastric carcinoma cells, and we validate this association in a series of gastric carcinoma tissues. The expression of CEA/SLe^x in gastric carcinoma, detected by PLA, was associated with the presence of venous invasion, supporting the role of SLe^x for an aggressive behavior of the tumor cells. Our results open new avenues for addressing CEA altered glycosylation with SLe^x in order to improve diagnosis and potential therapeutic decisions in gastric cancer.

In summary, we performed a comprehensive characterization of aberrant protein glycosylation in gastric carcinogenesis, both using serum samples from patients and cell line models, that allows the identification of putative new targets that can be used in clinical practice. The identified

proteins may be particularly valuable for the development of novel glycan-based strategies for cancer diagnosis.

FUTURE PERSPECTIVES IN GLYCAN-BASED SEROLOGICAL ASSAYS IN GASTRIC CANCER

Currently, the clinical application of the existing serological assays in gastric cancer screening lacks the specificity and sensitivity necessary for its application in early diagnosis, being used mostly for monitoring treatment and relapses. Considering the role of glycans during gastric carcinogenesis, the design of new glycan-based approaches constitute a distinct starting point for the identification of newly identified serological targets for cancer detection at the early stages of the disease.

Based on the results presented in this thesis, we intend to validate the glycan structures, namely STn and SLe^x, and the glycan sites on the identified proteins plasminogen and CEA, respectively. Ultimately, we will consider the development of glycan-based assays that specifically recognize the identified proteins and the associated glycan antigens in patients with gastric preneoplastic lesions and with gastric carcinoma.

Recently, it was reported an efficient model to study protein *O*-glycosylation using zinc-finger nuclease-glycoengineered SimpleCell lines technology (Steentoft *et al.,* 2011; Steentoft *et al.,* 2013a; Steentoft *et al.,* 2013b). Using this approach it is possible to knock out Cosmc chaperone that will impair *O*-glycosylation elongation resulting in the synthesis of Tn and STn antigens in *O*-glycoproteins. Taking advantage of this technology we propose to use gastric carcinoma cell lines knock out for Cosmc chaperone to study plasminogen sites of *O*-glycosylation. To achieve this, gastric carcinoma cell lines knocked out for Cosmc can be further transfected with the full length human plasminogen gene, and plasminogen protein enriched by SNA (recognizes α2,6SA residues) and VVA (recognizes Tn antigens) lectin affinity chromatography. Further plasminogen purification steps can be performed, and the specific *O*-glycosylation sites can be determined by mass spectrometry. This strategy will be useful for validating the glycosylation sites identified in serum plasminogen from gastric disease patients. The ultimate goal will be to develop a monoclonal antibody that specifically recognizes one site-specific STn antigen in plasminogen to be used in a ELISA serological assay for screening of patients with gastric lesions.

Regarding our results on CEA, to our knowledge there are no descriptions about SLe^x expression in CEA molecules in gastric cancer. In addition, the relevance of CEA glycosylation in cancer has been described for colon cancer. So, as future work we envision to confirm by mass spectrometry technology the presence of SLe^x in CEA molecules using both cell models and CEA from serum samples. Particularly interesting would be to isolate and characterize the glycosylation of CEA molecules from gastric carcinoma vs non-neoplastic gastric mucosa and define the presence of SLe^x. Finally, we want to evaluate the presence of CEA/SLe^x in serum from gastric cancer patients vs normal individuals, and to develop a novel serological assay for gastric cancer diagnosis.

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SUMMARY AND CONCLUSIONS

Gastric cancer incidence and mortality have remained high over the last past decades, and gastric cancer persists as one of the leading causes of cancer-related death worldwide. This high mortality rate reflects the late diagnosis due to the lack of early screening biomarkers and the appearance of clinical symptoms only when the cancer has already progressed.

Glycosylation alterations of glycoconjugates are a hallmark of cancer, and in gastric cancer many alterations have been described during gastric carcinogenesis. The development of new glycoproteomic strategies to detect glycan alterations observed in cancer has contributed to the improvement of biomarker identification.

This work aimed to identify new possible glycobiomarkers in gastric cancer, based on alterations in the normal glycosylation pattern of glycoproteins. In order to accomplish this aim, we have employed different strategies that allowed us to reach the following conclusions:

1) We have developed a glycoproteomic approach that specifically detected cancer associated truncated *O*-glycans in serum glycoproteins from patients with percursor lesions of gastric cancer nad in patients with gastric cancer. The employed glycoproteomic strategies allowed the identification of glycoproteins presenting truncated *O*-glycans, namely plasminogen.

2) We have characterized the STn structure present on plasminogen in the serum of patients with intestinal metaplasia. Furthermore, we have shown that enriched sialopeptides exhibit mass peak that matched with peak of plasminogen peptides containing STn structures. The sialopeptides analysis has also demonstrated the presence of STn-containing glycopeptides in gastritis, in complete and in incomplete intestinal metaplasia.

These findings highly suggest that plasminogen display *O*-glycosylation modifications during gastric carcinogenesis, and this altered glycosylation may be particularly important for future development of new glycan-based serological assays.

3) We have demonstrated, in a gastric carcinoma cell model, the role of ST3Gal IV in the biosynthesis of SLe^x structures on glycoproteins. Furthermore, we have performed a comprehensive characterization of the biological role of SLe^x expression on gastric carcinoma cells, and have demonstrated the increased invasive capacity of these cells both *in vitro* and *in*

vivo, and an enhanced adhesion capacity to extracellular matrix proteins such as collagen IV and vitronectin.

4) We have evaluated the contribution of SLe^x expression in tyrosine kinase receptor activation, and demonstrated an increased activation of c-Met and downstream effectors such as Src, FAK and GTPases (RhoA, Rac and Cdc42). In addition, inhibition of c-Met and Src activation completely abolished the increased invasive capacity on SLe^x expressing cells.

5) We have identified the glycoproteins expressing SLe^x in our cell model and found that CEA is a protein carrier of SLe^x. Moreover, this co-expression was also demonstrated in gastric carcinoma tissues by PLA, and was correlated with venous invasion of cells.

These results show the involvement of SLe^x in the invasive capacity of gastric cancer cells through activation of c-Met and downstream effectors, and demonstrate the potential application of CEA altered glycosylation as a potential biomarker of invasive gastric carcinomas.

Overall, our results have extended the current knowledge on altered glycosylation observed in gastric cancer as source for biomarker discovery and understanding cancer cell behavior. The identification of glycobiomarkers is opening new avenues for the development of new glycan-based assays leading to the improvement of cancer diagnosis.

Chapter 5

Other Contributions

Content

5.1

Alterations in Glycosylation as Biomarkers for Cancer Detection

Alterations in glycosylation as biomarkers for cancer detection

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ABSTRACT

Glycoconjugates constitute a major class of biomolecules which include glycoproteins, glycosphingolipids and proteoglycans. Glycans are involved in several physiological and pathological conditions, such as host—pathogen interactions, cell differentiation, migration, tumour invasion and metastisation, cell trafficking and signalling. Cancer is associated with glycosylation alterations in glycoproteins and glycolipids. This review describes various aspects of protein glycosylation with the focus on alterations associated with human cancer. The application of these glycosylation modifications as biomarkers for cancer detection in tumour tissues and serological assays is summarised.

INTRODUCTION

Glycosylation is a common post-translational modification of proteins, and variation in oligosaccharide structures is associated with many normal and pathological events: host—pathogen interactions, differentiation, migration, tumour invasion and metastisation, cell trafficking and signalling. Cancer is associated with aberrations in glycolipids and glycoproteins.[1 2] In glycoproteins, about half of which are glycosylated in eukaryotes, both *N*-glycans and *O*-glycans can be synthesised, and both can be affected during cancer progression. *N*-glycans have a functional role in cell adhesion, and modifications in cancer cells are associated with invasion and metastisation.[3] O-Glycosylation of glycoproteins, of which mucin glycoproteins are a major component because of their high content of serine and threonine and the fact that they are highly overexpressed in carcinomas, contributes to a substantial part of cancer biomarkers and will be the focus of the present review. The review is directed to non-specialised scientific readers assumed to be familiar with cancer nomenclature and concepts. It is intended to give an overview of the normal process of glycosylation and the alterations associated with cancer and their usefulness as tumour markers.

GLYCOSYLATION IN HUMAN CELLS

Glycosylation is the covalent attachment of a carbohydrate to a protein, lipid, carbohydrate or other organic compound, catalysed by glycosyltransferases, using specific sugar donor substrates. Glycans are found in several types of biomolecule which can be classified into different families of glycoconjugates: glycoproteins, glycosphingolipids, proteoglycans and glycosylphosphatidylinositol-linked proteins (figure 1).

As mentioned above, there are two types of glycan in glycoproteins: *N*-glycans and *O*-glycans. Both types of glycosylation often coexist in the same protein and in the same cell. N-Glycosylation consists of an oligosaccharide chain N-linked to asparagine in the sequence context Asn-X-Ser/Thr, where X is any amino acid except proline. In rare cases, the sequence Asn-X-Cys is also used. N-Glycosylation requires the production of an oligosaccharide precursor which is transferred en bloc to nascent proteins in the endoplasmic reticulum (ER). After the transfer of the oligosaccharide precursor structure to the nascent protein, several subsequent processing reactions occur in the ER, including cycles of glucose removal and addition, which contribute to protein folding. In addition, *N*-glycan chains can be further diversified in the Golgi apparatus, with terminal saccharide residues.

O-Glycosylation is the other type of glycosylation found in glycoproteins and consists of a glycan O-linked to a serine or a threonine residue (figure 2). The frequency of O-glycosylation on glycoproteins is high, particularly on secreted or membrane-bound mucins, which are rich in serine and threonine. The first step in mucin-type O-glycosylation is the transfer of GalNAc from a sugar donor UDP-GalNAc to serine and threonine residues and is controlled by UDPGalNAc-polypeptide *N*-acetylgalactosaminyl-transferases (ppGalNAc-Ts).[4–6] To date, more than 15 distinct members of the mammalian ppGalNAc-T family have been identified and characterised,[7–20] and in silico analysis indicates that as many as 20 ppGalNAc-Ts may exist.[6] They control the first level of complexity of mucin glycosylation—that is, the sites and density of *O*-glycan occupancy of the mucin tandem repeat. This is because ppGalNAc-Ts, although catalysing the same enzymatic step, display different tissue expression specificity[21 22] and have different kinetic properties and acceptor substrate specificities.[5 11 23] This enzymatic specificity may lead to different functions depending on the cell type and organ in which it is expressed.[9 10 24–26] Altered expression of ppGalNAc-Ts may be one of the mechanisms involved in changes in mucin O-glycosylation during malignant transformation.[21 22 27–30]

A second level of complexity in mucin O-glycosylation is the processing of carbohydrate chains by other glycosyltransferases. After the first glycan (GalNAc) is added forming the Tn antigen, the core 1 structure is synthesised by Gal-transferase (C1GalT-1), which adds Gal to GalNAc, forming the core 1 (T antigen). Alternatively, Tn and T antigens can be sialylated by sialyltransferases forming the sialyl-Tn, sialyl-T and disialyl-T

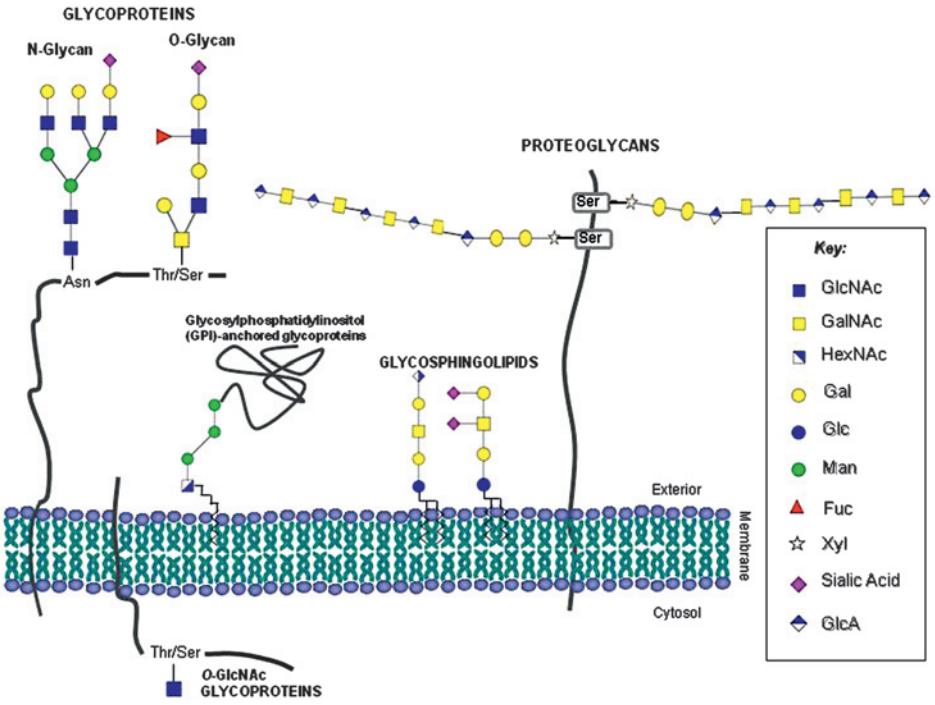

Figure 1 Schematic representation of common classes of glycoconjugates expressed in human cells. Protein O-glycosylation and N-glycosylation can occur in both membrane-associated and secreted glycoproteins.

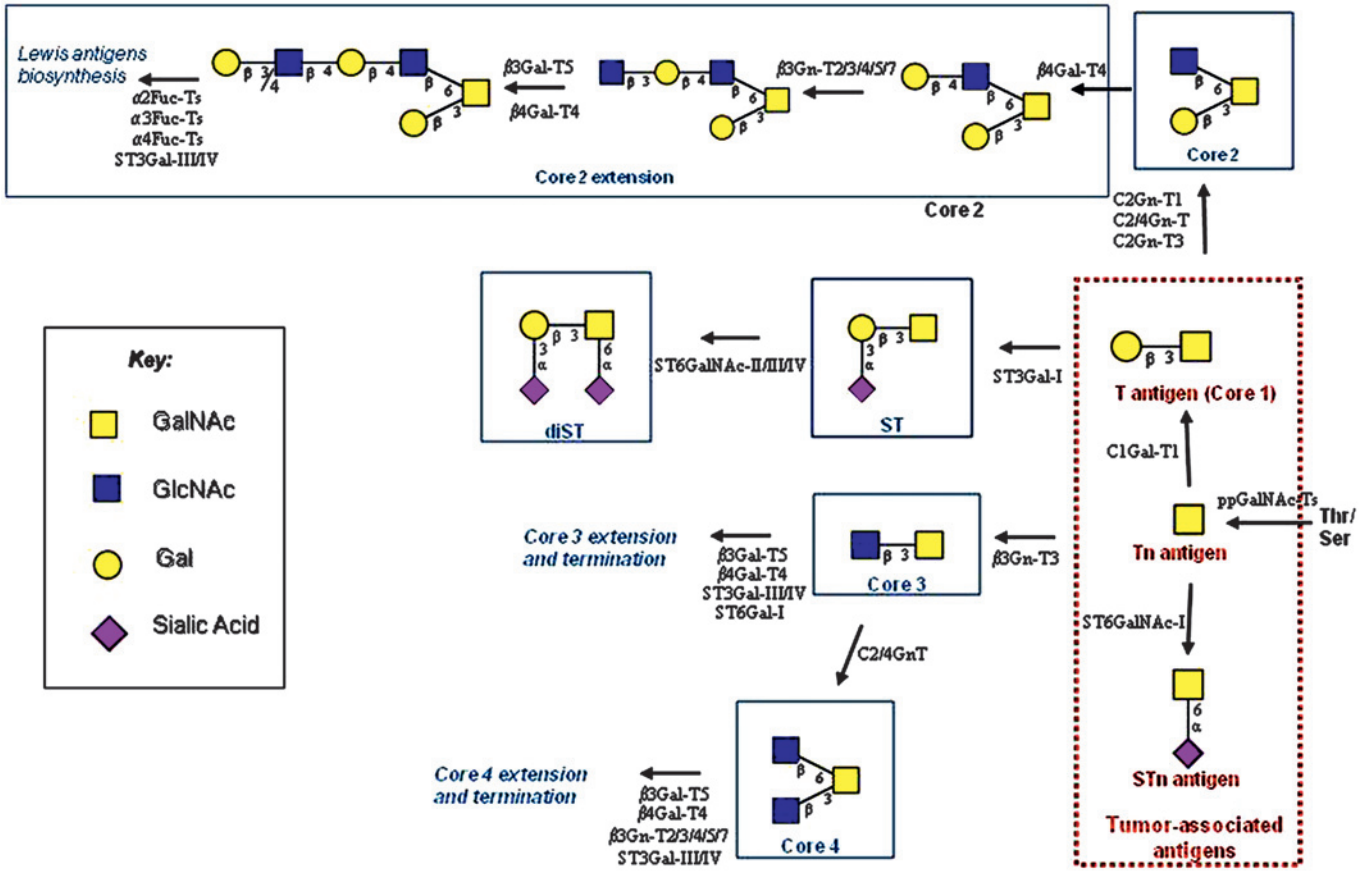

Figure 2 Schematic representation of the biosynthetic pathways of most common mucin-type *O*-glycans. Glycosyltransferases involved in the enzymatic steps are indicated. The major tumour-associated antigens are highlighted. b3Gal-T, β1-3-galactosyltransferase; b4Gal-T, β1-4-galactosyltransferase; b3Gn-T, β1,3-N-acetylglucosaminyltransferase; C1Gal-T1, core 1 β1-3-galactosyltransferase; C2GnT, core 2 β1–6 *N*-acetylglucosaminyltransferase; ppGalNAc-T, UDPGalNAc-polypeptide *N*-acetylgalactosaminyltransferase; ST3Gal, α2,3-sialyltransferase; ST6GalNAc-I, GalNAc α2,6-sialyltransferase.

antigens.[31–34] Formation of the sialyl-Tn antigen stops any further processing of the oligosaccharide chain[31 32 35] (figure 2).

Another common core structure present in normal cells contains a branching GlcNAc attached to core 1 and is termed core 2 (figure 2).[36] Core 2 is produced in many epithelial and haematopoietic cells. The enzyme responsible for core 2 synthesis is core 2 β1–6 *N*-acetylglucosaminyltransferase (C2GnT).[37] At least three genes encode this subfamily (C2GnT1 to C2GnT3) of a larger family of β1–6 *N*-acetylglucosaminyltransferases.[38] There are two major types of C2GnTs. The L type (leucocyte type, C2GnT1 and C2GnT3) synthesises only the core 2 structure, whereas the M type (mucin type, C2GnT2) is also involved in the synthesis of core 4 and other GlcNAcβ1–6-linked branches (figure 2). The C2GnT1 and C2GnT3 enzymes are active in many tissues and cell types, but the C2GnT2 enzyme is found only in mucin-secreting cell types.[38 39] The expression and activity of C2GnTs are altered in certain tumours. Because of their branched nature, core 2 *O*-glycans can block the exposure of mucin peptide epitopes. There are other types of core structures, and most of them show tissue specificity expression.

The extension of the core structures is catalysed by β3/4 Gal-Ts and β3/4 Gn-Ts (figure 2) leading to the formation of type 1 and type 2 chains. The Lewis and ABO glycan-based blood group antigens are common terminal structures which are present in mucins as in other glycoconjugates. The families of glycosyltransferases that catalyse the addition of these terminal structures are described in detail below. In contrast with *N*-glycans, *O*-glycans do not have sialic acid α2-6Gal linkages, although the sialic acid α2-6GalNAc moiety is common, for example, in the sialyl-Tn antigen. Thus, in most mammalian mucin-producing cells, α2–6 sialyltransferases act on GalNAc, and α2–3 sialyltransferases act on galactose. Some of the sialyltransferases prefer *O*-glycans as their substrate, but many of these enzymes have an overlapping specificity and also act on *N*-glycan structures as acceptor substrates. There are other types of non-mucin *O*-glycans, such as *O*-GlcNAc in proteins found in the cytoplasm and nucleus; these are not the focus of this review.

Another important group of glycoconjugates are proteoglycans. These consist of a core protein and covalently attached glycosaminoglycan chains, which are linear polysaccharides (figure 1). Proteoglycans are expressed in a tissue-specific manner and have been shown to participate in several cellular and extracellular interactions.

ALTERATIONS IN GLYCOPROTEIN O-GLYCOSYLATION IN CANCER AND PRENEOPLASTIC LESIONS

Altered glycosylation of cell surface glycolipids, membrane-associated glycoproteins and secreted glycoproteins is a quasi-universal modification in cancer.[40] This was first demonstrated by showing that antibodies raised against cancer cells often recognise abnormal glycan structures.[41] Despite there being no evidence for a role of altered glycosylation in cancer initiation, and despite information on the mechanisms that generate abnormal glycosylation still being limited, it is well established that it can contribute from early stages to invasion and metastisation.[2 42–44]

We will focus on glycan alterations of glycoproteins, in particular mucins that are major carriers of glycan structures in carcinomas, characterised by O-glycosylation initiated by addition of a GalNAc on serine or threonine residues. Of the various changes, the two most important ones from the standpoint of biomarker signatures are generation of truncated versions of normal oligosaccharides and generation of unusual forms of terminal structures, namely sialylated versions of the normal counterparts (figure 2). Most modifications are generated by upregulation/downregulation of glycosyltransferases, and one study has implicated a mutation in the Cosmc chaperone protein as the underlying mechanism for the absence of a functional enzyme, leading to accumulation of cancer-associated precursors.[45] Therefore it is reasonable to assume that the glycan signature at the cancer cell surface is unstable, at variance with what happens to most cancer-associated alterations, which are clonal because of their genetic origin. This fits into the mosaicism of glycan expression in tissue sections, reflecting variations in differentiation along cancer progression.[46] However, despite their non-clonal nature, it is clear that they stabilise during cancer progression,[47] probably because of the positive selective properties they confer on the cell populations, by facilitating invasion and metastisation.

It is relevant in considering altered glycosylation central to the cancer biomarker field that it is visible on the cell surface of cancer cells (and therefore easily accessible to antibodies or lectins as tissue biomarkers) and often expressed in the circulation, either on secreted products or by shedding from cell surfaces (and therefore identifiable as serum biomarkers).

As mentioned above, mucins are major carriers of cancer-associated carbohydrates and they amplify alterations at the surface of the cancer cell because they are highly overexpressed in cancer and have repetitive sequences rich in serine and threonine, the potential O-glycosylation sites.[48] They are major carriers of the modified glycans secreted or anchored at cancer cell membranes. However, they can themselves be biomarkers due to modifications induced by altered glycosylation. Such an example is the differential recognition of the MUC1 mucin by different monoclonal antibodies according to glycosylation.[49] Mucins can be either secreted or membrane-bound and contain both O-linked and N-linked oligosaccharides and share a common structural feature, the presence of a tandem repeat (VNTR) domain.[50–59] Tandem repeats are rich in serine and threonine, which can be O-glycosylated. The polymorphic nature of mucins at the VNTR was first recognised in 1987[50] and later shown, for the MUC1 mucin, to have implications for the risk of gastric cancer development,[60] partly by modulating glycosylation.[61] Mucins show restricted, tissue-specific, expression in normal epithelial cells, but are overexpressed and aberrantly expressed in cancer. As an example, MUC2 mucin, which is expressed in normal intestine, can be aberrantly expressed in intestinal metaplasia, a precursor lesion of gastric carcinoma,[62] and in 25% of gastric carcinomas.[63] Also MUC4 is expressed in premalignant and malignant lesions of the pancreas despite it not being expressed in normal pancreatic cells.[64] In some cases, modifications of mucin expression are strictly linked to modified glycosylation. For example, MUC2 mucin in intestinal metaplasia colocalises with expression of sialyl-Tn in goblet cells.[47 65 66] Future studies should address the putative coordination of mucin/glycosyltransferase regulation to clarify if, at least to some extent, mucin expression can 'instruct' glycosylation or if they are independently but coordinately regulated.

SIMPLE MUCIN-TYPE CARBOHYDRATE ANTIGENS

One of the most common cancer-associated modifications is poor glycosylation of glycoproteins, leading to expression of truncated *O*-glycans at the cell surface[67–69] (figure 1). These are Tn, sialyl-Tn and T antigens (figure 2), which are pan-carcinoma antigens.[70 71] Many studies have identified aberrant expression

of these so-called simple mucin-type antigens in carcinomas from breast,[72] oesophagus,[73] colon,[74] pancreas,[75 76] stomach,[47] lung,[77] endometrium,[78 79] ovary[80] and bladder.[81] Other studies have shown an association of expression of these antigens with poor prognosis in patients with breast,[82] colon[83] and stomach[84 85] carcinoma.

Modifications stem from disorganisation of secretory pathway organelles (ER and Golgi) in cancer cells and altered glycosyltransferase expression. As mentioned above, these modifications can also occasionally depend on mutations in a chaperone essential for glycosyltransferase function.[45] Another, more common, mechanism for cancer-associated expression of truncated *O*-glycans is absence of glycosyltransferases responsible for the synthesis of core structures used as substrates for chain elongation.[31] Alternatively, or in combination with the previous mechanism, is overexpression of sialyltransferases responsible for the synthesis of sialyl-Tn and sialyl-T antigens.[35 86] In fact, transfection of cells that do not express sialyl-Tn with the ST6GalNAc I (GalNAc α2,6-sialyltransferase) is induced to express sialyl-Tn,[33 87] and, in human breast cancer, expression of ST6GalNAc I colocalises with expression of sialyl-Tn.[34] Furthermore, induced expression of sialyl-Tn in cell lines increases their tumorigenicity.[88 89]

Future studies will be directed at identifying specific *O*-glycan/core-protein combinations to increase the specificity of the biomarkers for cancer detection. In the case of MUC1, it has been shown that specific glycopeptide combinations can generate antibodies with increased specificity in cancer reactivity.[90]

LEWIS CARBOHYDRATE ANTIGENS (BIOSYNTHESIS AND EXPRESSION)

Lewis-type blood group antigens, such as sialyl Lewis A (SLea) and sialyl Lewis X (SLex) (figure 3), are expressed in cancer cells, mimicking their normal expression on blood cells (monocytes and neutrophils) and also mimicking their potential for migration through binding to endothelial cell selectins. They are expressed on carbohydrate chains, type 1 and type 2, according to the linkage between the galactose residue and the GlcNAc residue, β1,3 and β1,4, respectively. The presence or absence of type 1 Lewis antigens in a given individual depends initially on the presence of active enzymes responsible for the addition of the fucose monosaccharide. The α1,2-fucosyltransferase, product of the secretor gene (Se), acts on the terminal galactose and produces the H type 1 structure which forms the substrate for the α1,4-fucosyltransferase, the product of the Lewis gene (Le), which synthesises the difucosylated Le^b antigen (figure 3). People with inactivating mutations of the Se gene are unable to synthesise H type 1 and Le^b antigen; they are called non-secretors and constitute 20% of the human population. The secretor and Lewis status of individuals are implicated in susceptibility to several diseases, mostly infections, with almost complete absence of gastrointestinal infections from calicivirus in non-secretors[91] and an implication of BabA+ *Helicobacter pylori* infection.[92]

The relevance of Lewis sialylated structures in cancer was first revealed in the 1980s, when monoclonal antibodies raised against cancer cells were shown to recognise $SLe^{a/x}$.[93–95] The biosynthetic basis for sialylated Lewis antigens also started to be

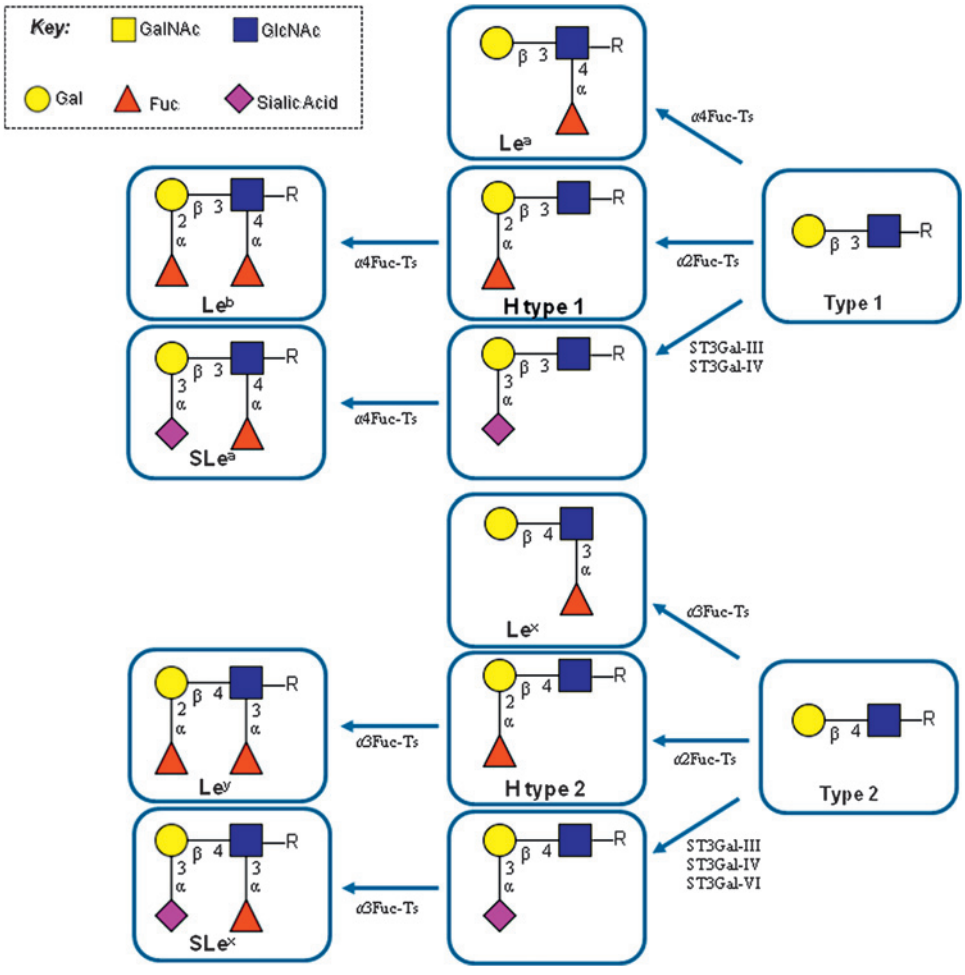

Figure 3 Schematic representation of the biosynthesis of Lewis antigens. R represents precursor carbohydrate chain. Fuc-T, fucosyltransferase; Le^a, Lewis A; Le^b, Lewis B; Le^x, Lewis X; Le^y, Lewis Y; SLe^a, sialyl Lewis A; SLe^x, sialyl Lewis X; ST3Gal, α2,3-sialyltransferase.

determined in the 1980s,[96 97] and it is now recognised as depending on increased α2,3-sialyltransferase and/or α1,3/4-fucosyltransferase activities.[98 99] In leukaemias, a viral gene product of human T-cell lymphotropic virus type 1 transactivates fucosyltransferase VII, an α1,3-fucosyltransferase with rate-limiting activity for the synthesis of sialyl-Le^x in leucocytes, and induces strong constitutive expression of sialyl-Le^x in leukaemic cells.[100]

Mucins can be carriers of these glycan structures,[101] and MUC1 was specifically identified as one such carrier.[102] In 1991, it was demonstrated that SLe^a and SLe^x were recognised by endothelial leucocyte adhesion molecule 1 (ELAM-1) in endothelial cells[103] and also that cancer cells use these structures to adhere to activated endothelial cells[104] and facilitate establishment of haematogenous dissemination and metastisation. In agreement with this, overexpression of SLe^x and SLe^a is common in carcinomas of several origins (eg, lung, colon, gastric and pancreas) and is associated with increased metastatic ability[105–108] and poor survival of the patients.[109–113]

The relevance of SLe^a and SLe^x to cancer dissemination led to attempts to use them not only as cancer biomarkers but also as therapeutic targets. One therapeutic strategy is to reduce synthesis of SLe^x by using competitive disaccharide substrates as decoys.[114] Antisense strategies, directed to α1,3/4-fucosyltransferase, were successful in reducing liver metastisation in a mouse model.[115] Similarly, increased and cancer-associated expression of these antigens has been used for in vivo bioimaging.[116]

Mechanisms controlling gene expression, including methylation and identification of transcription factors, are under investigation to better understand aberrant expression of Lewis antigens in cancer and to improve their usefulness as cancer biomarkers.

GLYCAN-BASED SEROLOGICAL ASSAYS IN CANCER

Glycosylation changes on glycoconjugates either present on the surface or secreted by cancer cells are a major potential source of cancer biomarkers. At present, most serological assays used for cancer detection, prognosis and monitoring are based on quantifying glycoconjugates in the serum of patients with cancer. These serological assays detect carbohydrate antigens such as SLe^a (CA19-9) and STn (CA72-4) or mucin glycoproteins such as MUC1 (CA15-3) and MUC16 (CA125).[57 117–119]

The use of these biomarkers for cancer screening is limited because of their broad expression by various types of cancer, precluding identification of the organ in which the cancer has originated.[120–122] In addition, these biomarkers can also be produced in some non-neoplastic and inflammatory diseases,[123] reducing the specificity of the assays for screening purposes.[124] Nevertheless, sound data support the use of the CA125 assay for detection of ovarian cancer. Raised CA125 concentrations are found in 50% of patients with stage I ovarian cancer and in 25% of serum samples collected 5 years before diagnosis of ovarian cancer.[125]

In general, the detection of these biomarkers in the serum of patients with cancer has been shown to be particularly useful for evaluation of prognosis and for monitoring purposes. This is the case for the assay of CA125, which is detected in 80% of patients with ovarian cancer[117]; furthermore, increases and decreases in CA125 correlate with regression and progression of the disease. In addition, preoperative evaluation of CA125 has been shown to aid evaluation of prognosis for patients with ovarian cancer.[126 127]

Similarly, the aberrantly glycosylated MUC1 mucin, which is produced by cancer cells and shed into the circulation, can be detected by the CA15-3 assay. Raised CA15-3 concentrations

Take-home messages

- Glycoconjugate modifications are a quasi-universal hallmark of cancer which makes them important cancer biomarkers.
- Some modified glycoconjugates detected in serum are in clinical use for follow-up of patients.
- New developments are expected to increase the scope of their clinical application, particularly at the diagnostic level.

have been shown to be useful for prognosis evaluation in early-stage breast cancer and for monitoring the course of the disease,[128–130] including monitoring patients with metastatic disease during active therapy.[131] In the absence of readily measurable disease, an increasing CA15-3 concentration may indicate treatment failure.[124 132] Evaluation of the clinical utility of CA15-3 in other cancers is under investigation.

The aberrant expression of other carbohydrate antigens on glycoconjugates has also been shown to be useful for evaluating prognosis and for monitoring purposes in cancer. Serological detection of SLe^a on glycolipids and glycoproteins by the CA19-9 assay has been performed in patients with an established diagnosis of pancreatic, colorectal, gastric or biliary cancer and used to monitor clinical response to therapy.[133 134] In addition, in colon cancer, a raised CA19-9 concentration before surgery has independent prognostic value: patients with increased concentrations had a fourfold increase in death rate at 3 years compared with those with lower concentrations. In gastric carcinoma, preoperative CA19-9 concentration remains one of the best prognostic factors,[135 136] and preoperative positivity for CA19-9 is an independent risk factor for recurrence of gastric carcinoma.[137]

Another carbohydrate antigen, sialyl-Tn, which is expressed in glycoproteins such as mucins, can be detected by the CA72-4 assay. Raised CA72-4 concentration has been shown in patients with gastric, colorectal and pancreatic carcinomas.[136 138] In gastric carcinoma, CA72-4 has been shown to be useful as an independent prognostic factor: patients positive for CA72-4 show a 3.8-fold higher risk of death.[139] The CA72-4 assay is also useful for monitoring gastric carcinoma, where positivity is considered to be a predictor of tumour recurrence.[136] CA72-4 has also been shown to be an independent prognostic factor in pancreatic cancer.[140]

Determination of carcinoembryonic antigen (CEA) is another serological assay widely used in clinics. CEA glycoproteins are rich in *N*-glycans, and these glycoproteins are produced by normal and carcinoma cells. In colorectal and some other cancers, CEA is expressed at high level and shed into the circulation.[129–131] The clinical significance of serum CEA in patients with colorectal carcinoma is in the evaluation of prognosis and follow-up of patients.[141 142] Increases in serum concentrations of CEA can also have non-cancer-related causes.[140] Because of its lack of sensitivity in the early stages of colorectal cancer, CEA measurement is unsuitable for population screening.

The glycosylation alterations observed in cancer, particularly the putative glycopeptide specificities been identified above, constitute a major target for the development of novel serological-based assays for early cancer detection with major screening and clinical implications.

PERSPECTIVES

Glycoconjugate modifications are a universal hallmark of cancer, which makes them important cancer biomarkers. Many

of the current biomarkers used in clinics, in both tissue and serum assays, are based on these carbohydrate modifications. Their basis and precise structure are, however, largely not understood by those who use them in the clinical setting. This stems from the molecular complexity of the expression of these biomarkers and the still largely unknown regulatory pathways. Given the importance of these biomarkers because of both their high frequency and high accessibility at the cell surface and in serum, this review is an attempt to give the clinical/pathological expert a relevant biochemical understanding of the field.

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Alterations in glycosylation as biomarkers for cancer detection

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5.2

ST6GalNAc-I Controls Expression of Sialyl-Tn Antigen in Gastrointestinal Tissues.

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ST6GalNAc-I controls expression of sialyl-Tn antigen in gastrointestinal tissues

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1. ABSTRACT

Sialyl-Tn is a simple mucin-type carbohydrate antigen aberrantly expressed in gastrointestinal adenocarcinomas and in the precursor lesion intestinal metaplasia. Sialyl-Tn tumour expression is an independent indicator of poor prognosis. We have previously shown *in vitro* that ST6GalNAc-I and ST6GalNAc-II sialyltransferases can synthesize sialyl-Tn. The aim of the present study was to establish whether ST6GalNAc-I is the major enzyme responsible for the expression of sialyl-Tn. We used a model of CHO-ldlD cells producing only MUC1-Tn glycoform and showed that ST6GalNAc-I is the key-enzyme leading to sialyl-Tn biosynthesis. We developed novel monoclonal antibodies specific for ST6GalNAc-I and evaluated its expression in gastrointestinal tissues. ST6GalNAc-I was detected in normal colon mucosa co-localized with *O*-acetylated sialyl-Tn. Expression was largely unaltered in colorectal adenocarcinomas. In contrast, we found that ST6GalNAc-I is weakly expressed in normal gastric mucosa, but over-expressed in intestinal metaplasia, co-localized with sialyl-Tn. In gastric carcinomas ST6GalNAc-I was also associated with sialyl-Tn, but with heterogeneous staining and partial co-localization. Our results showed ST6GalNAc-I as the major enzyme controlling the expression of cancer-associated sialyl-Tn antigen in gastrointestinal tissues.

2. INTRODUCTION

Altered glycosylation is a common feature of cancer cells. The sialyl-Tn (STn) antigen (Neu5Acα2-6GalNAcα-*O*-Ser/Thr), also known as CD175s, is a simple mucin-type carbohydrate antigen which has attracted much attention because it is highly expressed in most gastric (1-3), colorectal (4), ovarian (5), breast (6, 7) and pancreatic (8) carcinomas, but has limited expression in normal tissues. The expression of sialyl-Tn is an established indicator of poor prognosis in patients with gastric (9-11), colorectal (12, 13), and ovarian cancer (14) and contributes to the aggressive phenotype of carcinoma cells by altering their behaviour (15-18). Sialyl-Tn expression is also aberrantly expressed in precursor and early lesions of carcinomas of the gastrointestinal tract, such as intestinal metaplasia (IM) of the stomach (2), adenomatous polyps (12), chronic ulcerative colitis (13) and pancreatic intraepithelial neoplasias (8).

The synthesis of the mucin-type carbohydrate antigens depends upon expression of the enzymes that initiate *O*-glycosylation: UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-transferases) (19). GalNAc-transferases control the first step in mucin glycosylation and controls the level of complexity of mucin glycans, i.e., the sites and density of *O*-glycan occupancy

of the mucin tandem repeats (20, 21). Processing of GalNAcα-*O*-Ser/Thr, also known as the Tn antigen, by different glycosyltransferases is dependent on the cell type and tissue of origin. Monosaccharides are added sequentially to the GalNAc in the Golgi apparatus, forming a variety of *O*-glycan stuctures that include the Core1 (Galβ1-3GalNAcα-*O*-Thr/Ser), also known as T antigen, and the Core2 (GlcNAcβ1-6 (Galβ1-3)GalNAcα-*O*-Thr/Ser) glycans. These glycoforms may be glycosylated further forming larger and more complex structures. A common feature in carcinoma cells is the activation of an alternative glycosylation pathway that caps the GalNAcα-*O*-Thr/Ser structures with α2,6-sialic acid. Once sialylated, the resulting sialyl-Tn glycoform, Neu5Acα2-6GalNAcα-*O*-Ser/Thr, cannot be glycosylated further thereby preventing normal glycan elongation (22-24). The underlying mechanisms responsible for the induction of the sialyl-Tn glycoform in cancer cells are not fully understood. Two α2,6-sialyltransferases have been shown to be capable to sialylate the GalNAc residue O-linked on proteins: ST6GalNAc-I, and ST6GalNAc-II (25-27). However, over-expression of the ST6GalNAc-I glycosyltransferase in cells can override the normal *O*-glycosylation pathways leading to the formation of sialyl-Tn *O*-glycans as the dominant mucin phenotype (28-30). We have previously shown that both ST6GalNAc-I and ST6GalNAc-II can synthesize sialyl-Tn, *in vitro*; however, only ST6GalNAc-I could create large amounts of sialyl-Tn when over-expressed in a cancer cell line (28). Previous studies have also correlated the amount of ST6GalNAc-I mRNA with the amount of sialyl-Tn expression in breast cancer (30) and several cell lines (31), while the level of ST6GalNAc-II mRNA did not correlate with the expression of sialyl-Tn in gastric (unpublished results) and breast cancer cell lines (26) or in breast tumours (30). However, the role of each sialyltransferase, ST6GalNAc-I and II, in the synthesis of the cancer-associated sialyl-Tn structure in cells and pathological tissues remains unclear.

A novel monoclonal antibody (MAb) directed to ST6GalNAc-I was used to evaluate its expression in normal gastric and colonic mucosa, in intestinal metaplasia of the stomach, and in gastric and colorectal adenocarcinomas. The results show that over-expression of ST6GalNAc-I in intestinal metaplasia of the stomach co-localizes with the expression of sialyl-Tn. ST6GalNAc-I was not over-expressed in colon adenocarcinomas when compared to normal mucosa, suggesting that the acetylation status of sialyl-Tn may mask this antigen in normal colonic mucosa and exposed in adenocarcinomas. In contrast, in gastric carcinomas expression of sialyl-Tn was partially, but not exclusively, associated with ST6GalNAc-I. Our results demonstrate that ST6GalNAc-I is the major regulator of expression of cancer-associated sialyl-Tn *O*-glycosylation.

3. MATERIAL AND METHODS

3.1.Recombinant expression of soluble ST6GalNAc-I

An expression construct of the human ST6GalNAc-I were designed to encode an enzyme lacking the cytoplasmic and the transmembrane region. This soluble construct of ST6GalNAc-I encoding amino acid residues 43–601was prepared as described previously (28). The soluble construct of ST6GalNAc-I was expressed in Sf9 cells using the Baculo-virus expression system, and purified to near homogeneity (28).

3.2. Generation of monoclonal antibodie

The recombinant ST6GalNAc-I enzyme was used as immunogen. Balb/c mice were immunized with one subcutaneous injection of 10 µg native protein in Freunds complete adjuvant, followed by two injections with Freunds incomplete adjuvant, and finally an intravenous boost without adjuvant. Eye bleeds were taken 7 days after the third immunization, and the titre and specificity of antibodies were evaluated by immunocytology with baculo-virus infected Sf9 cells expressing recombinant human ST6GalNAc-I or irrelevant enzymes. Spleen cells from one immunized mouse were fused to NS-1 myeloma cells to produce hybridomas and the cloning procedure was performed as described previously (32, 33). Hybridomas were selected by initial screening on baculo-virus infected Sf9 cells expressing either one of the following human sialyltransferases: ST6GalNAc-I, ST6GalNAc-II or ST3Gal-I. Further characterization of the hybridoma clones was done on human cell lines expressing full-length coding ST6GalNAc-I or ST6GalNAc-II enzymes as previously described (28). Antibodies were also tested by SDS-PAGE Western blot analysis.

3.3. CHO ldlD-MUC1F cells stably transfected with ST6GalNAc-I or ST6GalNAc-II

CHO ldlD cells were kindly provided by M. Krieger (MIT, USA) (34). Stable CHOldlD cell line expressing full length FLAG-epitope tagged MUC1 (MUC1F) has been previously established (CHO ldlD-MUC1F) (35). Based on existing cDNA constructs (28, 30), full length human ST6GalNAc-I and ST6GalNAc-II-Myc constructs were produced by PCR, using PfuUltra™ polymerase (Stratagene). Regions encoding the membrane anchoring (ST6GalNAc-I; aa 1-293 and ST6GalNAc-II; aa 1-80) and catalytic domains (ST6GalNAc-I; aa 294-601 and ST6GalNAc-II; aa81-374) were generated using two sets of primers. For ST6GalNAc-I membrane anchor ST6I-1 (5′-GCG GAT CCA CCA TGA GGT CCT GCC TGT GGA GAT GCA GGC-3′)/ST6I-3 (5′-GAG CGC TCT AGA CCA CAG CGA CTT GGA GGC TTT GAT CTT C-3′) and catalytic domain ST6I-2 (5′-GAG CGC TCT AGA AAA CTC TTT CTG CCC AAC CTC ACT CTC-3′)/ST6I-4 (5′-GCG GCG GCC GCT CAG TTC TTG GCT TTG GCA GTT CCG GG-3′) were used. For ST6GalNAc-II membrane anchor ST6II-1 (5′-GCG GGA TCC ACC ATG GGG CTC CCG CGC GGG TCG TTC TTC-3′)/ST6II-3 (5′-GAG CGC TCT AGA GTG GGG GTG CCG CTG AAT GGC CAG-3′) and catalytic domain ST6II-2 (5′-GAG CGC TCT AGA GGC CTG TTC AAT CTC TCC ATT CCA GTG-3′)/ST6II-4 (5′-GGC GGC CGC GCG CTG GTA CAG CTG AAG GAT GCC GGC C-3′). Single XbaI sites were introduced in the membrane anchoring domain preceding the catalytic domain leading to L293S and Q294R mutations in ST6GalNAc-I and F80S mutation in ST6GalNAc-II-Myc, sites underlined in primers shown above. Dual insert (membrane anchor and catalytic domain) were cloned

Table 1. Monoclonal antibodies, their specificity and references

MAb	Specificity	Dilution	Reference
2C3	ST6GalNAc-I	1:2	This paper
1C9	ST6GalNAc-I	1:2	This paper
HB-STn	sialyl-Tn	1:15	Dako
B72.3	sialyl-Tn	1:50	(38)
TKH2	sialyl-Tn	1:10	(39)
PMH1	MUC 2	1:10	(40)
CLH2	MUC 5AC	1:10	(41)
5E5	MUC1-Tn/STn	1:10	(42, 43)

directionally into the BamHI and NotI sites of the pcDNA3-zeo (Invitrogen™). For ST6GalNAc-II the C-terminal Myc-tag construct was introduced by inserting a double stranded Myc-oligo into the Not-I site of ST6GalNAc-II-pCDNA3-zeo. All constructs were sequence verified using an ABI Avant sequenator. CHO ldlD-MUC1F cells were transfected with full length human ST6GalNAc-I or ST6GalNAc-II-Myc and grown in the presence of GalNAc.

3.4. Patients and tissues

Gastric carcinomas and gastric mucosa adjacent to carcinomas were obtained from individuals undergoing surgery at Hospital S. João, Medical Faculty (Porto, Portugal). Study was performed with the approval of the local HSJ ethical committee (from 05-09-2007). Specimens were frozen at – 80^{o}C. Serial sections were cut and used for immunofluorescence. We evaluated 22 gastric mucosas with normal morphology, 24 IM lesions adjacent to the carcinoma cases that were classified as complete IM (n =14) and incomplete IM (n =10) according to the pattern of mucin expression (described below) and 31 cases of gastric carcinoma. Gastric carcinomas were classified according to Laurén's classification (36). Colorectal adenocarcinoma cases were obtained from the KAM cohort based on the screening group of the Norwegian Colorectal Cancer Prevention study in the county of Telemark, Norway (37) and a series of colorectal cancer cases recruited to the KAM cohort from routine clinical work at Telemark Hospital, Skien and Ulleval University Hospital, Oslo. The KAM study is approved by the Regional Ethical Committee and the Norwegian Data Inspectorate. We evaluated 15 colorectal adenocarcinomas. A sample of control tissue was taken from the surgically removed tissue close to the adenocarcinoma or as far away from the tumor as possible. The histology of adjacent normal tissue was examined independently by two specialist histopathologists, and found to be normal. The histology of the adenocarcinomas was also examined independently by two specialist histopathologists in order to determine the tumor stage. All colon cancer cases were histomorphologically classified as moderately differentiated.

3.5. Immunofluorescence

Details of MAbs used are shown in Table 1. Cells were fixed in ice-cold acetone for 5 min and kept at -20ºC before staining. Tissue slides were preserved at -80ºC and fixed in 4% paraformaldehyde for 15 min at RT, prior to procedure.

Double staining was used for co-localization of ST6GalNAc-I (MAb 2C3) and sialyl-Tn (MAb TKH2 and HB-STn) and ST6GalNAc-I and MUC2 (MAb PMH1). MUC5AC and MUC2 staining was performed for the characterization of IM types (44). Deacetylation (saponification) of selected samples was accomplished by treating slides immediately after fixation with 0.1N NaOH for 20 min at RT prior to performing the immunofluorescence (45). Samples designed for MUC2 detection with PMH1 were pre-treated with neuraminidase as previously described (40).

Samples were washed twice in PBS and incubated for 20 min with rabbit non-immune serum (DAKO) diluted 1:5 in PBS/10%BSA. Samples were incubated overnight at 4ºC with the monoclonal antibodies 2C3 or CLH2 (Table 1) diluted in PBS/5%BSA. Sections were washed in PBS and incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO) diluted 1:70 in PBS/5%BSA, for 45 min. Then the samples were washed in PBS and blocked with non-immune goat serum (DAKO) diluted 1:5 in PBS/10%BSA for 20 min. Sections were incubated with the monoclonal antibody PMH1 or TKH2 (Table 1) diluted in PBS/5%BSA overnight at 4ºC. Sections were washed with PBS and incubated for 30 min with Texas-Red-conjugated goat anti-mouse IgM (in the case of PMH1) or Texas-Red-conjugated goat anti-mouse IgG1-specific (in the case of TKH2) (Jackson Immunoresearch) diluted 1:70 in PBS/5%BSA and DAPI diluted 1:100, for 30 min in the dark. Samples were washed in PBS and mounted in Vectashield (Vector Laboratories, Inc).

Results are based on assays analyzed by two independent observers. Statistical analysis was performed using the chi-square test with Yates correction using Statview 4.01 software.

4. RESULTS

4.1 Monoclonal antibodies to ST6GalNAc-I

Two monoclonal antibodies 2C3 (IgG2a) and 1C9 (IgG1) were selected for their specific reactivity with ST6GalNAc-I. MAb 2C3 reacted with Sf9 cells expressing ST6GalNAc-I but no reactivity was observed with Sf9 cells expressing ST6GalNAc-II. MAb 2C3 was also found to react with the human cell line K562, which expresses high levels of ST6GalNAc-I transcripts and sialyl-Tn antigen (31) (Figure 1, Panel II G, J). MAb 2C3 did not show reactivity with the denatured form of ST6GalNAc-I when analyzed by SDS-PAGE Western blot (not shown). On the other hand MAb 1C9 was selected for their specific of the reactivity with ST6GalNAc-I in reduced SDS-PAGE Western blot analysis (Figure 1, Panel I). These results are in agreement with our previous findings that MAbs to glycosyltransferases tend to react either with the native conformation of the protein or with the denatured protein (32, 46). We have generally been unable to produce MAbs that react with both the native protein in unfixed cells or tissues and by SDS-PAGE Western blot analysis.

Further analysis of MAb 2C3 showed some immunoreactivity in immunocytology with the human

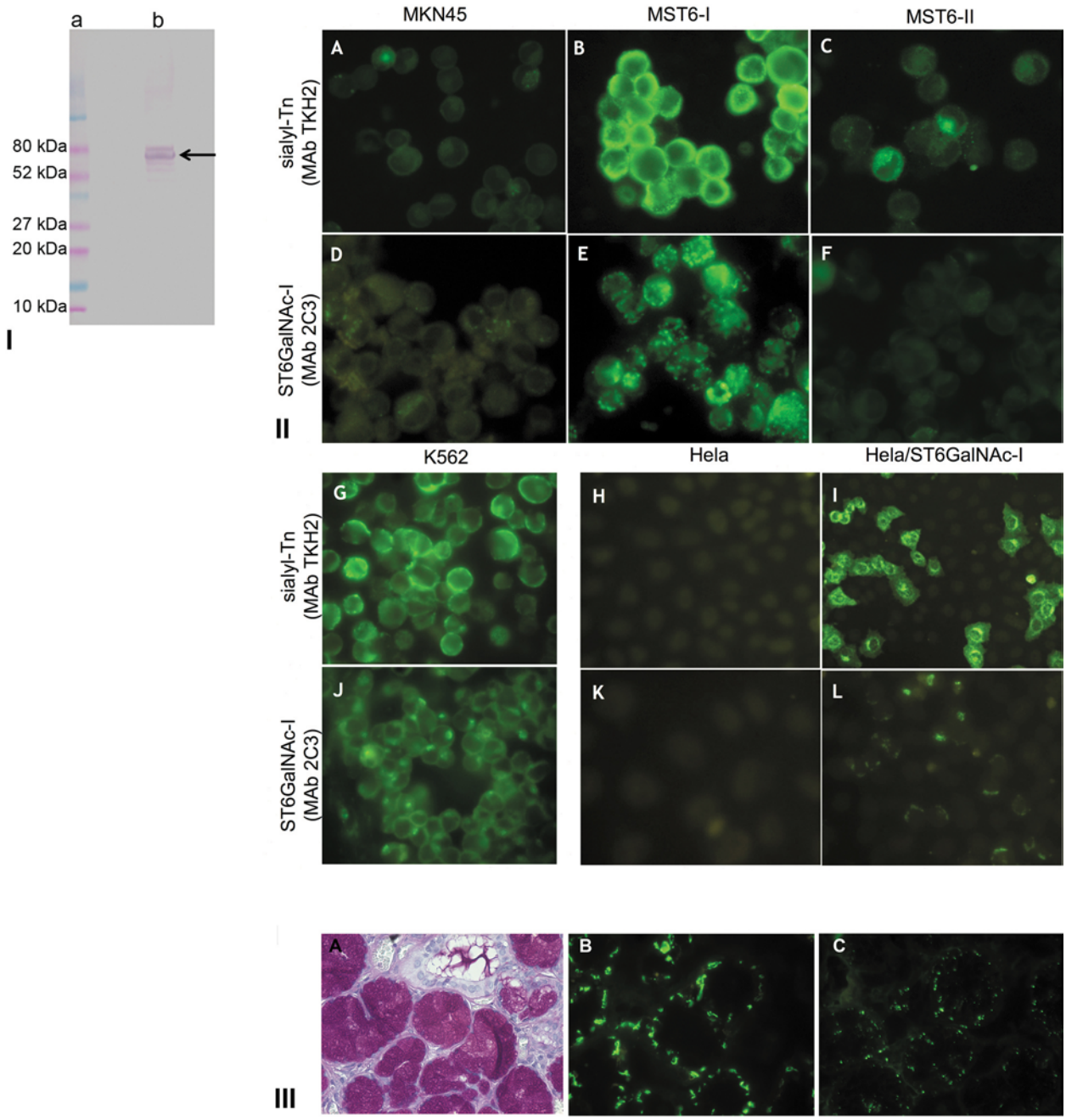

Figure 1. Characterization of monoclonal antibodies to ST6GalNAc-I. Panel-I: SDS-PAGE and Western blot analysis of the immunoreactivity of monoclonal antibody 1C9 with total cell lysate of transfected Sf9 cells expressing a secreted construct of ST6GalNAc-I (b). Molecular weight markers are indicated as (a) in the figure. Arrow indicates the detection ST6GalNAc-I. Additional band corresponds to N-glycosylated form of the protein. Panel-II: Expression of ST6GalNAc-I and sialyl-Tn in cell lines. MAb 2C3 reacts exclusively with MKN45 cells transfected with ST6GalNAc-I (MST6-I) (E) and shows no reactivity with *wt* MKN45 (D) nor with MKN45 cells transfected with ST6GalNAc-II (MST6-II) (F). The same pattern is observed for Hela cells (K, L). K562 hematopoietic cell line naturally expresses ST6GalNAc-I (J). Sialyl-Tn staining with MAb TKH2 correlates with ST6GalNAc-I (A-C, G-I). Panel-III: Expression of ST6GalNAc-I and sialyl-Tn in minor salivary glands from oral cavity. PAS&E staining (A). MAb 2C3 to ST6GalNAc-I stains salivary glands (B) and co-localizes in the same regions as sialyl-Tn, MAb HB-STn (C). Magnification 200x.

tumour cell lines MKN45 (31) and Hela, which express low levels of endogenous ST6GalNAc-I. However, we observed strong immunoreactivity with these cells when transfected with full coding human ST6GalNAc-I (Figure 1, Panel II). The staining pattern observed in these cells was a distinct perinuclear punctuate staining typical of

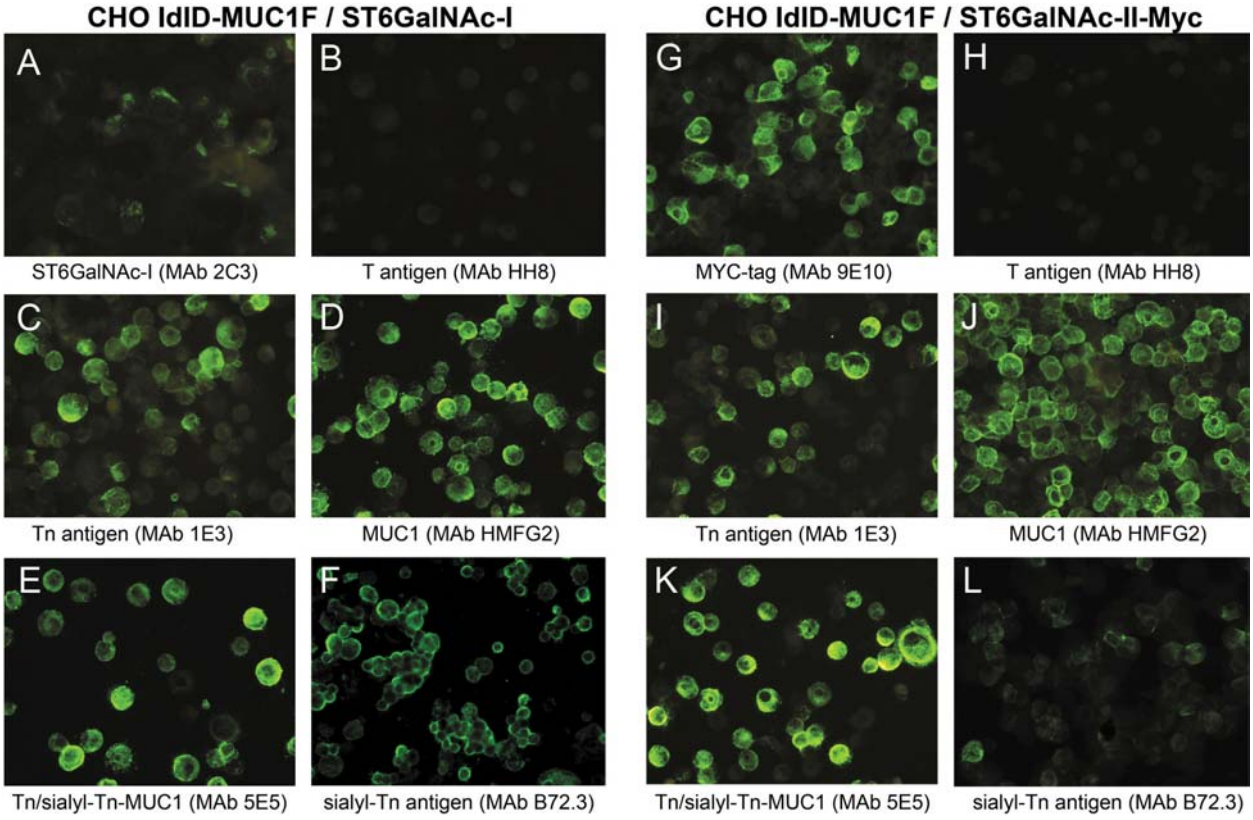

Figure 2. Stably transfected CHO ldlD-MUC1F cells with full coding human ST6GalNAc-I and ST6GalNAc-II-MYC grown in the presence of GalNAc. Cell staining images from CHO ldlD-MUC1F +ST6GalNAc-I (A-F) or +ST6GalNAc-II (G-L) are shown in left and right panels, respectively. Primary antibodies used are shown in the figure.

Golgi vesicles. MAb 2C3 furthermore produced the same staining pattern in glandular epithelial cells of salivary glands (Figure 1, Panel III), which are known to express ST6GalNAc-I mRNA.

4.2 Glycophenotype of CHO ldlD-MUC1F cells stably transfected with ST6GalNAc-I and ST6GalNAc-II

Glycosylation-defective CHO ldlD cells lack the epimerase that transforms UDP-Glc and -GlcNAc to UDP-Gal and -GalNAc, respectively (34). This defect blocks *O*-linked glycosylation of proteins. However, the addition of exogenous Gal or/and GalNAc to the media of ldlD cells overcame this defect and corrected the abnormal glycosylation phenotype (34). Addition of only GalNAc to the cells produced a truncated mucin limited to Tn. No sialyl-Tn was observed. This indicated that CHO ldlD cells have no competing glycosylation pathway after the Tn glycoform was produced. Interestingly, both the parental cell line CHO K1 and the CHO ldlD cell line grown in the presence of Gal and GalNAc are known to produce mono- and to a lesser extent di-sialylated T-antigen glycoforms (47), and indicates that an ST6GalNAc-transferase activity must be present. Stably transfectanted cells (CHO ldlD-MUC1F) of a full coding MUC1 have been produced and shown to express MUC1 on the cell membrane (35, 42).

CHO ldlD-MUC1F cells cultured in the presence of GalNAc expressed the Tn antigen (GalNAc-*O*-Ser/Thr) (Figure 2). There was no sialyl-Tn found indicating that the endogenous sialyltransferases were not capable of producing sialyl-Tn. A lack of the presence of the sialyl-T antigen was confirmed by treating the cells with neuraminidase and then staining the cells using controlled conditions with an anti-T antibody. Stable transfection of ST6GalNAc-I into these cells resulted in production of the sialyl-Tn glycoform as detected by B72.3 (Figure 2) and other sialyl-Tn Mabs (not shown). In contrast, stable transfection with ST6GalNAc–II did not result in substantial production of sialyl-Tn, although a few faint cells (<25%) could be visualized (Figure 2). Interestingly, while the cytolocalization of ST6GalNAc-I detected by MAb 2C3 was clearly supranuclear and Golgi-like, the staining of myc-tagged ST6GalNAc-II was localized throughout the cell with no apparent Golgi localization. While tagged constructs may mislocalize recombinant proteins, previously we have shown co-localisation of native and tagged constructs of several other glycosyltransferases. A 6X His tagged ST6GalNAc-II construct has also been expressed as a secreted functional enzyme in insect cells (unpublished). We are currently trying to develop Mabs to ST6GalNAc-II and characterize the cytolocalization in more detail.

4.3 Expression of ST6GalNAc-I and sialyl-Tn in normal gastric mucosa

Gastric mucosa with normal morphology showed

Table 2. Immunoreactivity of MAb 2C3 in normal gastric mucosa, intestinal metaplasia,gastric carcinoma, and colon carcinoma

	ST6GalNAc-I (Mab 2C3 immunoreactivity)			
Gastric mucosa and Intestinal metaplasia	**Negative**	**Weakly positive**	**Strongly positive**	
Normal gastric mucosa (n=22)	0	21	1	
IM complete [a] (n=14)	0	0	14	
IM incomplete [a] (n=10)	0	0	10	
	ST6GalNAc-I (Mab 2C3 immunoreactivity)			
Gastric Carcinomas (n=31)	**Negative**	**< 50% of pos. cells**	**> 50% of pos. cells**	**P value**
Histologic type [b]				
Intestinal (n=18)	4	10	4	0.600
Diffuse (n=9)	2	6	1	
Atypical (n=4)	1	1	2	
Sialyl-Tn expression				
Negative (n=4)	3	1	0	0.009
< 50% of positive cells (n=9)	1	8	0	
> 50% of positive cells (n=18)	3	8	7	
	ST6GalNAc-I (Mab 2C3 immunoreactivity)			
Colon Carcinomas (n=15)	**Negative**	**< 50% of pos. cells**	**> 50% of pos. cells**	**P value**
Clinical stage				
A (n=4)	0	2	2	0.897
B (n=4)	0	3	1	
C (n=7)	0	3	4	
Sialyl-Tn expression				
Negative (n=1)	0	1	0	0.005
< 50% of positive cells (n=7)	0	7	0	
> 50% of positive cells (n=7)	0	0	7	

(a) Classification based on MUC2 and MUC5AC mucin expression, (b) Classification according to Laurén (34)

a complete absence of expression of sialyl-Tn antigen, as expected. Immunodetection of ST6GalNAc-I was observed in all normal gastric mucosa (Table 2) and was characterised by a weak immunofluorescence signal limited to the perinuclear area of cells from the foveolar epithelium, corresponding to a Golgi-like staining (Figure 3A-G). The process of deacetylation did not alter the expression of sialyl-Tn in normal gastric mucosa, which remained negative after treatment (data not shown).

4.4 Expression of ST6GalNAc-I and sialyl-Tn in intestinal metaplasia

The 24 foci of IM, 14 of complete IM and 10 of incomplete IM, were classified according to the pattern of expression of mucins (44). Cases with metaplastic areas which co-expressed gastric mucin MUC5AC together with intestinal mucin MUC2 were classified as incomplete IM, whereas cases with MUC2 but lacking MUC5AC were classified as complete IM.

Expression of sialyl-Tn antigen was observed in the mucin vacuoles of goblet cells of all cases of complete and incomplete IM (Figure 3C and 3D, respectively). All IM cases showed a strong expression of ST6GalNAc-I (Table 2, Figure 3A-D), clearly contrasting with the faint staining of normal mucosa areas. The staining was also restricted to goblet cells and the pattern was perinuclear (Golgi-like). Therefore, both in complete and incomplete IM, ST6GalNAc-I showed increased staining and co-localized with MUC2 (Figure 3A and 3B) and sialyl-Tn (Figure 3C and 3D).

4.5 Expression of ST6GalNAc-I and sialyl-Tn in gastric carcinoma

Expression of ST6GalNAc-I was observed in 24/31 (77%) of cases of gastric carcinoma (Table 2; Figure 3 E-G).The expression levels of enzyme (intensity) could not be assessed due to the heterogenous intensity observed within each gastric carcinoma case. However, differences were observed between the pattern of expression in gastric carcinoma and normal mucosa. A punctuated Golgi-like staining restricted to foveolar epithelium was seen in normal mucosa, opposite to the widespread perinuclear and diffuse cytoplasmic staining observed in gastric carcinoma. Due to this fact and to the focal and patchy nature of sialyl-Tn and ST6GalNAc-I expression in carcinomas, a semi-quantitative scale of percentage of positive cells was used to achieve a more informative classification.

We observed a significant association between ST6GalNAc-I expression and sialyl-Tn expression, but not with the histopathologic type of gastric carcinoma (Table 2). In the majority of carcinoma cases where co-expression of sialyl-Tn and ST6GalNAc-I was observed, both sialyl-Tn and ST6GalNAc-I were largely expressed in the same cells. However, in 8 cases the score for sialyl-Tn expression exceeded the score observed for ST6GalNAc-I. In addition, there were 4 cases with expression of sialyl-Tn where we did not observe ST6GalNAc-I (Table 2).

4.6 Expression of ST6GalNAc-I and sialyl-Tn in normal colorectal mucosa

In all 15 cases, normal appearing colorectal mucosa showed a strong perinuclear, Golgi-like, staining pattern of ST6GalNAc-I throughout all compartments of the colonic crypts (Figure 4B). Before deacetylation with sodium hydroxide, sialyl-Tn was only expressed sporadically in lower compartments of the crypts in about half of the cases (Figure 4C). After deacetylation, all cases expressed sialyl-Tn intracellularly in lower compartments; however, in some cases the expression of sialyl-Tn was also observed in goblet cells throughout the crypts (Figure 4D).

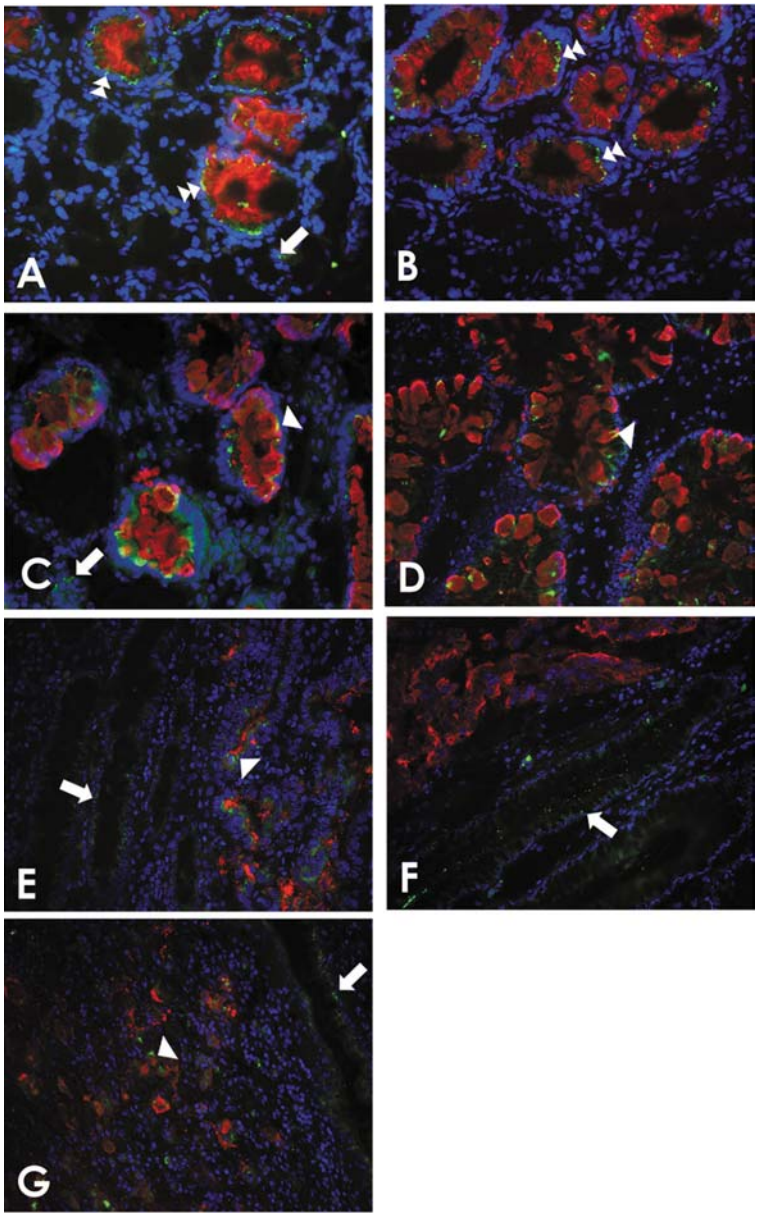

Figure 3. Double labelling of ST6GalNAc-I (Mab 2C3) and sialyl-Tn (Mab TKH2) or MUC2 (MAb PMH1) in gastric tissues. Normal gastric mucosa adjacent to intestinal metaplasia and carcinomas shows weak expression of ST6GalNAc-I in the foveolar epithelium (A, C, E, F, G, arrows). MUC2 (red) and ST6GalNAc-I (green) are overexpressed in the goblet cells in complete intestinal metaplasia (A, double arrowheads) and incomplete intestinal metaplasia (B, double arrowheads). Sialyl-Tn (red) and ST6GalNAc-I (green) are overexpressed in the goblet cells in complete intestinal metaplasia (C, arrowhead) and incomplete intestinal metaplasia (D, arrowhead). Gastric carcinoma of the intestinal type showing co-localization of sialyl-Tn and ST6GalNAc-I (E, arrowhead). Gastric carcinoma of the intestinal type showing expression of sialyl-Tn and absence of expression of ST6GalNAc-I (F). Gastric carcinoma of the diffuse type showing co-expression of sialyl-Tn and ST6GalNAc-I (G, arrowhead). Magnification 200x.

4.7. Expression of ST6GalNAc-I and sialyl-Tn in colorectal adenocarcinoma

ST6GalNAc-I was observed in 15 cases of colorectal adenocarcinoma (Table 2, Figure 4E and 4F). Expression of ST6GalNAc-I was observed in 50% of cases and in >50% of the tumour cells. Generally the staining intensity was weaker in tumour cells than in the normal cells. Exceptionally strong staining of both ST6GalNAc-I and sialyl-Tn was observed in transitional tissue, a tissue which is immediately adjacent to tumour edge but with no histological features of malignancy. Transitional tissue was observed in 4 of the colon biopsies (not shown).

Sialyl-Tn was expressed in the same cases as

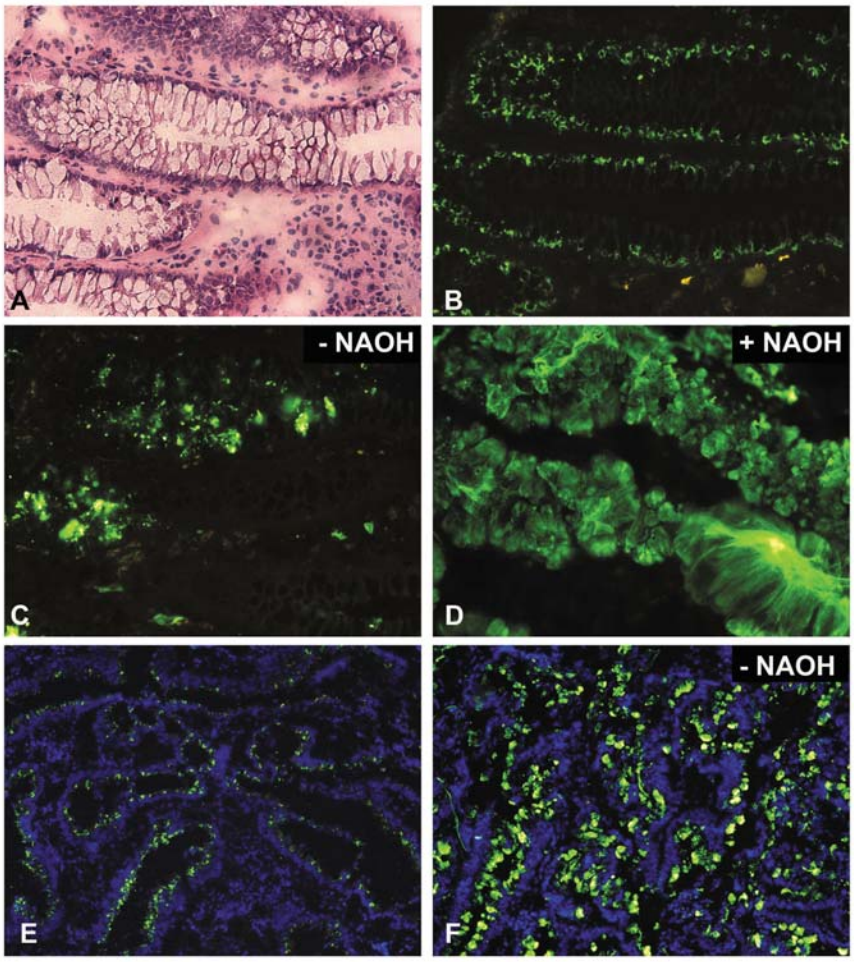

Figure 4. Normal colon stained with H&E (A), with MAb 2C3 (ST6GalNAc-I) (B) and MAb TKH2 (Salyl-Tn) before deacetylation (C) and after deacetylation by treatment with NAOH (D) as described in Mat. & Methods. Colon carcinoma stained with MAb 2C3 (ST6GalNAc-I) (E) and MAb TKH2 (Sialyl-Tn) (F). ST6GalNac-I and sialyl-Tn co-localize in colon carcinomas. In normal colon this co-localization is observed only when the tissue sample is submitted to deacetylation, exposing the Sialyl-Tn antigen. Magnification 200x.

ST6GalNAc-I and in the same areas. Deacetylation of the tissue enhanced the staining of sialyl-Tn in most of the cases, but this effect was far less pronounced than in normal colon samples. Sialyl-Tn was observed in the apical cell membranes, cytoplasm, and luminal contents of the colorectal adenocarcinomas. There was no correlation of Duke's clinical staging with the expression of ST6GalNAc-I; however, a strong association with sialyl-Tn expression was observed (Table 2). We could not detect tumour cells that expressed sialyl-Tn and does not express ST6GalNAc-I. On the other hand we could occasionally detect few tumour cells that expressed ST6GalNAc-I and lacking expression of sialyl-Tn.

5. DISCUSSION

Sialyl-Tn antigen is a simple mucin-type carbohydrate antigen whose aberrant expression is common in several human carcinomas and precursor lesions of cancer (1-8, 12, 13). Sialyl-Tn antigen is the product of an abnormal glycosylation pathway, corresponding to the early α2,6-sialylation of GalNAcα-*O*-Ser/Thr. The molecular mechanism leading to the activation of this pathway in carcinomas remains to be clarified.

We have previously shown *in vitro* that ST6GalNAc-I and ST6GalNAc-II can synthesize sialyl-Tn, but only ST6GalNAc-I can induce high amounts of sialyl-Tn when over-expressed in cancer cell lines (28). In most cells multiple glycosylation pathways compete for the same substrate and hence concordance between *in vitro* activity and *in vivo* function may not be evident. We used the CHO ldlD cell system to determine if the function of ST6GalNAc-II was inhibited by other glycosyltransferases competing for the same substrate. Indeed, ST6GalNAc-II did not produce substantial amounts of sialyl-Tn in a cell capable of only producing the enzymes substrate, the Tn glycoform as part of the abundant MUC1 mucin. In

contrast, ST6GalNAc-I produced sialyl-Tn under the same conditions. Therefore, it may be concluded that the low activity of ST6GalNAc-II at producing sialyl-Tn in cells was not due to competing glycosylation. Different molecular mechanisms leading to sialyl-Tn biosynthesis have been previously described. In the LS174T colon carcinoma cell line model, Brockhausen and co-workers demonstrated that the expression of sialyl-Tn in a subset of cells is associated with a lack of core1 β3Gal-transferase activity, and not with α2,6Sialyl-transferase activity (22). Whereas in the LMCR colon carcinoma cell line, they showed that the reverse was true, i.e., sialyl-Tn expressing cells had increased α2,6Sialyl-transferase activity (23). Therefore, using two cell lines from colonic origin, it was shown two different mechanisms leading to sialyl-Tn synthesis: one due to ST6GalNAc-transferase activity overexpression, and another due to the inability of synthesizing the core1 glycoform (T-antigen), as a result of inactivation of the molecular chaperone, *Cosmc* (48). The *Cosmc* chaperone is required for expression of an active β3Gal-transferase and, subsequently, core1 synthesis and proper *O*-glycosylation (49). *Cosmc* mutations have been found in cancer cell lines and primary cervical cancers where its inactivation has been associated with sialyl-Tn expression (48, 49). *Cosmc* mutations in gastric carcinomas remain unidentified. In fact, the action of competing glycosyltransferases, namely core 1 β3Gal-transferase that uses the same substrate as ST6GalNAc-I (GalNAc-*O*-Ser/Thr), and core 3 (β3GnT6), may be a mechanism interfering with sialyl-Tn biosynthesis. Another factor may be the sub cellular localization and Golgi compartment localization of these glycosyltransferases. Previous studies have shown that sialyl-Tn synthesis in colorectal adenocarcinoma was localized throughout the Golgi apparatus, including the early compartments (50). This result was further supported by another study showing that ST6GalNAc-I was found in all Golgi sections in carcinoma cells (30). In fact, the biosynthetic pathway leading to the production of disialyl-T structures (27-29) involves the assembly of the monosialyl-T glycoform (NeuAcα2-3Galβ1-3GalNAcα-*O*-Thr/Ser) prior to the action of ST6GalNAc-transferase. This sequence of glycan addition to the T-antigen is supported by the topological location of ST6GalNAc-I in the Golgi compartment; whereas, core 1 β3Gal-transferase and α2,3sialyltransferases, are located in earlier cellular compartments. Studies showing that transfection of cells with ST6GalNAc-I can result in overriding of the natural *O*-glycosylation pathway with resulting truncated sialyl-Tn *O*-glycans therefore suggest that it is the overexpression and likely the resulting altered topology of the enzyme that drives the premature α2,6sialylation of Tn before core 1 extension (30). This interpretation would be in agreement with the recent finding that ST6GalNAc-II, while capable *in vitro* of using Tn substrates, is incapable of inducing substantial amounts of sialyl-Tn synthesis in cells with abundant Tn substrates and without competitive alternative glycosylation pathways (28). Presumably this is due to an entirely different subcellular topology (Figure 2).

In the present study, we have produced and characterized two novel MAbs that specifically react with either the native or the denatured ST6GalNAc-I enzyme protein and have utility in immunohistology applications and SDS-PAGE Western blot. Although the homologous enzymes ST6GalNAc-I and ST6GalNAc-II share a degree of sequence similarity we did not identify clones with cross-reactivity between the enzymes. This result is similar to our past experience with the immunogenicity of homologous glycosyltransferases (32). In agreement with previous studies we have observed the absence of sialyl-Tn expression in normal gastric mucosa. Nevertheless, weak immunoreactivity for the ST6GalNAc-I enzyme was observed in the foveolar epithelium of gastric mucosa. The ST6GalNAc-I staining was restricted to the perinuclear region suggesting Golgi localization (Table 2; Figure 3). A recent study has shown that ST6GalNAc-I mRNA is weakly expressed in various normal tissues despite that these tissues generally do not express the sialyl-Tn antigen (30). In addition, we have previously shown that gastric cell lines devoid of sialyl-Tn, still express basal levels of ST6GalNAc-I transcripts (31). It is therefore clear that expression of low levels of ST6GalNAc-I mRNA as well as basal enzyme levels, as shown here, may not be sufficient *per se* to induce sialyl-Tn expression and further supports the importance of the levels of expression of the ST6GalNAc-I enzyme as well as the Golgi compartment localization.

The expression of sialyl-Tn in colonic mucosa appears to represent a different scenario. ST6GalNAc-I is expressed in normal colon both at the mRNA and protein levels. Nevertheless, sialyl-Tn is not normally detectable at the surface colon cells, being restricted to perinuclear staining at the base of the crypt in few cases (Figure 4). In normal colonic epithelial cells, sialic acid residues are often modified by *O*-acetyl groups, thereby precluding antibody recognition of the sialyl-Tn antigen. Deacetylation leads to sialyl-Tn exposure and consequent detection, and MAb staining largely increases after this procedure, as can be seen in Figure 4. Yet, this mechanism is not true for normal gastric mucosa. We observed no sialyl-Tn expression after deacetylation of normal gastric mucosa (data not shown). *O*-acetylation in the human stomach is very rare and limited to pathological conditions (51) so the absence of sialyl-Tn detection in normal gastric mucosa cannot be explained by such mechanism.

In the human stomach, Intestinal Metaplasia is a pre-malignant lesion characterized by a global trans-differentiation of gastric epithelium into intestinal epithelium and is associated with an increased risk for gastric carcinoma development. Intestinal metaplasia expresses several intestinal markers that are foreign to normal gastric epithelial cells, such as MUC2 and sialyl-Tn, which co-localize at the mucinous vacuoles of goblet cells (44, 52). We observed that ST6GalNAc-I was expressed in all intestinal metaplasia cases (24/24), and this expression co-localizes both with MUC2 and sialyl-Tn (Figure 3). The ST6GalNAc-I staining was in the perinuclear area of goblet cells observed in all metaplastic glands, regardless of the histological sub-type, and was noticeably more intense than in normal gastric mucosa (Figure 3). It is therefore clear that an overexpression of

ST6GalNAc-I exists in intestinal metaplasia when compared to normal gastric mucosa, and supports the hypothesis that overexpression of ST6GalNAc-I is a mechanism leading to sialyl-Tn antigen expression in intestinal metaplasia.

In the present study, the abundant sialyl-Tn expression observed in intestinal metaplasia without deacetylation indicated that *O*-acetylation did not interfere with sialyl-Tn detection in intestinal metaplasia as it does on normal colon (51). Nevertheless, our results showed that the intestinal metaplasia model recapitulates normal colon as ST6GalNAc-I and sialyl-Tn are co-expressed in the same cells.

In gastric carcinoma, sialyl-Tn expression was detected in 87% of the cases similarly to previously published series (2), and ST6GalNAc-I expression was found on 77% of gastric carcinoma cases. ST6GalNAc-I showed a heterogeneous staining, either clustered in focal areas or widely dispersed, and showed variable intensity of staining within each case, thus justifying for a different classification criteria than in intestinal metaplasia cases, based on the percentage of positive cells in the tumour. Using this classification, ST6GalNAc-I expression was significantly associated with sialyl-Tn expression, independent of the histologic type of the tumour. Expression of ST6GalNAc-I in >50% of tumour cells was observed in 7/31 cases, all of which also expressed sialyl-Tn in >50% of cells. This equals saying that all gastric carcinoma cases that were high enzyme expressers were also so for sialyl-Tn. However, the inverse was not true, i.e. there were several cases that expressed sialyl-Tn in >50% of their tumour cells but have <50% cells with ST6GalNAc-I (8/18) or are even negative (3/18). This means that, although the majority of gastric carcinoma cases expressed sialyl-Tn and ST6GalNAc-I in the same cells, there were areas/cases where sialyl-Tn was expressed without ST6GalNAc-I detection. Therefore, although there was an association between sialyl-Tn and ST6GalNAc-I at the case level, the expression of both did not always co-localize at the cellular level. This apparent discrepancy at the cellular level may be explained by different hypothesis. One possibility is that ST6GalNAc-I may be present but technically undetectable due to low levels of expression. Supporting this hypothesis, Sewell and co-workers have found that, in breast carcinomas, ST6GalNAc-I expression correlated at the mRNA level with sialyl-Tn expression. Nevertheless, cases that were weak to moderate positives for sialyl-Tn (50% of cases) did not express any detectable ST6GalNAc-I RNA by Northern Blot analysis, but were positive by RT-PCR (30). These results suggest that enzyme detection at the protein level may only occur in cases with abundant sialyl-Tn expression. Another possibility is that ST6GalNAc-I may not be constitutively expressed in the tumour cells, resulting in a temporary presence of the enzyme and longer expression of sialyl-Tn bearing glycoprotein. Finally, we could not exclude the possibility that ST6GalNAc-II, which was demonstrated to be enzymatically capable of synthesizing sialyl-Tn in this study and in a previous one (28), may co-adjuvate ST6GalNAc-I in tumour cells.

A clear scenario was observed in colon adenocarcinomas where a major overlap of enzyme and glycan staining was observed. Colon adenocarcinomas showed a significant association between ST6GalNAc-I expression and sialyl-Tn expression that is independent of the clinical stage (Table 2). The presence of both enzyme and product was similar to normal colon mucosa with the difference that for a complete overlap, deacetylation procedures must be applied. In line with this, a previous study has shown that normal and cancerous colon tissues have similar ST6GalNAc enzymatic activity (53). Interestingly enough, transitional tissues have been shown to display increased enzymatic activity when compared to normal mucosa and cancer (54). We found identical results reflected by the exceptionally strong staining of ST6GalNAc-I in our transitional colon tissues.

In conclusion our results demonstrate that the novel monoclonal antibody 2C3 is highly specific for the ST6GalNAc-I enzyme and detects this protein in cells and tissues expressing it. We also demonstrate that ST6GaNAc-I expression is associated with sialyl-Tn expression in gastrointestinal tissues supporting the ST6GalNAc-I as the major regulator of expression of cancer-associated sialyl-Tn antigen on *O*-linked carbohydrate chains.

6. ACKNOWLEDGMENTS

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Key Words: ST6GalNAc-I, Sialyl-Tn, Glycosylation, Gastric Carcinoma, Intestinal Metaplasia

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5.3

Challenging the Limits of Detection of Sialylated Thomsen-Friedenreich Antigens by In-gel Deglycosylation and nano-LC-MALDI-TOF-MS.

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Short Communication

Challenging the limits of detection of sialylated Thomsen–Friedenreich antigens by in-gel deglycosylation and nano-LC-MALDI-TOF-MS

The identification of sialylated Thomsen–Friedenreich antigens in proteins poses much interest in the context of cancer research. MALDI-TOF-MS is a powerful technique for this purpose; still it shows considerable low sensitivity for sialylated molecules due to in-source and metastable decomposition. Herein, we report a target-driven strategy to identify these antigens in minute amounts of glycoproteins isolated in polyacrylamide gels. The glycans were recovered from gel spots by reductive β-elimination, permethylated and analyzed by nano-LC-MALDI-TOF-MS. A computational algorithm was developed to filter spectral noise and enhance/isolate the signals of interest. Sialylated antigens were identified in minute amounts of fetuin (0.1 μg) and plasminogen (1.0 μg) by this approach. MS assignments were further validated by enzymatic methods. This methodology allowed a fivefold decrease in the current LOD of fetuin sialylated *O*-glycans by MALDI-TOF-MS.

Keywords:
Nano-LC-MALDI-TOF-MS / Permethylation / Sialic acids / Simple mucin-type antigens / Thomsen–Friedenreich antigens DOI 10.1002/elps.201300148

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Sialylated Thomsen–Friedenreich-related antigens are a class of short-chain glycans that result from a premature stop in the *O*-glycosylation of proteins [1]. It includes the sialyl Tn (Neu5Acα2–6GalNAcα-O-Ser/Thr; sTn), sialyl T (Neu5Acα2–3Galβ1–3GalNacα-O-Ser/Thr and/or Galβ1–3[NeuAcα2–6]GalNAcα-O-Ser/Thr; sT), and di sialyl T (NeuAcα2–3Galβ1–3[NeuAcα2–6]GalNAcα-O-Ser/Thr; dsT). These antigens are often overexpressed by human cancers, reflecting alterations in glycosylation pathways [2]. Its expression modulates protein function and consequently the cell behavior and immune recognition [3, 4]. They are also important cancer biomarkers [1] and present potential for immunotherapy [5]. Thus, major efforts have been put in the identification of these glycans in proteins.

MALDI-MS is a powerful, highly sensitive, tool for the structural analysis of complex glycans [6, 7] and glycopeptides [8]. However, this type of ionization induces a high degree of vibrational excitation in the generated ions leading to a significant dissociation of sialic acids [9, 10]. Sialylated molecules also experience metastable decay [7, 11], particularly in the reflector mode [12]. Thus, high amounts of the targeted

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Abbreviations: dsT, di sialyl T; **NEIC**, normalized extracted ionic current; **sT**, sialyl T; **sTn**, sialyl Tn; **TIC**, total ionic current

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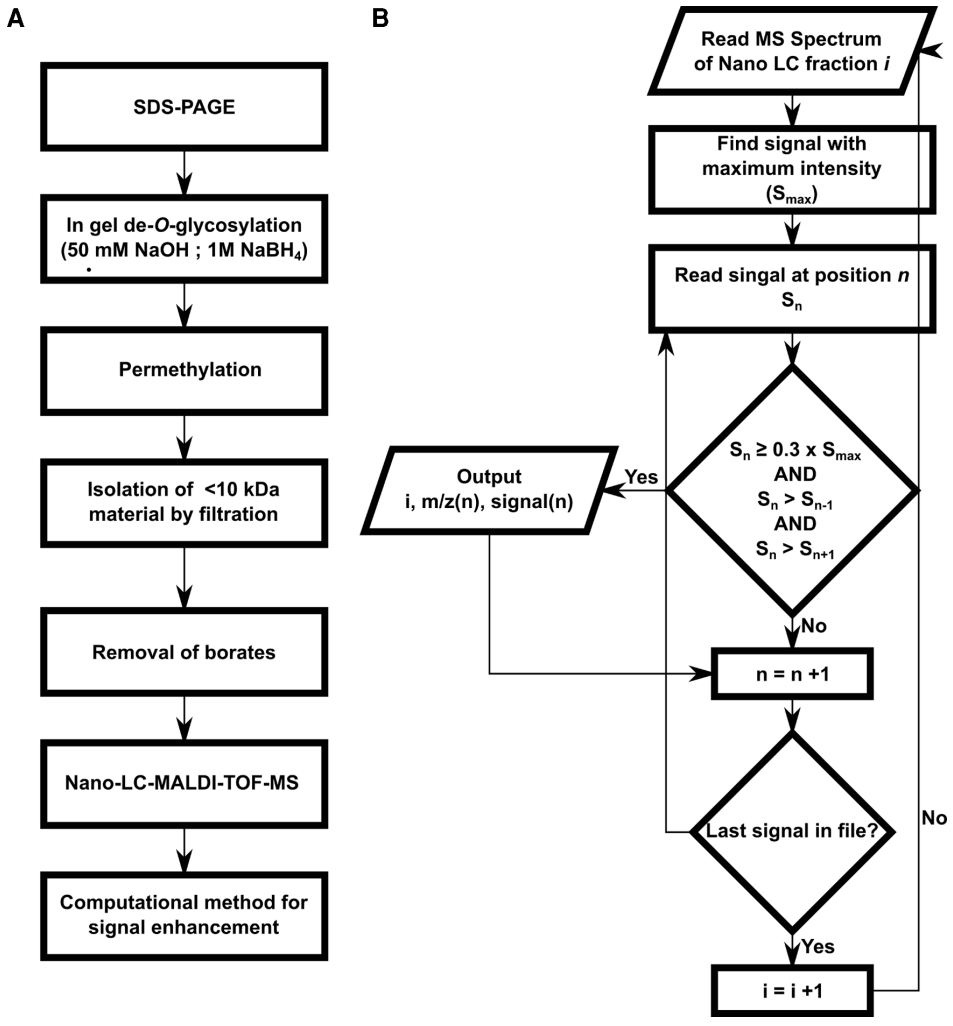
Colour Online: See the article online to view Figs. 1 and 2 in colour.



Scheme 1. (A) Overview on the analytical approach developed for the analysis of trace amounts of sialylated Thomsen–Friedenreich-related and (B) the "in house" algorithm used to enhance/identify the signals of interest.

glycoproteins are needed to achieve structural assignments, making the introduction of enrichment strategies mandatory [13].

The permethylation of the glycans is considered the best strategy to overcome these limitations [4, 14–21]. Permethylation reduces the liability of sialic acids [7], allowing their detection together with neutral sugars by MALDI-MS in positive ion mode [14]. By equalizing their chemical properties it also allows a semiquantitative comparison between glycans [15]. Most laboratories use a modification of the method described by Ciucanu and Kerek [16] introduced by Ciucanu and Costello [17]. This involves the reaction of glycans with methyl iodide in the presence of sodium hydroxide in dimethyl sulfoxide in nonanhydrous conditions, which significantly reduces the oxidative degradation of glycans [17]. Permethylation in small/capillary columns packed with sodium hydroxide (solid-phase permethylation) has been shown capable of improving sample recovery, a critical matter when dealing with trace samples [18]. This approach has enabled the identification by MALDI-TOF-MS of sT and dsT antigens in 0.5 μg of fetuin from fetal bovine serum [19].

In a recent study, we combined in-gel de-*O*-glycosylation followed by permethylation with nano-LC-MALDI-TOF-MS to identify sTn antigens in plasminogen of individuals with gastric precancerous lesions [20]. However, this was done with about 10 μg of plasminogen previously isolated from human serum by Lysine-sepharose affinity chromatography. Herein, we challenge the LOD of this technique using fetuin from bovine serum, which is known to express mainly the sT antigen [21, 22] and lower amounts of dsT and other glycans [23]. Since fetuin is the most used model protein for glycomics studies, this allows a comparison with previous reports.

Briefly, 0.1, 0.5, and 1.0 μg of fetuin were separated by SDS-PAGE and the corresponding bands (50 and 75 kDa) were revealed with Coomassie Colloidal Blue thereafter. The bands were then excised from the gels and incubated with 50 mM NaOH and 1 M $NaBH_4$ at 45°C for 16 h to promote the release of *O*-glycans (Scheme 1A). The reaction was stopped with a few drops of glacial acetic acid. The reducing agent kept the glycan from "peeling" after being released and allowed tagging the nonreductive end. The samples were then prepurified with 10 kDa MWCO filters (Millipore) to remove

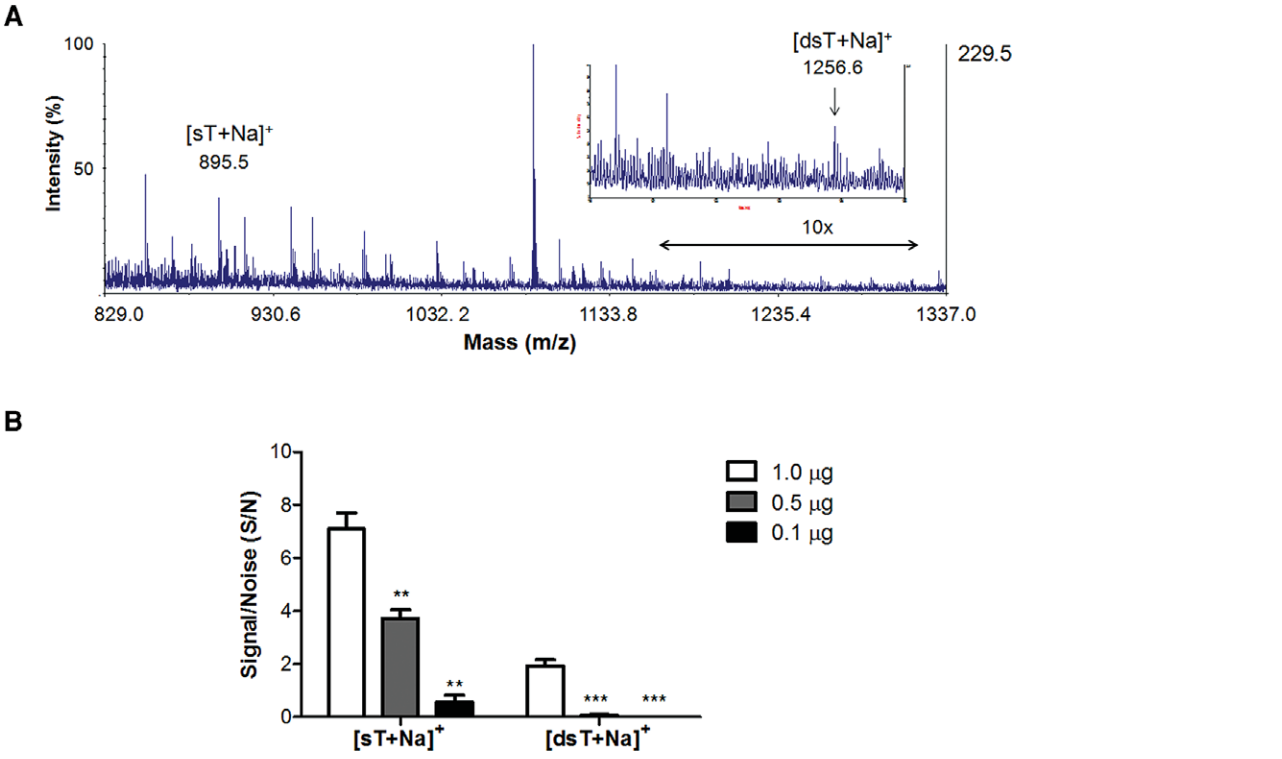
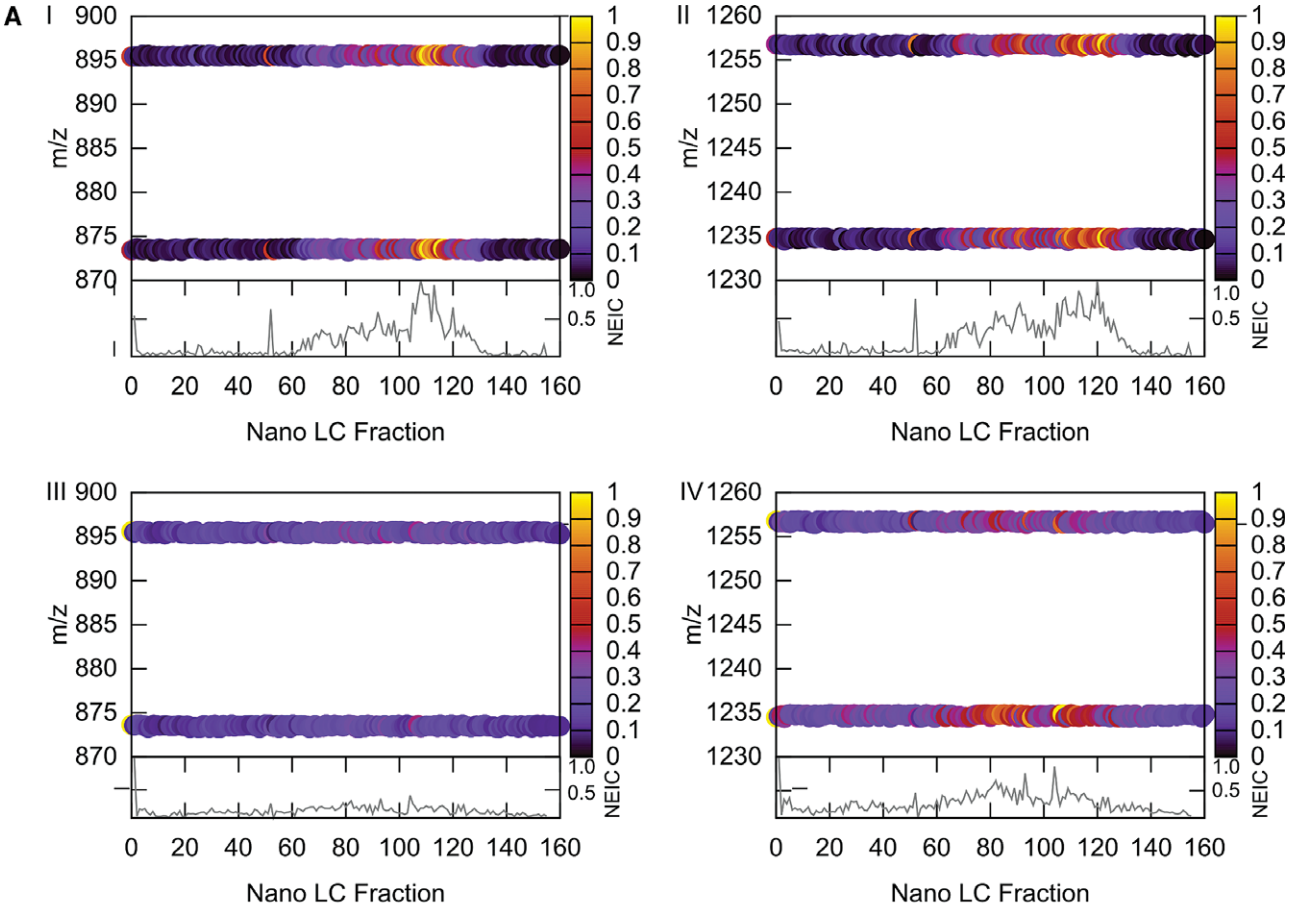


Figure 1. (A) MALDI-MS spectra of reduced and permethylated *O*-glycans isolated from 1.0 μg of fetuin, highlighting the ions at *m/z* 895.5 ± 0.2 $[sT + Na]^+$ and 1256.6 $[dsT + Na]^+$. The other signals in the MALDI-MS spectrum did not match the *O*-glycans deposited in CFG, Carbbank, GlycomeDB, and Glycosciences databases has shown by the GlycoWorkBench 2 software [24]. (B) S/N of these ions for different amounts of fetuin. The MS spectra of desialylated samples did not exhibit these ions, which confirmed the structural assignment (data not shown). The results are presented as average ± SD of three independent replicates. "**" $p < 0.01$; "**" $p < 0.001$ versus preceding concentration. The S/N was calculated according to the standard definition: $S/N = P_{signal}/P_{Noise}$; where *P* stands for the average TIC in the named region of the mass spectra.

proteins and high molecular weight peptides, generated by the harshness of the deglycosylation conditions. The filtrate, containing the reduced *O*-glycans but also low molecular weight peptides, was incubated several times with methanol containing 5% (v/v) acetic acid under a stream of nitrogen to remove borates as methyl esters. The samples were permethylated adopting a modification of the method by Ciucanu and Costello [17]. Briefly, native *O*-glycans were dissolved in 78.0 μL of DMSO containing trace amounts of water (0.3 μL) and fine grinded NaOH was added to the reaction medium. The mixture was sonicated for 10 min and frozen prior to the addition of 10 μL of CH_3I and then incubated under mild stirring for 10 min at room temperature. The permethylated glycans were then dissolved in dichloromethane and extensively washed with 10 mM HCl solution to avoid the base-induced hydrolysis the permethylated sialic acids carboxymethyl groups. The organic phase was evaporated under vacuum. Equivalent amounts of fetuin previously digested with neuraminidase from *Clostridium perfringens* to remove sialic acids that were used as controls. All experiments were performed in triplicates.

The permethylated samples were then analyzed in a MALDI TOF/TOF mass spectrometer (4800 Proteomics Analyzer, AB SCIEX, Foster City, CA, USA) in the positive ion reflector mode. The mass spectra were obtained in the mass range from 600–4500 Da with 1200 laser shots using as a matrix 2,5-dihydroxybenzoic acid (DHB; 10 mg/mL in 70% ACN/0.1% TFA). The MS spectra for 0.5 and 1.0 μg of fetuin exhibited the ions at *m/z* 895.7 and 1256.6 consistent with $[sT+Na]^+$ and $[dsT+Na]^+$, respectively (Fig. 1A and B). The higher signal-to-noise ratio (S/N) of sT in comparison to dsT (Fig. 1B) was in agreement with its abundance in fetuin [22, 23]. Due to the low abundance of the ions it was not possible to run MS/MS experiments. Nevertheless, these species were not observed in samples digested with neuraminidase, suggesting that the ions at *m/z* 895.7 and 1256.6 belong to sialylated species, which reinforces our structural assignments. However, none of these ions were detected using 0.1 μg of fetuin (Fig. 1B).

In an attempt to decrease the LOD, 0.1 μg of fetuin samples were dissolved in 70% ACN and separated in a nano-HPLC Ultimate 3000 system (Dionex, Amsterdam) equipped with a C18 reverse phase capillary column (Pepmap100 C18; 3 μm particle size, 0.75 μm internal diameter, 15 cm in length). The separation was performed using a linear gradient of 3–50% B (90% ACN, 0.1% TFA) for 45 min, 50–70% B for 10 min, and 70–32% A (0.1% TFA) for 5 min. The eluted glycans were applied directly on a MALDI plate in



B

Plot	Designation	Background noise Average NEIC	Background noise standard deviation NEIC	NEIC Average	NEIC Standard deviation	NEIC SNR[(a)]
I	sT	0.07	0.04	0.74	0.17	10.6
II	dsT	0.08	0.04	0.58	0.18	7.6
III	Desialylated sT	0.16	0.04	0.22	0.04	1.5
IV	Desialylated dsT	0.25	0.04	0.32	0.09	1.3

Figure 2. (A) Plots of the sparse matrix for 0.1 μg of fetuin (nano-LC fraction versus *m/z* versus NEIC) for the signals at *m/z* 873.5 ± 0.2 $[sT + H]^+$ and 895.5 ± 0.2 $[sT+Na]^+$ (plots I and III) and *m/z* 1234.7 $[dsT + H]^+$ and 1256.6 $[dsT + Na]^+$ (plots II and IV). The top part of each graph represents the NEIC of each extraction window, whereas the lower part represents the combined NEIC of protonated and sodiated species as a function of the nano LC fraction. Plots I and II refer to reduced and permethylated glycans recovered from native fetuin, while III and IV result from desialyated fetuin. A chromatographic envelop, characterized by a cluster of high-intensity signals and lesser dispersion of *m/z* over the envelope region, is visually detected in plot I, spanning fractions 105–115, and in plot II, spanning fractions 115–125. This chromatographic profile is absent from desialylated samples (plots III and IV). (B) Signal average and S/N for NEIC of the combined sodiated and protonated species presented in Fig. 2A). In all cases, LC factions 20–40 where used to sample the background noise. The signal window considered for sT spanned LC fractions 105–115 and for dsT LC fractions 115–125. The S/N was calculated according to the standard definition: $S/N = P_{signal}/P_{Noise}$; where *P* stands for the average NEIC in the named region of the mass spectra.

10 s fractions using an automatic fraction collector Probot (Dionex, Amsterdam) under a continuous flow rate of 270 nL of DHB with 15 fentmole of Glu-1-Fibrinopeptide B (Protea Biosciences) as an internal standard.

An "in house" algorithm (Scheme 1B) was applied to nano-LC-MALDI-MS spectra to filter noise and reduce the original dataset to a sparse matrix containing only candidate signals. This algorithm combined the MALDI-MS data from each chromatographic run into a 3D data array comprehending the nano-LC fractions versus *m/z* versus MALDI-MS normalized extracted ionic current (NEIC). The NEIC results from a normalization of the total ionic currents (TIC) of each run. The distribuition of the signals within 0.2 Da of the ions of interest were comprehensively analyzed. The surveyed ions included both protonated and sodiated forms of sT (*m/z* 873.5 and 895.7) and dsT (*m/z* 1234.7 and 1256.6).

As shown by the plots in Fig. 2A, all the surveyed ions presented sparse matrix plots with clusters of high-intensity signals. These patterns showed a lesser dispersion of m/z values over these particular regions, thus consistent with chromatographic envelopes, which strongly suggests the presence of the targeted compounds. This also allowed the identification of LC fractions enriched in the species of interest that were not evident in the original spectra (data not shown). The average NEIC for the species of interest was then evaluated and compared with the corresponding intensity of the background signals (nano-LC fractions 40–20; Fig. 2B). The NEIC of the combined signals of both protonated and sodiated was 0.74 ± 0.18 (S/N 10.6) and 0.58 ± 18 (S/N 7.6), for sT and dsT, respectively. The desialylation of fetuin led to the disaperance of the chromatographic envelopes, as highlighted by the decrese in combined NEIC to 0.22 ± 0.18 (S/N 1.5) for sT and 0.32 ± 0.18 (S/N 1.3) for dsT (Fig. 2B), which reinforced the structural assignments. Having established the technique, we attempted to decrease the amount of fetuin required for analysis, however without success. This strategy was then applied to plasminogen isolated from the serum of healthy individuals as described by Gomes et al. [20]. This allowed a tenfold decrease in the amount of protein required for analysis (Supporting Information Fig. 1S).

In summary, in-gel de-*O*-glycosylation, permethylation and nano-LC-MALDI-MS backed by comprehensive data mining allows the detection of sialylated T-related antigens in trace amounts of glycoprotein isolated in SDS-PAGE gels. The LOD of sialylated T-related antigens recovered from fetuin was decreased approximately 5 times in comparison to previous reports [18]. To our knowledge, this is the first attempt to analyze such low amounts of glycans recovered from gel spots. This approach may now be adopted to study other classes of glycans.

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The authors have declared no conflict of interest.

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Appendix

Published Papers

Journal of proteome research

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Glycoproteomic Analysis of Serum from Patients with Gastric Precancerous Lesions

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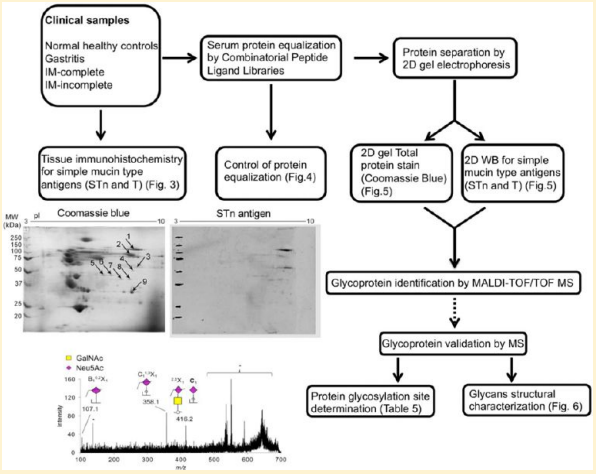
Supporting Information

ABSTRACT: Gastric cancer is preceded by a carcinogenesis pathway that includes gastritis caused by *Helicobacter pylori* infection, chronic atrophic gastritis that may progress to intestinal metaplasia (IM), dysplasia, and ultimately gastric carcinoma of the more common intestinal subtype. The identification of glycosylation changes in circulating serum proteins in patients with precursor lesions of gastric cancer is of high interest and represents a source of putative new biomarkers for early diagnosis and intervention. This study applies a glycoproteomic approach to identify altered glycoproteins expressing the simple mucin-type carbohydrate antigens T and STn in the serum of patients with gastritis, IM (complete and incomplete subtypes), and control healthy individuals. The immunohistochemistry analysis of the gastric mucosa of these patients showed expression of T and STn antigens in gastric lesions, with STn being expressed only in IM. The serum glycoproteomic analysis using 2D-gel electrophoresis, Western blot, and MALDI-TOF/TOF mass spectrometry led to the identification of circulating proteins carrying these altered glycans. One of the glycoproteins identified was plasminogen, a protein that has been reported to play a role in *H. pylori* chronic infection of the gastric mucosa and is involved in extracellular matrix modeling and degradation. Plasminogen was further characterized and showed to carry STn antigens in patients with gastritis and IM. These results provide evidence of serum proteins displaying abnormal *O*-glycosylation in patients with precursor lesions of gastric carcinoma and include a panel of putative targets for the non-invasive clinical diagnosis of individuals with gastritis and IM.



KEYWORDS: *biomarkers, O-glycosylation, gastritis, intestinal metaplasia, sialyl-Tn, T antigen, plasminogen*

INTRODUCTION

The development of gastric cancer is associated with a long carcinogenesis pathway that is initiated by *Helicobacter pylori* (*H. pylori*), a Gram-negative bacterium that causes gastritis, and may lead to the development of a chronic atrophic gastritis, intestinal metaplasia (IM), and ultimately gastric adenocarcinoma.[1−6] *H. pylori* infects more than 70% of the population in some countries,[7] but only a fraction of those individuals develop more severe gastric conditions, such as atrophic gastritis and IM, a precancerous lesion.[1,3] The current diagnosis for these precursor lesions relies almost exclusively in endoscopy followed by biopsy, which is both invasive and costly to apply for screening strategies. Therefore, biomarkers that can aid in the screening and identification of individuals with silent gastric pathologies are highly needed.

Glycosylation is a common post-translational modification of proteins with more than 50% of eukaryotic proteins thought to be glycosylated.[8] The pattern of protein glycosylation is cell- and tissue-specific and closely reflects the physiological status of the cell. Thereby, changes in glycan expression are frequently observed in several pathological conditions[9] and in particular in

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the gastric context.[10−12] In gastric pathologies the glycosylation alterations include aberrant expression of simple mucin-type carbohydrate antigens, namely, T (Galβ1−3GalNAcα-O-Ser/Thr), Tn (GalNAcα-O-Ser/Thr), and sialyl-Tn (Neu5Acα2−6GalNAcα-O-Ser/Thr).[10,11,13,14] An overexpression of sialylated Lewis antigens[15] and the decreased expression of terminal α1,4-linked *N*-acetylglucosamine residues (αGlcNAc) has also been reported.[11,16,17]

The biosynthesis of the carbohydrate structures in glycoproteins relies on a number of competitive and concerted processes involving several glycosyltransferases. Mucin (GalNAc)-type *O*-glycosylation (hereafter called *O*-glycosylation) is one of the most common types of glycosylation found in glycoproteins and consists of a glycan *O*-linked to a serine or a threonine residue. The first step in *O*-glycosylation is the transfer of GalNAc from a sugar donor UDP-GalNAc to a serine or threonine residue and is controlled by UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases (GalNAc-Ts).[18,19] These Golgi enzymes control the site of *O*-glycosylation. After the first glycan (GalNAc) is added forming the Tn antigen, the action of other glycosyltranferases leads to the synthesis of the various core structures depending on the cell context. In gastric epithelial cells, a Gal-transferase (C1GalT-1) leads to the biosynthesis of the core 1 (T antigen), which can be further branched, extended, and terminated by Lewis and ABO blood group antigens. Alternatively, Tn and T antigens can be sialylated by sialyltransferases forming the sialyl-Tn (STn), sialyl-T (ST), and disialyl-T (Figure 1) (for a review see Reis et al.[9]).

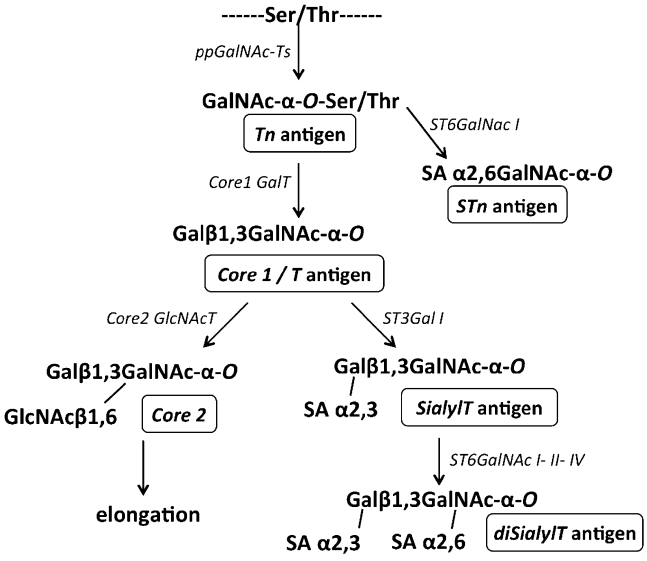

Figure 1. Schematic representation of the biosynthesis of core *O*-glycan structures and formation of simple type carbohydrate antigens Tn, STn, T, and ST.

The alterations in glycosylation observed in pathologic conditions are mostly due to modifications at the glycosylation cell machinery, disorganization of secretory pathway organelles (ER and Golgi), and altered glycosyltransferase expression. The aberrant expression of glycoconjugates bearing these glycans either present on the surface or secreted by cells are a major potential source of biomarkers representing most serological assays used for cancer detection and monitoring. These serological assays detect carbohydrate antigens such as SLe[a] (CA19−9) and STn (CA72−4) or mucin glycoproteins such as MUC1 (CA15−3) and MUC16 (CA125).[9]

The glycoproteins carrying immature glycans, such as simple mucin-type *O*-glycan antigens, can be present in circulating proteins in pathological conditions and are being targeted for early detection approaches. In this study we assess the immunohistochemical presence of truncated *O*-glycans STn and T antigens in gastric tissues, including normal gastric mucosa, gastritis, and IM. We have further screened sera from the same patients searching to identify proteins bearing these truncated glycans. This approach resulted in the identification of fine alterations in the *O*-glycosylation of serum glycoproteins, such as the presence of STn, which is known to be aberrantly expressed in the gastric lesions under study. Specifically, we identified serum proteins carrying abnormal *O*-glycans that can be candidate targets for the noninvasive diagnosis of precursor lesions of gastric cancer.

MATERIALS AND METHODS

Tissue Samples and Histology

Stomach biopsies and serum from individuals without gastric lesions and *H. pylori* infection (n = 6), gastritis (n = 5), and complete (n = 5) and incomplete (n = 3) IM were used. The individuals are part of a cohort from northern Portugal (Viana do Castelo) that have been studied and characterized as previously described for gastric pathologies and *H. pylori* infection.[20]

Paraffin sections were used for histochemistry and immunohistochemistry. Tissue sections were stained with hematoxylin and eosin (H&E) for histopathology. Clinical data, including *H. pylori* infection status, of every case was considered for selection of the cases and controls.

Immunohistochemistry

Paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min, and then sections were incubated with normal rabbit serum diluted 1:5 in PBS containing 10% BSA. Incubation with the monoclonal antibodies was performed overnight at 4 °C. Slides were washed in PBS and incubated for 30 min with secondary biotinylated rabbit anti-mouse antibody (E0354-DakoCytomation, Glostrup, Denmark) diluted 1:200 in PBS containing 5% of BSA. The slides were subsequently washed in PBS and incubated for 30 min with avidin−biotin complex (Vectastain Elite ABC kit, Burlingame, CA, USA) according to the manufacturer's recommendations. Staining was performed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) containing 0.02% hydrogen peroxide. Counterstaining of the nucleus was done with Mayer's hematoxylin. Monoclonal antibodies used in this study, their specificities, and their references are listed in Table 1.

Table 1. Specificity of Monoclonal Antibodies Used for Immunohistochemistry

MoAbs	isotype	dilution	antigen and ref
TKH2	IgG1	1:20	STn[73]
3C9	IgM	1:50	T[74]
CLH2	IgG2	1:750	MUC5AC[75]
PMH1	IgM	1:40	MUC2 GalNAc[76]

Serum Sample Collection and Protein Equalization by Combinatorial Peptide Ligand Library

Serum samples from the same individuals as the biopsies were pooled according to the clinical data (without gastric lesions and *H. pylori* infection, gastritis, and complete and incomplete IM). Equalization of the amount of proteins within each serum sample

group was done using a combinatorial peptide ligand library, CPLL (ProteoMiner, BioRad, CA),[21] according to the manufacturer's recommendations.

2D Gel Electrophoresis

Equalized protein samples from CPLL were precipitated (ProteoExtract, Calbiochem), resuspended in rehydration buffer (7 M urea, 2 M thiourea, 4% (v/v) CHAPS, and 0.0002% bromophenol blue) with 0.2% of ampholyte and quantified (2D Quant Kit from GE Healthcare). Passive rehydration of the strips was performed overnight with 100 μg of sample using IPG strips of pH 3−10 NL (ReadyStrip; 0.5 mm × 3 mm × 70 mm, Bio-Rad, Hercules, CA) at room temperature. Isoelectric focusing was performed on Protean IEF cell (Bio-Rad) with an initial voltage of 250 V for 15 min and then by applying a voltage gradient up to 4000 V with limiting current of 50 μA per strip and temperature set at 20 °C. The first dimension was concluded at 14−20 kVh.

Following the isoelectric focusing proteins were reduced and alkylated by incubation with 2% DL-dithiothreitol (DTT) followed by 2.5% iodoacetamide in an equilibration buffer (6 M urea, 2% SDS, 0.002% bromophenol blue, 75 mM Tris pH 8.8, 29.3% glycerol) for 10 min each under gentle agitation. The strips were then packed in a 1% low gelling (1% agarose in running buffer: 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, pH 8.3; Bio-Rad) on top of a 10% acrylamide gel (acrylamide/bisacrylamide 37.5:1, 2.6% from Bio-Rad). Second dimension electrophoresis was performed in a Mini-Protean tetra cell system (Bio-Rad) using 1xTris/glycine/SDS buffer at constant voltage of 125 V.

Western Blot Analysis

Gels were transferred to nitrocellulose membranes (Amersham) in a semidry system according to manufacturer's recommendations (TE 77 PWR Amersham) and were blocked with 5% BSA in PBS-Tween (PBS-T) 0.05%. Primary antibodies were incubated overnight at 4 °C with 5% BSA in PBS-T 0.05%. Membranes were washed three times with PBS-T 0.05% before secondary antibody incubation. Secondary antibodies were isotype-specific (Jackson immunoresearch) anti-IgM (dilution 1:100,000) and anti-IgG1 (dilution 1:50,000) and were incubated for 1 h in 1% BSA in PBS-T 0.05%. Signal detection was obtained by enhanced chemiluminescence (ECL plus, Amersham). The primary antibodies were the same as those used for immunohistochemistry (Table 1) and used undiluted.

Protein Selection and In-Gel Tryptic Digestion

2D gels were stained with Coomassie Blue (Bio-Safe Coomassie from Bio-Rad, CA) overnight, and images were acquired with a Gel Doc XR system (Bio-Rad, CA).

The spots highlighted in the Western blots were matched in the Coomassie Blue gels, and proteins were excised with a spotpicker (OneTouch 2D gel spotpicker, 1.5 mm diameter, Gel Company, USA). The selected protein spots were then processed for MALDI MS analysis in agreement with the trypsin manufacturer instructions (Promega, USA): the protein gel plugs were washed with water, destained with methanol/50 mM NH_4HCO_3 and acetonitrile/50 mM NH_4HCO_3 (1:1 v/v each), reduced with 25 mM DTT in 50 mM NH_4HCO_3 for 20 min at 56 °C, alkylated with 55 mM IAA in 50 mM NH_4HCO_3 for 20 min in the dark, and in-gel digested with 10 μL of 2 ng/μL trypsin for 3 h at 37 °C in the presence of 0.01% surfactant (ProteaseMAX, Promega), and the resulting peptides were extracted with 20 μL of TFA 2.5% for 15 min.

Protein Identification by MALDI-TOF/TOF

Protein digests were desalted, concentrated, and spotted onto a MALDI plate using ZipTips (Millipore, USA) following the manufacturer's instructions. For the matrix preparation, a solution of 6−8 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA was used. Samples were analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (AB SCIEX, Framingham, MA). Peptide mass fingerprint (PMF) data were collected in positive MS reflector mode in the range of m/z 700−4000 and was calibrated with external standards and internally calibrated using trypsin autolysis peaks. If necessary, several of the highest intensity nontryptic peaks were selected for MS/MS analysis. The MS and MS/MS spectra were processed and analyzed using the software GPS Explorer (Version 3.6, AB SCIEX, Framingham, MA) and were searched together against the UniProt (release 2012_09) protein sequence database using the Mascot search engine (Version 2.1.04, Matrix Science, U.K.) limited to *Homo sapiens* taxonomy. The search included 65 peaks with a signal-to-noise ratio greater than 10 and allowed for up to two missed trypsin cleavage sites. The MS tolerance was 50 ppm for PMF analysis and 1.0 Da for MS/MS analysis; fixed modifications, carbamidomethylation of cysteine; variable modifications, oxidation of methionin; keratins were filtered out. To be considered a match, a confidence interval (CI), calculated by the AB SCIEX GPS Explorer/Mascot software, of at least 99% was required.

Data Mining for Glycosylation Sites in Glycoproteins

N-Glycosylation in human proteins was predicted by the NetNglyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc), an artificial neural network that examines the sequence context of Asn-Xaa-Ser/Thr (where Xaa is not Pro) sequences. *O*-Glycosylation was predicted by the NetOglyc 3.1 server (http://www.cbs.dtu.dk/services/NetOGlyc) that produces neural network predictions of mucin- type GalNAc *O*-glycosylation sites in mammalian proteins.[22]

Plasminogen Sialoglycopeptides Enrichment and Characterization

Plasminogen spots of a 2D gel from each clinical situation were excised and processed as described above. For the enrichment of plasminogen sialoglycopeptides the plasminogen peptide extract was subjected to titanium dioxide chromatography[23] as described by the manufacturer (GE Healthcare, USA). The sialoglycopeptides were eluted with the MALDI matrix 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP). The mass spectra acquisition was performed in linear positive mode in the instrument MALDI-TOF/TOF 4700 Proteomics Analyzer (AB SCIEX, USA). In order to search for sialoglycopeptides the mass spectra raw data was submitted to the software Glycomod (http://web.expasy.org/glycomod/) and MSBridge, ProteinProspector (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msbridgestandard). The mass error tolerance was 0.4 Da. Mass spectra were internally calibrated by mass plasminogen sialoglycoforms previously described.[24−27]

In-Gel *O*-Deglycosylation and Permethylation

Enrichment of plasminogen from serum samples of individuals without gastric lesions and *H. pylori* infection, gastritis, and complete and incomplete IM cases were performed using lysine-sepharose affinity chromatography.[28] The enriched plasminogen samples were run in SDS-PAGE gels and stained with Coomassie Blue. The plasminogen bands were removed from the SDS-PAGE gels, and the protein identity was confirmed by MALDI MS. Equivalent amounts of plasminogen previously digested

with neuraminidase from *Clostridium perfringens* (Sigma-Aldrich; Karlsruhe, Germany) were used as a control.

Plasminogen was then in-gel *O*-deglycosylated by reductive β-elimination upon incubation with 50 mM NaOH and 1 M $NaBH_4$ at 45 °C for 16 h. The reaction was stopped with glacial acetic acid until no fizzing was observed, and the samples were subsequently filtered using 10-kDa molecular weight cutoff (MWCO; Millipore). The filtrate, containing low molecular weight peptides, *O*-glycans, and borate salts, was recovered and incubated several times with methanol containing 5% (v/v) acetic acid under a stream of nitrogen to remove borates as methyl esters.

The *O*-glycans-enriched fractions were then permethylated adopting a modification of the method by Ciucanu and Kerek.[29] Briefly, the samples were dissolved in 100 μL of anhydrous DMSO, and powdered NaOH was added. The mixture was sonicated for 30 min and frozen prior to the addition of 100 μL of CH_3I and then incubated under mild stirring for 1 h. The permethylated samples were recovered from the reaction mixture by extraction with dichloromethane and extensively washed with acidified water (pH 2.0) to avoid base-induced hydrolysis of the carboxymethyl group of permethylated sialic acids. The samples were then desalted using Dowex ion-exchange resin (Dowex 50W-X8, Dow, USA).

Nano-HPLC-MALDI-TOF/TOF

The permethylated samples were separated in a nano-HPLC Ultimate 3000 system (Dionex, Amsterdam) equipped with a capillary column (Pepmap100 C18; 3 μm particle size, 0.75 μm i.d., 15 cm in length). The samples were dissolved in 5% acetonitrile (ACN) aqueous solution containing 0.1% formic acid (phase A). The separation was performed using a linear gradient of 32−50% B for 45 min, 50−70% B for 10 min, and 70−32% A for 5 min. The eluted glycans were applied directly on a MALDI plate in 10 s fractions using an automatic fraction collector Probot (Dionex, Amsterdam, Netherlands) under a continuous flow rate of 270 nL of DHB matrix solution (10 mg/mL in 70% acetonitrile/0.1% TFA and internal standard Glu-Fib at 15 ftmol). Mass spectra were obtained on a MALDI TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode and obtained in the mass range from 600 to 4500 Da with 1200 laser shots. For the experiment, Glu-Fib was used for internal calibrations. The MALDI-MS data from each chromatographic run was combined into a three-dimensional data array (LC fraction, m/z, total ionic current). A survey of plausible analytical signals at 691.36 $[STn + Na]^+$, was performed by determining the most prominent peaks occurring within 0.2 Da of the reference peak. The analysis of the distribution of the STn MS signals allowed the identification of chromatographic profiles. MS^2 were acquired under high-energy collisional ionization dissociation (CID) conditions. Peak assignments MS^2 spectra and database searches were performed using the GlycoWorkBench platform.[30]

RESULTS

The proteomic strategy applied in this study is schematically represented in Figure 2.

Expression of Aberrant Simple Mucin-Type Carbohydrate Antigens in Gastric Tissues

The pattern of expression of STn and T antigens was assessed in normal gastric mucosa, in gastric mucosa with gastritis, and in IM (complete and incomplete types). The pattern of mucin expression (MUC5AC *and* MUC2) allowed the classification of IM in the gastric mucosa as previously described.[31] Table 2 shows the overall results of STn and T antigen expression, and Figure 3 displays representative cases. Normal gastric mucosa showed absence of expression of STn and T antigens, with the exception of a single case that displayed a faint staining for T antigen in few cells. As expected, mucin expression in normal mucosa was limited to MUC5AC detection in the foveolar

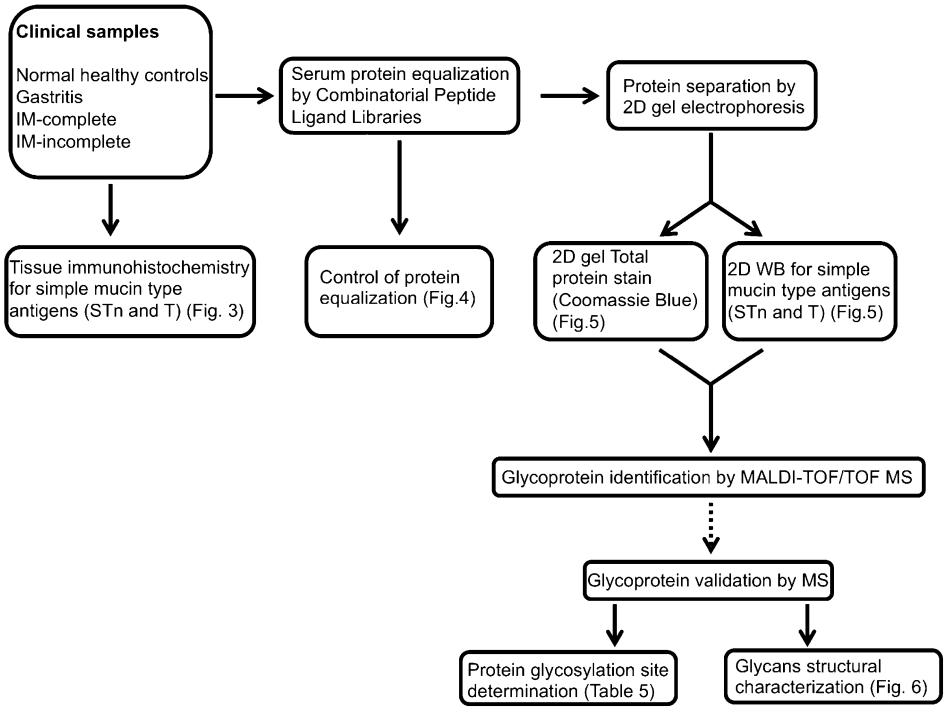

Figure 2. Schematic representation of the proteomic strategy applied in this study.

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Table 2. *In Situ* Analysis of Mucins and Simple Mucin-Type Carbohydrate Antigens by Immunohistochemistry in Gastric Tissues

cases	immunostaining			
	MUC2	MUC5AC	STn	T
control (n = 5)	0	5	0	1
gastritis (n = 6)	0	6	0	1
complete intestinal metaplasia (n = 4)	4	0	4	1
incomplete intestinal metaplasia (n = 3)	3	3	3	0

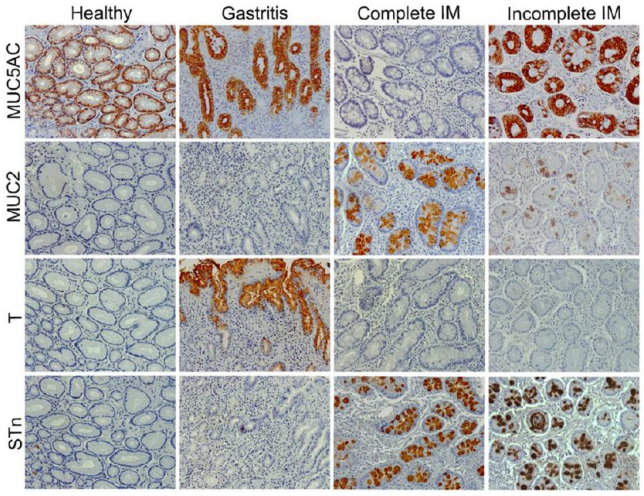

Figure 3. Immunohistochemical staining of normal mucosa and gastric lesions with MUC5AC, MUC2, and the truncated glycoforms T and STn antigens. The figure shows normal expression of MUC5AC and absence of expression of MUC2 in mucosa from healthy individuals, as well as lack of truncated glycoforms (except one normal case that also expressed T antigen; Table 2, not shown in figure). In gastric lesions we see the presence of truncated glycans (although T antigen was also seen in only one case; Table 2 and Figure 3) and also *de novo* expression of MUC2 in intestinal metaplasia.

superficial epithelium of the gastric mucosa. No expression of MUC2 was observed in normal gastric mucosa (Table 2, Figure 3).

Gastric mucosa displaying gastritis showed no expression of STn, but staining for the T antigen was observed in one case (Table 2, Figure 3). Mucosa with gastritis showed expression of MUC5AC with a strong cytoplasmic staining detected in every case. Mucosa with gastritis showed absence of MUC2 expression (Table 2, Figure 3).

Among the seven cases with IM, three showed a mixed expression of MUC5AC and MUC2 characteristic of the incomplete type of IM. Four cases showed only positive staining for MUC2 consistent with the complete type of IM. Both types of IM showed high levels of STn antigen expression detected mostly in the vacuole of goblet cells (Table 2, Figure 3) of the metaplastic glands. Absence of expression of T antigen was observed in IM with the exception of one case of the complete type that displayed expression of T antigen in few cells (Table 2).

Serum Protein Equalization by Combinatory Peptide Ligand Library

Serum is a highly complex biofluid comprehending proteins spanning a wide range of dynamic concentrations.[32] In addition, a few proteins account for approximately 70−90% of the overall proteome, which is known to mask subtle alterations associated with pathological events.[33] To overcome this limitation, the proteome of each clinical group was equalized by CPLL. This

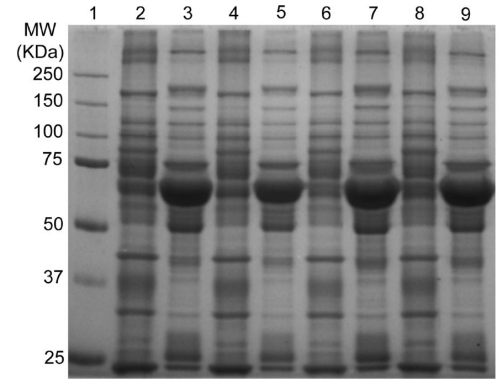

Figure 4. SDS-PAGE of sera before and after CPLL treatment. In the gel are represented serum samples of the four different groups before and after protein equalization using the CPLL technique. Lanes 2, 4, 6, and 8 represent the eluted sample of the ligand library for healthy, gastritis, complete, and incomplete intestinal metaplasia sera, respectively. Lanes 3, 5, 7, and 9 represent the unbound fraction of the same samples in the same order. Lane one shows molecular weight standards.

proteome equalization resulted in the gel electrophoretic profile presented in Figure 4, which displays the bound and unbound fractions of the CPLL. The equalized fractions of the different clinical groups showed a normalized amount of proteins characterized by an increased ratio of low abundant proteins and a decreased ratio of high abundant proteins (Figure 4).

Serum Protein Separation by Two-Dimensional Gel Electrophoresis and Simple Carbohydrate Antigen Detection

To identify proteins in the serum displaying simple mucin-type carbohydrate antigens a glycoproteomic analysis was performed. This approach included protein separation by two-dimensional (2D) gel electrophoresis combined with a Western blotting directed for the detection of simple mucin-type carbohydrate antigens using specific monoclonal antibodies.

The equalized serum protein samples from the different clinical groups were subjected to two-dimensional gel electrophoresis with a first dimension separation according to the protein isoelectric point and a second dimension based on the protein molecular weight. The patterns of protein distribution in 2D gels were similar among the different clinical groups as revealed by Coomassie Blue staining of the gels (Figure 5, left panel). These protein maps showed profiles compatible with a good resolution separation of protein isoforms.

2D gels replicates of the same clinical groups that were immunoblotted for simple mucin-type carbohydrate antigens showed reactivity for T and STn antigens. This immunoreactivity was restricted to few proteins in the proteomic map (Figure 5, center and right panels).

The 2D immunobloting revealed that the immunoreactivity for the T antigen was higher in the gastritis group with lower detection in IM groups. Furthermore, STn antigen immunoreactivity showed to be higher in proteins from the 2D gel maps of the IM groups and in gastritis when compared with normal control group (Figure 5, right panel).

Protein Identification by MALDI-TOF/TOF Mass Spectrometry

Identification of the proteins that were labeled in Western blots for simple mucin-type carbohydrate antigens was obtained using the excised spots from the Coomassie Blue stained 2D gel (Figure 5). Table 3 shows the list of identified proteins by

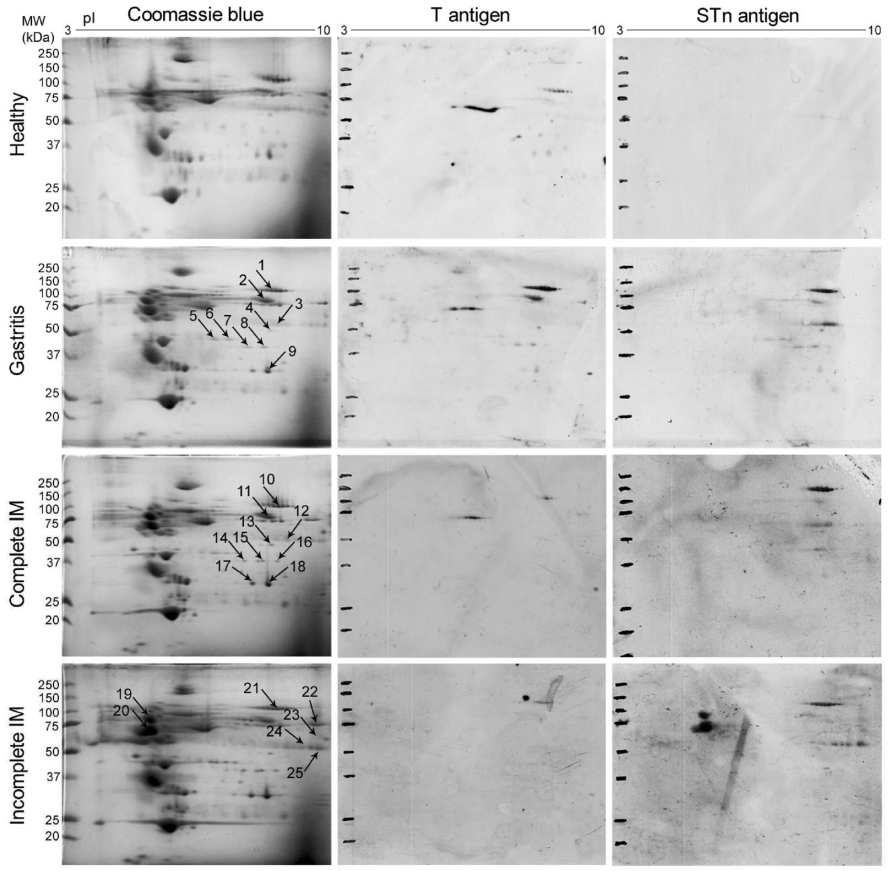

Figure 5. 2D gel electrophoresis and Western blot analysis for T and STn antigens of serum from healthy individuals, individuals with gastritis, and individuals with intestinal metaplasia (complete and incomplete type). In the left side of the figure are represented Coomassie Blue gels of serum samples equalized with CPLL, and in the middle and right side are represented Western blots against T and STn antigens, respectively. The spots that were highlighted in the Western blots were matched on Coomassie blue gels and excised for protein identification by MALDI-TOF TOF analysis.

Table 3. Proteins Identified in Sera of Gastritis, Complete Intestinal Metaplasia and Incomplete Intestinal Metaplasia According to the Immunoreactivity with Antibodies for T and STn Antigens

spot ID	protein description	accession no.	mascot Protein C.I. %	peptide count	% cov	peaks matched	MOWSE score
1	plasminogen	PLMN_HUMAN	100	44	58	49	454
2	histidine-rich glycoprotein	HRG_HUMAN	100	21	36	21	156
3	IGH protein	Q6GMX6_HUMAN	99.8	9	22	9	79
4	complement factor H	F8WDX4_HUMAN	100	18	44	18	133
5	complement factor H-related protein 1	FHR1_HUMAN	100	13	40	14	95
6	complement factor H-related protein 1	FHR1_HUMAN	99.4	7	21	9	66
7	complement factor H-related protein 1	FHR1_HUMAN	99.3	10	30	11	74
8	complement factor H-related protein 1	FHR1_HUMAN	99.4	11	37	12	74
9	complement C4 gamma chain	B4DDH0_HUMAN	100	17	34	18	105
10	plasminogen	PLMN_HUMAN	100	43	59	49	442
11	histidine-rich glycoprotein	HRG_HUMAN	100	22	40	22	169
12	IGH protein	Q6GMX6_HUMAN	99.1	11	27	11	72
13	complement factor H	CFAH_HUMAN	100	27	65	32	262
14	complement factor H-related protein 1	FHR1_HUMAN	100	14	43	15	107
15	complement factor H-related protein 1	FHR1_HUMAN	100	15	42	17	119
16	complement factor H-related protein 1	FHR1_HUMAN	100	15	37	16	118
17	complement C4-B gamma chain	B4DDH0_HUMAN	99.8	15	25	15	79
18	complement C4-B gamma chain	B4DDH0_HUMAN	100	20	31	22	133
19	vitronectin	VTNC_HUMAN	100	17	36	19	121
20	vitronectin	VTNC_HUMAN	99.8	13	30	15	79
21	plasminogen	PLMN_HUMAN	100	42	54	48	424
22	complement C4-B	B4DIE5_HUMAN	100	25	30	25	170
23	complement C4-B	B4DIE5_HUMAN	100	10	13	10	86
24	IGH protein	Q6GMX6_HUMAN	99.9	9	24	9	83
25	IGH protein	Q6GMX6_HUMAN	100	10	17	11	106

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Table 4. Glycosylation Information of the Identified Proteins

	glycosylation[a]		predicted glycosylation		T and STn expression in serum by Western blot			
protein	*O*-linked	*N*-linked	Net-*O*-Glyc	Net-*N*-Glyc	healthy	gastritis	complete IM	incomplete IM
plasminogen	Ser^{248}	Asn^{289}	Thr^{171}	no	T	T/STn	T/STn	T/STn
	Ser^{339}		Thr^{340}					
	Thr^{346}		Thr^{346}					
vitronectin	no	Asn^{86}	Thr^{113}	Asn^{86}				STn
		Asn^{169}	Ser^{137}	Asn^{169}				
		Asn^{242}	Thr^{141}					
complement factor H	no	Asn^{511}	No			STn	STn	STn
		Asn^{700}						
		Asn^{784}		Asn^{529}				
		Asn^{804}		Asn^{882}				
		Asn^{864}		Asn^{1029}				
		Asn^{893}		Asn^{1095}				
		Asn^{1011}						
		Asn^{1077}						
histidine-rich glycoprotein	no	Asn^{45}				T		
		Asn^{69}	Ser^{307}	Asn^{63}				
		Asn^{107}		Asn^{125}				
		Asn^{184}		Asn^{344}				
		Asn^{326}						
		Asn^{327}						

[a]Glycosylation sites reported in previous studies.

MALDI-TOF/TOF mass spectrometry. Proteins identified due to T antigen detection in samples from gastritis patients were plasminogen (spot ID 1) and histidine-rich glycoprotein (spot ID 2). Plasminogen was also identified based on T antigen immunoreactivity in both types of IM (spot ID 10 and 21). Proteins identified according to the immunoreactivity with STn antigen included plasminogen, which was detected in all disease groups (spot ID 1, 10, 21). Vitronectin was also identified based on STn immunoreactivity in incomplete IM (spot ID 19 and 20). Other proteins indentified in the 2D maps showing immunoreactivity with STn are displayed in Table 3.

Glycosylation Characterization of the Identified Proteins

Table 4 depicts the simple mucin-type carbohydrate antigens detected by Western blotting in the identified proteins from the sera in the different clinical groups, as well as the bioinformatical prediction of glycosylation in these proteins. Additionally, Table 4 shows available information on the *N*- and *O*-glycosylation of these proteins previously described, as well as the sites of *O*-glycosylation and *N*-glycosylation predicted bioinformaticaly by NetOGlyc and NetNGlyc, respectively. We observed expression of T antigen in plasminogen from all clinical groups and a strong STn immunoreactivity in gastritis and IM. Conversely, the expression of T antigens was considerably decreased in the plasminogen from patients with IM. The sites of *O*-glycosylation known to date for plasminogen include Thr346,[25] Ser248,[27] and Ser339,[26] as well as *N*-glycosylation in Asn289.[24] NetOGlyc (Table 4) has further highlighted Thr290, Thr359, and Thr365 as putative *O*-glycosylation sites.

Vitronectin, which has been described until now as an *N*-glycosylated protein,[34] was also found among the proteins showing simple mucin-type *O*-glycans immunoreactivity. Still, its expression was restricted to STn in the context of incomplete IM. In agreement with these observations, NetOGlyc has also predicted possible *O*-glycosylation sites for Vitronectin.

Additionally, we observed STn antigen reactivity in complement factor H in sera from gastritis and IM. No sites of *O*-glycosylation have been described, but at least 8 sites of *N*-glycosylation have been shown in complement factor H.[35] Histidine-rich glycoprotein was identified as displaying T antigen reactivity. No sites of *O*-glycosylation have been described, but at least six sites of *N*-glycosylation have been described in this protein[36] (Table 4). Noteworthy, despite the absence of reports of *O*-glycosylation in histidine-rich glycoprotein, theoretically this protein can carry this type of post-translational modification as predicted by NetOGlyc.

In summary, among the proteins found carrying simple mucin-type carbohydrate antigens, plasminogen showed the most differentiated pattern of *O*-glycosylation for IM. Namely, it presented decreased levels of T antigen accompanied by the abnormal overexpression of STn.

Structural Characterization of STn Antigen from Plasminogen and Glycosylation Site Determination

Structural analysis was performed in order to validate immunoblotting assignments regarding the expression of STn in plasminogen. The protein purified by affinity chromatography using lysine-sepharose and further separated by SDS-PAGE electrophoresis was chemically de-*O*-glycosylated in gel. The released *O*-glycans from plasminogen were then permethylated to avoid desialylation by MALDI in-source and metastable decay and analyzed by MALDI-TOF/TOF. This allowed the distinction of a chromatographic profile for the ion at m/z 691.36 corresponding to the sodium adduct of STn antigen (Figure 6A and B). This assignment was further supported by the absence of the signal upon desialylation of plasminogen with a α-neuraminidase. Moreover, the product ion spectrum exhibited the ions at m/z 107.1 resulting from $B_1{}^{0,2}X_1$ fragmentations, at m/z 358.1 from $C_1{}^{1,3}X_1$ and at m/z 416.2 from $^{2,5}X_1$ or C_1 (nomenclature according to Domon and Costello[37] (Figure 6C), therefore confirming the presence of STn in plasminogen. The prevalence of cross-ring fragmentations is in keeping with previous observations for high CID conditions.[38] In agreement with previous reports concerning the *O*-glycosylation of

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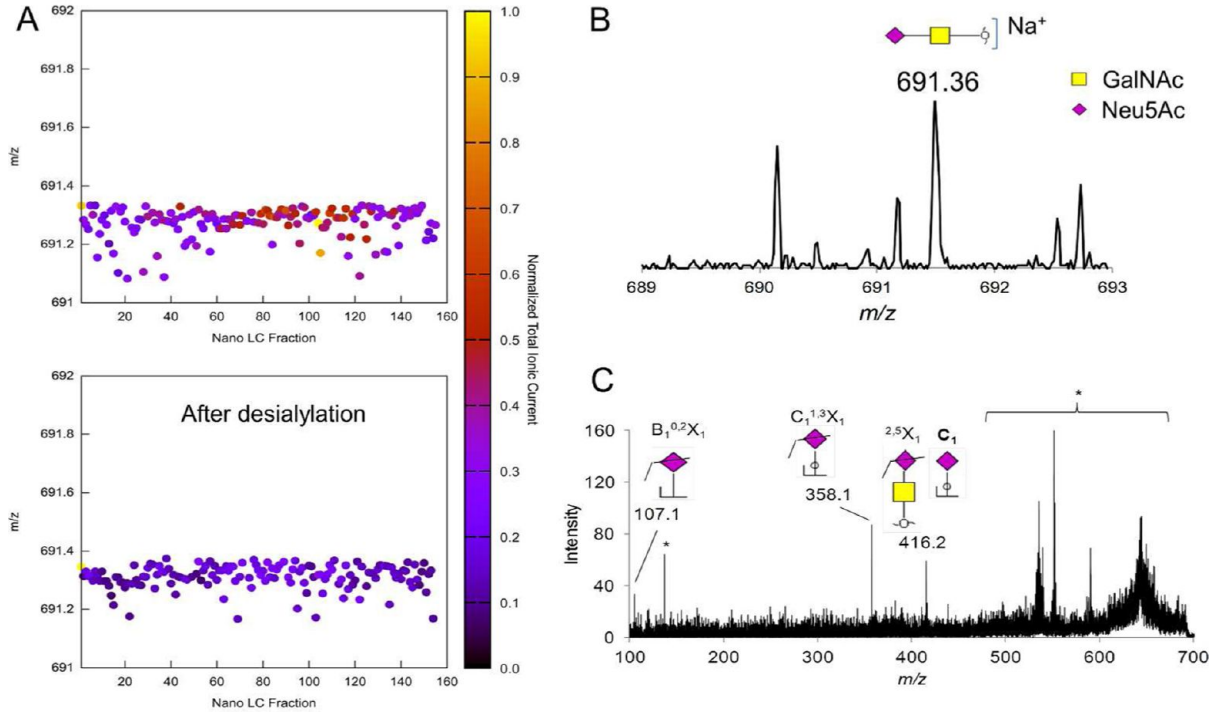

Figure 6. Positive mode MALDI-TOF/TOF identification of permethylated STn in serum plasminogen isolated from patients with intestinal metaplasia. (A) Relative ionic current of nano-HPLC fractions for the ion at m/z 691.36 corresponding to the monoisotopic mass of $[STn + Na]^+$, before and after desialylation. The analytical signals in each plot were normalized to allow the comparison of both sets of data. A chromatographic envelope was observed in the samples for fractions 80−100 that is absent after desialylation. (B) MS spectrum showing the ion at m/z 691.36 corresponding to the monoisotopic mass of $[STn + Na]^+$. (C) MS^2 spectrum of the ion at m/z 691.36 highlighting reporter ions resulting from combined sialic acid cross-ring fragmentations (X_1) and glycosidic bound cleavages B1 and C1 fragments (nomenclature according to ref 37) and symbology adapted from Glycoworkbench. Asterisk (*) denotes overlapping signals resulting from cross-ring fragmentations at the sialic moiety and/or at the reduced GalNAc residue and/or the loss of methoxy groups,[72] induced by high CID conditions.[38]

plasminogen,[25−27] low abundant ions belonging to mono- (m/z 895.5) and disialylated T (m/z 1256.6) antigens have also been detected (data not shown).

In order to characterize the plasminogen glycosylation sites containing STn, sialoglycopeptides from plasminogen of the different clinical groups were enriched by titanium dioxide chromatography[23] as described in Materials and Methods. The methodology was optimized for human plasminogen using a commercial plasminogen sample. Using this method we have detected the three *O*-glycosylation sites and one *N*-glycosylation site previously described in human plasminogen[24−27] (Supplementary Table 1 and Supplementary Figure 1). Using this approach we found 1 STn-containing glycopeptide in healthy controls, 5 in gastritis, 4 in complete IM and 8 in incomplete IM (Table 5).

DISCUSSION

Alterations of glycosylation are commonly observed in pathological conditions, including gastric cancer,[10,15] constituting a major source of biomarkers.[9] These changes in glycosylation are also observed during the process of gastric carcinogenesis, such as in gastritis and in IM, a precursor lesion of gastric carcinoma characterized by the expression of simple mucin-type carbohydrate antigens, such as T and STn.[10,11,13,14]

Serum remains the ideal biofluid for biomarker identification due to the easy collection and because it frequently displays proteins expressed by pathological tissues. The present study identified serum proteins displaying altered *O*-glycosylation as determined by the expression of the antigens T and STn in patients with gastric pathologies, such as gastritis and IM of the complete and incomplete types, as compared with control individuals without any gastric mucosa lesion.

In order to perform this glycoproteomic analysis we applied an equalizing tool for serum protein content. The CPLL[21] equalized sera from the different clinical groups showed a normalized amount of proteins characterized by an increased ratio of low abundant proteins and a decreased ratio of high abundant proteins. This tool showed to be quite efficient in avoiding the overrepresentation of serum albumin, immunoglobulins, as well as the other 20 most abundant serum proteins.[39] The search for serum proteins bearing T and STn antigens was performed using a combination of 2-D gel electrophoresis for protein separation and further detection by Western blotting of T and STn using specific monoclonal antibodies. This strategy directed this serum *O*-glycoproteome search for the protein targets displaying altered simple mucin-type carbohydrate antigens T and STn in the different clinical groups. The first dimension of the 2D gel electrophoresis using separation gradients of pH 3−10 and pH 4−7 were applied in the present study; however, the pH 3−10 gradient allowed better resolution of proteins localized in the upper limit of the gradient and showing immunoreactivity with the monoclonal antibodies against the STn and T glycan antigens.

The patterns of total protein distribution in 2D gel electrophoresis were similar among the different clinical groups, with protein maps showing good resolution separation of various protein isoforms (Figure 5, left panel). The Western blotting analysis showed T and STn antigens detection in few proteins (Figure 5, center and right panel) of the different clinical groups.

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Table 5. Characterization of Serum Plasminogen[a] Sialoglycopeptides

	exptl mass (Da)	theor mass (Da)	no. of STn glycoforms	glycoform mass	tryptic missed cleavages	sialoglycopeptides[b]
control	2782.59	2782.80	3	1483.36	0	494−504 HSIFTPETNPR
gastritis	3189.28	3189.15	4	1977.81	0	108−117 WSSTSPHRPR
	4656.01	4656.00	1	494.45	0	330−367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR
	2382.30	2382.46	2	988.91	0	379−389 C*QSWSSM§TPHR
	2782.40	2782.80	3	1483.36	0	494−504 HSIFTPETNPR
	2319.88	2319.56	1	494.45	2	777−791 VSRFVTWIEGVM§RNN
complete IM	2352.49	2352.68	1	494.45	2	71−85 DVVLFEKKVYLSEC*K
	2783.07	2782.80	3	1483.36	0	494−504 HSIFTPETNPR
	855.63	855.88	1	494.45	0	777−779 VSR
	2814.02	2814.02	2	988.91	2	777−791 VSRFVTWIEGVM§RNN
incomplete IM	1528.63	1528.54	2	988.91	0	94−98 GTM§SK
	3189.50	3189.15	4	1977.81	0	108−117 WSSTSPHRPR
	4656.33	4656.00	1	494.45	0	330−367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR
	2382.46	2382.46	2	988.91	0	379−389 C*QSWSSM§TPHR
	2782.59	2782.80	3	1483.36	0	494−504 HSIFTPETNPR
	4723.11	4722.93	3	1483.36	0	720−750 VQSTELC*AGHLAGGTDSC*QGDSGGPLVC*FEK
	3187.30	3187.62	1	494.45	0	753−776 YILQGVTSWGLGC*ARPNKPGVYVR
	855.49	855.87	1	494.45	0	777−779 VSR

[a]Plasminogen aminoacid numbering excludes the 19 aa signal peptide. [b]C* = carbamidomethylation of cysteine. M§ = methionine oxidation.

T antigen detection was observed in all groups with higher reactivity observed in gastritis, whereas STn antigen detection was observed in gastritis, and in complete and incomplete IM groups.

The detection of serum proteins expressing truncated glycans may reflect the aberrant glycosylation observed in proteins expressed by pathological tissues. This is the case in most serological assays that detect circulating glycoproteins derived from malignant tumors or benign lesions.[9,40] Our results showing the detection of truncated glycans STn and T in the glycoproteomic profiling of patients with gastritis and IM are in agreement with our previous findings that the STn and T antigens are expressed in the gastric mucosa of the patients displaying preneoplastic pathological conditions of the stomach,[10,11,41] particularly in IM, and in contrast to the absence (or almost absence) of expression of these truncated glycans in the normal gastric mucosa of control individuals.

The glycosylation modifications observed in gastric pathologic tissues may stem from altered expression of glycosyltransferases, previously shown in gastric epithelial cells induced by *H. pylori* infection,[42] and particular increased expression of specific glycosyltransferases.[14] The reasons underlying such glycosylation modifications are still largely unknown, but hypotheses such as deregulation of the glycosylation machinery in the Golgi apparatus of the cells[43] or as consequence of an altered differentiation program observed in IM are being tested.[20]

The proteomic mining of the serum for STn and T antigens expression in the different clinical groups tested in the present work has resulted in the identification of relatively abundant circulating proteins, namely, plasminogen, vitronectin, complement factor H, and histidine-rich glycoprotein. Even though the amount of these proteins did not vary significantly between groups, the pattern of simple mucin-type *O*-glycans immunoreactivity showed considerable alterations. The restricted number of proteins expressing the altered *O*-glycans are in agreement with the known specificity of the glycosyltransferases participating in *O*-glycans biosynthesis and that variation of expression of these enzymes in pathological conditions leads to altered glycosylation.[9,14,19]

Plasminogen was identified due to the immunoreactivity for T and STn antigen in all disease groups, but showed no immunoreactivity in healthy controls. Interestingly, among the identified proteins carrying simple mucin-type carbohydrate antigens, plasminogen showed the most differentiated pattern of *O*-glycosylation in IM, displaying decreased levels of T antigens accompanied by an increased expression of STn when compared with the other clinical groups. The detailed analysis of the *O*-glycans from purified plasminogen from IM patients was performed in order to validate the STn glycan detection at the molecular level. Plasminogen *O*-glycans released by reductive β-elimination were permethylated and analyzed by MALDI mass spectrometry. Our results showed the detection of ions compatible with STn antigens (Figure 6). Further MALDI-MS^2 analysis of the ion at m/z 691.4 Da exhibited glycosidic bond cleavages B, C and Z, and cross-ring fragmentation A and X product ions characteristic of STn glycan, therefore demonstrating the presence of STn in plasminogen from serum of incomplete IM patients.

In addition, the MALDI structural analysis of sialoglycopeptides from plasminogen enriched by titanium dioxide chromatography showed the presence of glycopeptides containing STn in all disease groups. The use of the sialoglycopeptides enrichment method by titanium dioxide followed by MALDI showed to be quite efficient with the identification of the three *O*-glycosylation sites and one *N*-glycosylation site previously described in human plasminogen[24−27] (Supplementary Figure 1 and Supplementary Table 1). Based on this approach the analysis of the clinical groups lead to the identification of 1 STn-containing glycopeptide in healthy control, 5 STn-containing glycopeptides in gastritis, 4 in complete IM and 8 in incomplete IM (Table 5). Some of the sites identified have not been described for plasminogen and may constitute potential novel biomarkers of precancerous gastric lesions.

Further analysis of enriched for sialic acid containing glycoproteins from gastritis, intestinal metaplasia, and carcinoma patients confirmed STn reactivity of plasminogen in an independent set of samples (Supplementary Figure 2 and Supplementary Table 2). These results using alternative glycoprotein enrichment approaches and different sample cohorts further demonstrate the potential application of the altered plasminogen STn glycosylation as a biomarker in these pathologies.

Plasminogen is released as a zymogen from the liver into circulation where it adopts a closed, activation resistant, conformation. Upon binding to blood clots, or to cell surfaces, plasminogen can adopt an open form that can be converted into active plasmin by a variety of enzymes, including tissue plasminogen activator and urokinase plasminogen activator.[44,45] Plasmin is a serine protease that acts dissolving fibrin clots and in other proteolysis functions in diverse systems. Two major glycoforms of plasminogen have been described in humans - type I plasminogen containing two glycosylation moieties (*N*-linked to Asn289 and *O*-linked to Thr346), and type II plasminogen containing a single *O*-linked sugar on Thr346.[24,25] However, additional sites of *O*-glycosylation of plasminogen have been reported in Ser248,[27] and Thr339.[26] Type II plasminogen has been shown to be preferentially recruited to the cell surface whereas type I plasminogen appears more readily recruited to blood clots.[46] Nevertheless, only one (Thr346) of these glycosylation sites could be theoretically predicted using the bioinformatics platform NetOGlyc. On the other hand this approach retrieved other putative glycosylation sites that are still lacking *in vivo* confirmation. The discrepancy between these findings has been previous highlighted for serum proteins[47] and suggests that *in vivo* processing and pathophysiological states may play a determinant role in the definition of the glycosylation of circulating glycoproteins.

The X-ray crystal structure analysis of closed plasminogen has revealed that *O*-glycosylation alter the position of a Kringle domain, partially explaining the functional differences observed between the type I and type II plasminogen glycoforms.[44] In closed plasminogen, the *O*-linked sugar on Thr346 is one of the requirements for the blocking of the cleavage by tissue plasminogen activator and urokinase plasminogen activator.[44]

Liver is the primary tissue for plasminogen synthesis[48] but other tissue sources, including the gut, have been described in animal models.[49] The truncated *O*-glycans observed in circulating plasminogen from gastritis and in IM patients may either reflect altered glycosylation in the liver response to inflammatory cytokines or altered glycosylation of locally expressed plasminogen. Pro-inflammatory cytokines produced within the gastric disease context,[50−52] may induce differential expression of glycosyltranferases in hepatocytes leading to alteration of glycosylation of circulating proteins.[53] This is in agreement with previous studies that have shown that modification of glycosylation characterized by increased expression of sialylated glycan structures, are observed in hepatocyte derived proteins during acute and chronic inflammatory diseases.[54−57] In addition, alterations of plasminogen may also be related with the infection by *H. pylori*. The increased expression of urokinase plasminogen activator has been described in *H. pylori*-associated gastritis.[58,59] Plasminogen-binding proteins in *H. pylori* with subsequent activation to plasmin[60,61] may provide proteolytic capacity and may contribute for the virulence of this bacterium.[60,61] These findings altogether may explain the importance of plasminogen activation in pathological conditions of the gastric mucosa.

Vitronectin was also identified based on STn immunoreactivity in the incomplete type of IM. Vitronectin is a glycoprotein known to be glycosylated. Human vitronectin has been shown to contain *N*-glycans, but no *O*-glycans have been described, contrary to vitronectin of other mammals that has been shown to contain both types of glycosylation.[62] Furthermore, it was demonstrated that despite the homology of about 73% between human and rat vitronectin, the sites of glycosylation are highly conserved and have been shown to be important for the protein function.[63]

Vitronectin is a multifunctional glycoprotein produced mainly by hepatocytes that is present mostly in plasma and the extracellular matrix of many tissues.[64] Vitronectin is involved in many functions such as the regulation of coagulation and fibrinolysis, cell adhesion, and invasion, as well as in matrix remodeling and humoral defense mechanisms.[65] Glycosylation has been reported to be important in the interaction of vitronectin with other molecules and on its functional activities.[66] Additionally, vitronectin oligosaccharide moiety was suggested to be relevant in *H. pylori* binding and in the mechanism of the bacterial immune escape.[67,68] Furthermore, vitronectin was shown to be important in the adhesion and migration of tumor-infiltrating lymphocytes.[69]

Our results showed STn antigen immunoreactivity for vitronectin in the context of incomplete IM. Despite the lack of reports on vitronectin *O*-glycosylation in humans we could predict three possible *O*-glycan sites (Thr113, Ser137, Thr141) using the NetOGlyc tool. Taking into consideration the importance of vitronectin glycosylation on its function and the reported bacterial interactions,[67,68] our findings may point toward a possible role in the context of gastric lesions development.

The present study also indicated the histidine-rich glycoprotein as a target expressing truncated *O*-glycans, presenting reactivity with T antigen in the gastritis group. Histidine-rich glycoprotein is a plasma glycoprotein produced by the liver and known to bind to a number of ligands in circulation, such as heparin, heparan sulfate, thrombospondin, and plasminogen. Histidine-rich glycoprotein acts as an adapter protein and has been implicated in regulating many processes such as immune complex and pathogen clearance, cell adhesion, angiogenesis, coagulation, and fibrinolysis.[70] Even though regarded as an *N*-glycosylated protein (6 described sites and 3 predicted) our study has found Western blotting reactivity for the T antigen in gastritis and the NetOGlyc tool identified Ser307 as a putative glycosylation site. This indicates that this type of glycosylation could also be present in histidine-rich glycoprotein and suggests that further structural insights on the *O*-glycan moiety may be required to complement previous studies focused on the analysis of the *N*-glycans.[70]

Similarly, our results showed immunoreactivity to STn antigen in all pathological conditions in proteins from the complement system, namely, complement factor H, complement H-related protein, and complement C4-B. The human complement pathway is a highly controlled effector mechanism of the immune system. Over 30 plasma proteins and membrane-bound molecules are involved in the complement system, and most of these proteins are glycosylated. The complement factor H, complement factor H-related protein, and the complement C4-B are proteins that have been described to be *N*-glycosylated.[71] Our results indicating that aberrant *O*-glycosylation can be detected in these members of the complement system warrants further structural characterization of the glycans in these proteins. Most of the protein modules that form the complement system have been crystallized and structural data provides evidence for the role

of *N*-glycans in this system. However, the structure and role of glycans in the resistance to proteolysis and functionality activation within pathological conditions is still incomplete. As these data become available, the glycans can be modeled at the appropriate locations and give further insights into the interaction between complement proteins and cofactors.

Our results provide novel glycobiomarkers in serum from patients with gastric pathologies, including gastritis, intestinal metaplasia, and gastric carcinoma. The present work opens new avenues for future targeted evaluation of these specific glycobiomarkers in additional immunoassay-based approaches enabling large scale individual analysis.

In summary, this work presents a set of proteins displaying altered *O*-glycosylation as detected by antibodies directed to STn and T antigens in the serum from patients with gastritis and IM in opposition to minor or no reactivity in the same proteins of healthy individuals without any gastric disease. We further demonstrated that circulating serum plasminogen from IM patients carries the truncated *O*-glycan STn antigen. These results warrant further studies to address the application of plasminogen STn glycosylation pattern as a serum biomarker of gastric pathologies.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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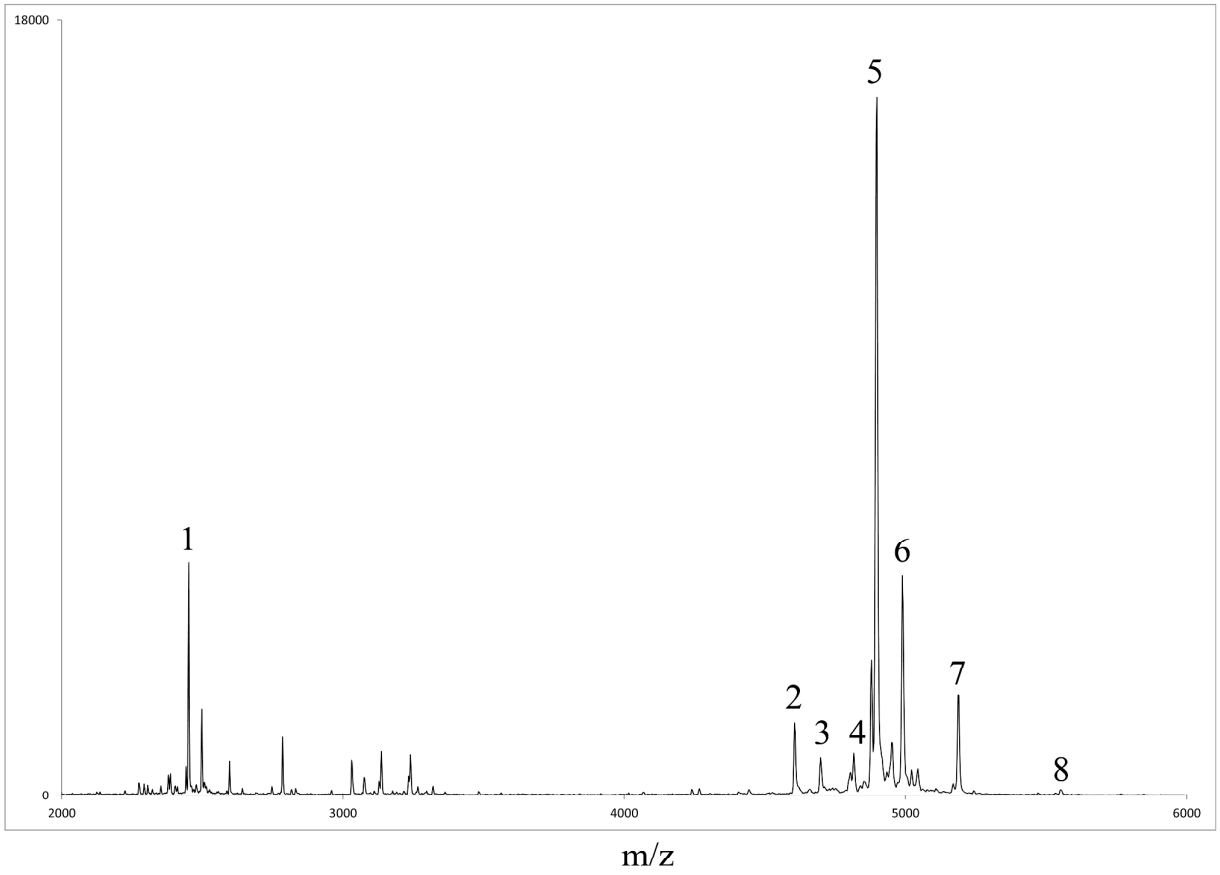
Supplementary Data

Supplementary data 1 – Characterization of plasminogen glycosylation by MALDI-TOF/TOF mass spectrometry

Supplementary Table 1: List of plasminogen glycopeptides detected by MALDI-MS. Serum plasminogen from a commercial sample was subjected to tryptic digestion followed by titanium dioxide enrichment of sialoglycopeptides (described in material and methods). MALDI mass spectra were acquired in linear positive mode using THAP (2',4',6'-Trihydroxyacetophenone monohydrate) as a matrix with a mass error tolerance of 0.06 %. Different already described plasminogen glycoforms have been detected.

Peak number	Experimental mass (Da)	Theoretical mass (Da)	Glycosylation type	Glycoform(s)	Sialoglycopeptides #	Reference
1	2451.99	2451.65	O	ST	243-258 C*TTPPPSSGPTYQC*LK(G)	27
2	4605.11	4606.87	O	T + Pi	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	
3	4697.11	4698.75	N	Hex2HexNAc2NeuAC1 + Man3GlcNAc2	266-290 GNVAVTVSGHTCQHWSAQTPHTHNR(T)	24
4	4815.91	4818.15	O	ST	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	25, 27
5	4896.60	4898.13	O	ST + Pi	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	27
6	4989.98	4987.07	N	Hex2HexNAc2NeuAC2 + Man3GlcNAc2	266-290 GNVAVTVSGHTCQHWSAQTPHTHNR(T)	24
7	5188.48	5189.39	O	diST + Pi	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	27
8	5554.99	5554.61	O	diST + T + Pi	330-367 IPSC*DSSPVSTEQLAPTAPPELTPVVQDC*YHGDGQSYR(G)	26



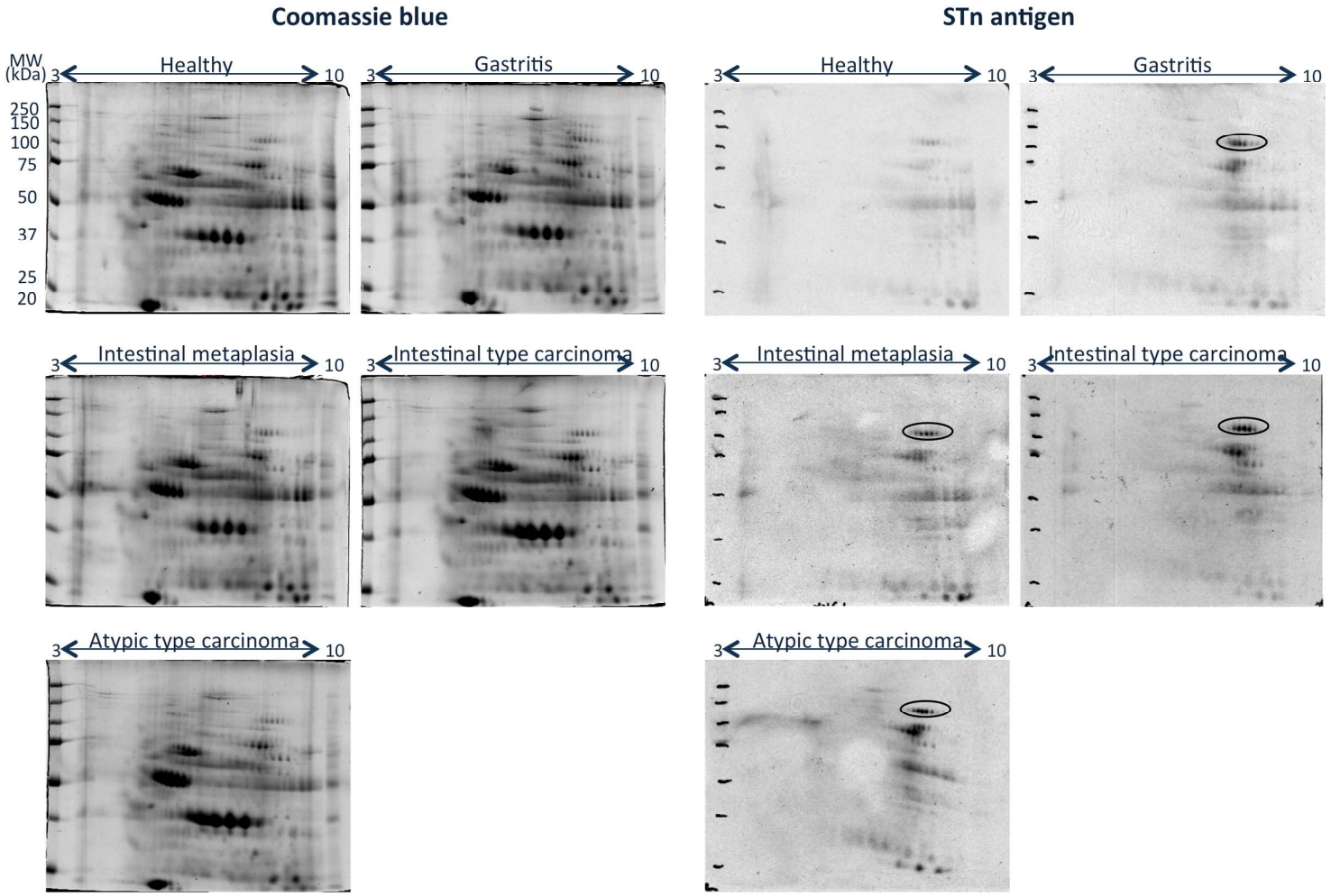
Supplementary Figure 1: MALDI MS spectrum of human plasminogen after titanium dioxide enrichment. The detected sialoglycopeptides are presented in the supplementary Table 1.

Supplementary data 2 - Identification of STn in Plasminogen from gastritis, intestinal metaplasia and gastric carcinoma of independent set of samples

Supplementary Material and methods

Sera sample collection, glycoprotein enrichment by lectin affinity chromatography and identification of STn containing proteins

A second set of sera sample from the University Hospital Santo António and Hospital São João were used for the identification of proteins carrying STn structures. Sera sample from ten individuals within each of the following clinical groups were used: without gastric lesions, gastritis, intestinal metaplasia and from two types of gastric carcinoma (intestinal and atypical). Sera sample were pooled and subjected to albumin and IgG depletion by a commercial kit (ProteoPrep from Sigma Aldrich). For the enrichment of STn containing glycoproteins, depleted sera were subjected to *Sambucus nigra* agglutinin (Vector laboratories) affinity chromatography that specifically recognizes and capture α2,6 sialic acids containing glycoproteins. The affinity chromatography eluted proteins were further subjected to 2D gel electrophoresis, Western blot analysis to detect STn containing proteins, and MALDI-TOF/TOF mass spectrometry protein identification was performed as described in materials and methods of this paper.



Supplementary Figure 2: Glycoproteomic approach to reveal serum proteins carrying STn antigen. Serum samples from healthy, gastric precursor lesions (gastritis and intestinal metaplasia) and two types of gastric carcinoma (intestinal and atypical type) individuals were subjected to albumin and IgG removal and lectin affinity chromatography to capture α2,6 sialic acid containing proteins. Affinity captured proteins were separated by 2D gel electrophoresis and STn highlighted by Western blot. The results show the presence of STn containing proteins in serum from individuals with gastric percursor lesions and with gastric carcinoma, and Plasminogen was identified by MALDI-TOF/TOF as STn protein carrier. Black highlighted areas indicate selected protein dots, reactive for STn antigen, and identified as plasminogen (see supplementary Table 2).

Supplementary Table 2: Proteins identified in sera of control, gastritis, metaplasia, intestinal type carcinoma and atypic type carcinoma according to the immunoreactivity with antibodies for STn antigen.

Spot ID	*Protein description*	*Accession number*	*MASCOT Protein C.I. %*	*Peptide count*	*% cov*	*Peaks matched*	*MOWSE score*
Control	Plasminogen	PLMN_HUMAN	100	31	32	34	243
Gastritis	Plasminogen	PLMN_HUMAN	100	32	35	32	259
Metaplasia	Plasminogen	PLMN_HUMAN	100	28	32	30	202
Intestinal Type Carcinoma	Plasminogen	PLMN_HUMAN	100	33	37	37	273
Atypic type carcinoma	Plasminogen	PLMN_HUMAN	100	30	33	34	232

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Expression of ST3GAL4 Leads to SLe^x Expression and Induces c-Met Activation and an Invasive Phenotype in Gastric Carcinoma Cells

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Abstract

Sialyl-Lewis X (SLe^x) is a sialylated glycan antigen expressed on the cell surface during malignant cell transformation and is associated with cancer progression and poor prognosis. The increased expression of sialylated glycans is associated with alterations in the expression of sialyltransferases (STs). In this study we determined the capacity of ST3GAL3 and ST3GAL4 sialyltransferases to synthesize the SLe^x antigen in MKN45 gastric carcinoma cells and evaluated the effect of SLe^x overexpression in cancer cell behavior both *in vitro* and *in vivo* using the chicken chorioallantoic membrane (CAM) model. The activation of tyrosine kinase receptors and their downstream molecular targets was also addressed. Our results showed that the expression of ST3GAL4 in MKN45 gastric cancer cells leads to the synthesis of SLe^x antigens and to an increased invasive phenotype both *in vitro* and in the *in vivo* CAM model. Analysis of phosphorylation of tyrosine kinase receptors showed a specific increase in c-Met activation. The characterization of downstream molecular targets of c-Met activation, involved in the invasive phenotype, revealed increased phosphorylation of FAK and Src proteins and activation of Cdc42, Rac1 and RhoA GTPases. Inhibition of c-Met and Src activation abolished the observed increased cell invasive phenotype. In conclusion, the expression of ST3GAL4 leads to SLe^x antigen expression in gastric cancer cells which in turn induces an increased invasive phenotype through the activation of c-Met, in association with Src, FAK and Cdc42, Rac1 and RhoA GTPases activation.

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Introduction

Alterations in cell surface glycosylation are considered a hallmark during carcinogenesis. These alterations usually lead to the expression of tumor-associated carbohydrates on glycoproteins or glycolipids that decorate cell surfaces [1]. One of the most common glycan alterations is the increase of sialylated Lewis-type blood group antigens, such as sialyl Lewis A (SLe^a (NeuAcα2,3-Galβ1-3(Fucα1-4)GlcNAc-R)) and sialyl Lewis X (SLe^x (NeuAcα2,3Galβ1-4(Fucα1-3)GlcNAc-R)). SLe^a and SLe^x are expressed in cancer cells, mimicking their normal expression on blood cells (monocytes and neutrophils) potentiating cancer cell migration through binding to endothelial cell selectins [2,3]. Therefore, SLe^a and SLe^x overexpression is a common feature of several carcinomas (e.g., lung, colon, gastric and pancreas) and it is associated with increased metastatic capacity [4,5,6,7] and poor patients survival [8,9,10,11,12].

The increased expression of sialylated glycans associated to carcinogenesis is the result of altered expression of sialyltransferases (STs) genes which encode for enzymes involved in the biosynthesis of the glycan antigens described above [13]. Up to 20 different sialyltransferases have been described to catalyse the transfer of sialic acid residues from a donor substrate CMP-sialic acid to the oligosaccharide side chain of the glycoconjugates. This sialic acid generally occupies the terminal non-reducing position on glycan chains [14]. Different STs show cell and tissue specific expression pattern and differ in substrate specificities and types of linkage formed [14]. Depending on these characteristics, STs are classified in four families - ST3Gal, ST6Gal, ST6GalNAc and ST8Sia. ST3Gal family are α2,3-STs which catalyze the transfer of sialic acid residues to terminal galactopyranosyl (Gal) residues and include six members from ST3Gal I to ST3Gal VI [15].

Among the six ST3Gal sialyltransferases, ST3Gal III, IV and VI have been described to contribute to SLe^x formation [16,17], with a substantial role attributed to ST3Gal IV [18,19].

The sialyl-Lewis antigens are synthesized on type 1 (Gal β1,3 GlcNAc) or type 2 (Gal β1,4 GlcNAc) disaccharide sequences. The sialyltransferase ST3Gal III preferentially acts on type 1 rather than on type 2 disaccharides and is involved in the synthesis of

SLe^a [20]. ST3Gal IV mainly catalyzes the α2,3 sialylation of type 2 disaccharides, leading to the biosynthesis of SLe^x [18,21].

We previously demonstrated the contribution of different ST3Gal sialyltransferases to the synthesis of sialyl Lewis antigens in gastric carcinoma cells, and described that ST3Gal IV is involved in the synthesis of SLe^x antigen [22]. In line with this report, other studies also found that high expression of ST3Gal IV, contributes to the expression of α2,3-linked sialic acid residues, and is associated with the malignant behavior of gastric cancer cells [23].

In gastric carcinoma tissues, the increased expression of ST3Gal IV [24] and of sialyl Lewis antigens have been associated with poor prognosis and metastatic capacity [8]. These reports highlight the role of STs and evidenced that the expression of crucial glycan determinants, such SLe^x, play an important role in tumor progression. However, the molecular mechanisms underlying the aggressive behavior of gastric cancer cells expressing SLe^x are not fully understood. Some studies pointed to the importance of tyrosine kinase receptor activation in STs overexpression models [25,26,27]. In the present study we assessed the effect of ST3GAL IV overexpression in the synthesis of SLe^x in gastric carcinoma cells and evaluated the functional role of SLe^x *in vitro* (proliferation, invasion and adhesion) and *in vivo* (angiogenesis, tumor growth and invasion). We further evaluated the contribution to cell behavior of tyrosine kinase receptors activation and identified the downstream effectors in the context of ST3Gal IV/SLe^x overexpressing gastric carcinoma cells.

Materials and Methods

Cell culture

The gastric cancer cell line MKN45 was obtained from the Japanese Cancer Research Bank (Tsukuba, Japan) and was stably transfected with full length human gene for ST3GAL3 (MST3Gal III), ST3GAL4 (MST3Gal IV) and the empty vector pcDNA3.1 (Mock) as shown previously [22]. The cells were grown in monolayer culture in T75cm^2 flasks and maintained at 37°C in an atmosphere of 5% CO_2, in Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX, HEPES medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S) and in the presence of 0.5 mg/mL G418 (all from Invitrogen). Culture medium was replaced every two days.

RNA isolation, cDNA synthesis and real-time PCR analysis

Total RNA was extracted from cell lysates of Mock, MST3Gal III and MST3Gal IV cell lines using TRI Reagent (Sigma) and converted to cDNA using the SuperScript® II Reverse Transcriptase (Invitrogen). Reverse transcription was performed using 3 μg of total RNA, random oligonucleotides primers and SuperScript II RT (Invitrogen) in a total volume of 20 μL as described by the manufacturer. For real-time PCR analysis, cDNA samples were diluted 50-fold with water and PCR amplified in triplicate with 10.0 μL Power SYBRGreen Master Mix (Applied Biosystems), 0.48 μL of each 10 μM primer and 4 μL cDNA using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The primers used were the following: ST3GAL3 for5'-ggtggcagtcgcaggattt-3'; rev5'-catgcgaacggtctcatagtagtg-3'; and ST3GAL4 for5'-cctggtagctttcaaggcaatg-3'; rev5'-cctttcgcacccgcttct-3'. Expression of 18S (for5'-cgccgctagaggtgaaattc-3'; rev5'-cattcttggcaaatgctttcg-3') and GAPDH (for5'-agtccctgccacactcag-3'; rev5'-tactttattgatggtacatgacaagg-3') was also measured in triplicate for each sample and used for normalization of target gene abundance. Specificity of amplification was confirmed by melting curve analysis. Standard curves were determined for each gene, and results are presented as ratio between target gene and housekeeping genes, 18S and GAPDH.

Proliferation assays

Cell growth was analyzed using the BrdU reagent (Roche) according to the manufacturer's directions. Cells (1×10^5) were seeded in slides on 24-well plates (Thermo Fisher Scientific) and grown in RPMI containing 10% FBS, 1% P/S in the presence of 0.5 mg/mL G418. When cells reached 50% of confluence, BrdU was incorporated in cell culture medium and incubated for 20 minutes. After incorporation cell culture medium was removed and cells fixed with methanol for 30 minutes. Cell labeling with anti-BrdU antibody and FITC secondary antibody was performed according to manufacturer's instructions. Three independent assays were performed and each assay was done in quadruplicates for all the cell lines. Percentage of dividing cells was calculated by measuring positive BrdU cells in relation to total cells with the help of ImageJ software. Results are presented as means ± SD for each sample, and proliferation levels obtained were compared with the Mock control cell line.

Invasion assay

Invasion assays were performed in a BD Biocoat Matrigel invasion chamber with an 8-μm diameter pore size membrane and a thin layer of Matrigel, in a 24-well plate. Inserts were rehydrated for at least 1 hour in RPMI medium. After detachment of confluent cells with trypsin/EDTA, cells (5×10^4) were seeded in the upper surface of Transwell plates and cultured in RPMI containing 10% FBS, 1% P/S in the presence of 0.5 mg/mL G418 for 6 hours, and the same culture medium was added in the lower part of the insert. After incubation, non-invading cells in the upper part of the insert were carefully removed, cells were fixed with methanol and membranes were removed from the inserts and mounted in a slide using Vectashield with DAPI (Vector labs). Three independent assays were performed and cells were seeded in duplicate for each cell line. Invading cells were counted under a fluorescence microscope, and measurement was done by counting cells in three different fields in each sample, with application of ImageJ software. Results are presented as means ± SD for each sample, and invasion levels obtained were compared with the Mock control cell line.

Cell-substrate adhesion assay

Cell adhesion assays were performed in a 96-well plate coated overnight at 4°C with 50 μL of different extracellular matrix (ECM) proteins: collagen IV, fibronectin and vitronectin in the concentration of 20 μg/mL, while bovine serum albumin (BSA) (Sigma-Aldrich) was used as negative control. After coating, the plate was incubated for 1 hour with 0.5% of BSA in phosphate buffer saline (PBS) and viable cells (2×10^4 cells/well) were introduced into the plate and allowed to adhere for 30 min in RPMI serum-free medium at 37°C and 5% CO_2. Removal of non-adherent cells was performed by washing the plate with PBS and adherent cells were fixed with methanol for 30 minutes. Cells were subjected to 0.5% crystal violet dissolved in 20% of methanol for 1 hour, and then washed several times with water and allowed to air dry. Crystal violet dye was solubilized with 10% acetic acid and absorbance was measured at $\lambda = 560$ nm. Results are presented as means ± SD for each sample, and adhesion levels obtained were compared with the Mock control cell line.

Phospho-RTK array analysis

Cells were cultured until reached confluence on T75 cm^2 flasks with RPMI medium supplemented with 10% FBS and 100 units/mL penicillin-streptomycin in the presence of 0.5 mg/mL G418. Cells were then lysed in NP40 lysis buffer (1% NP40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitor cocktail tablet (Roche), protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce) and 300 μg of total protein was used for the human Phospho-RTK array kit (R&D Systems). Phospho-RTK array protocol was performed according to manufacturer's instructions. Activated receptors were matched according to the phospho-RTK array coordinates: a1, a2: EphA6; a3, a4: EphA7; a5, a6: EphB1; a7, a8: EphB2; a9, a10: EphB4; a11, a12: EphB6; a13, a14: mouse IgG1 negative control; a15, a16: mouse IgG2A negative control; a17, a18: mouse IgG2B negative control; a19, a20: goat IgG negative control; a21, a22: PBS negative control; b1, b2: Tie-2; b3, b4: TrkA; b5, b6: TrkB; b7, b8: TrkC; b9, b10: VEGFR1; b11, b12: VEGFR2; b13, b14: VEGFR3; b15, b16: MuSK; b17, b18: EphA1; b19, b20: EphA2; b21, b22: EphA3; b23, b24: EphA4; c1, c2: Mer; c3, c4: c-Met; c5, c6: MSPR; c7, c8: PDGFRα; c9, c10: PDGFRβ; c11, c12: SCFR; c13, c14: Flt-3; c15, c16: M-CSFR; c17, c18: c-Ret; c19, c20: ROR1; c21, c22: ROR2; c23, c24: Tie-1; d1, d2: EGFR; d3, d4: ErbB2; d5, d6: ErbB3; d7, d8: ErbB4; d9, d10: FGFR1; d11, d12: FGFR2α; d13, d14: FGFR3; d15, d16: FGFR4; d17, d18: insulin R; d19, d20: IGF-IR; d21, d22: Axl; d23, d24: Dtk. Black dots represent phospho-tyrosine positive controls.

c-Met and Src inhibition assay

c-Met and Src inhibitors were used to evaluate the invasive capacity of the cells upon inhibition. c-Met inhibition was performed with 0.1 μM of PHA-665752 (Sigma) and Src inhibition with 20 μM of PP2 (Sigma) both during 10h. Inhibition was assessed by Western blot for the phosphorylation status of c-Met and Src, and invasion capacity of cells was evaluated as described above, after 10h of inhibitors incubation.

Immunoblotting

Proteins were obtained from total cell lysates of each cell line. Briefly, confluent T75 cm^2 flasks were incubated with NP40 lysis buffer and cells were scraped. Total cell lysates were centrifuge at 14000 rpm for 10 minutes to remove pellet cell debris. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce). Proteins from cell lysates were separated accordingly to protein molecular weight by gel electrophoresis in 7.5% acrylamide/bis acrylamide (Sigma) SDS-PAGE. For c-Met, phospho-Met, phospho-AKT, phospho-STAT3 and phospho-ERK detection, 25 μg of total protein extract were used and for phospho-Src and phospho-FAK detection we used 50 μg of total protein extract. Gels were then transferred onto a nitrocellulose membrane (Amersham) in a semi-dry system. Membranes were then blocked with 5% non-fat milk, washed three times with Tris buffer saline (TBS), and incubated overnight at 4°C with primary antibodies. After incubation, membranes were washed three times with TBS and incubated 1 hour with secondary antibodies. Analysis was done by chemiluminescence using the ECL Western blotting detection reagent and films (both from GE Healthcare).

Antibodies: anti-phosphorylated Akt Ser473, anti-phosphorylated FAK Tyr397, anti-phosphorylated Src Tyr416, anti-phosphorylated ERK Thr202/Tyr204 and anti-phosphorylated MET Tyr1234/1235, anti- phosphorylated STAT3 Tyr705 (all rabbit polyclonal antibody from Cell Signaling Technology) were used at 1:1000 dilution. Mouse monoclonal IgG2a antibody directed against human MET (Invitrogen) was used at 1:2000. Anti-SLe^x clone KM93 (Millipore) was used at 1:500 dilution. Goat anti-actin and rabbit anti-actin (Santa Cruz Biotechnology) were used at 1:8000 dilution. Secondary anti-rabbit and anti-goat antibodies, conjugated with horseradish peroxidase (DAKO), were used at 1:2000, while anti-mouse IgG2a and IgM antibodies, conjugated with horseradish peroxidase (Jackson immunoresearch) were used at 1:25000 and 1:10000, respectively.

Cdc42, Rac1 and RhoA GTPases pull down assay

Cells were cultured in serum free medium for 24 hours, and proteins were obtained from total cell lysates. Pull-down assays, using RhoA/Rac1/Cdc42 Activation Assay Combo Biochem Kit (Cytoskeleton, inc), were performed according to manufacturer's instructions, using 600 μg of total protein lysates. Briefly, rhotekin-RBD effector domain affinity beads were used to bind RhoA active (GTP-bound) protein and PAK-PBD effector domain affinity beads for Cdc42 and Rac1 active proteins. Total proteins were incubated with these beads for 2 hours, and pull down proteins were eluted with Laemmli buffer and separated on 12% acrilamide/bis acrilamide gels. Pull down negative and positive controls were performed according to manufacturer's instructions; briefly total cell lysates were incubated with GTPases inhibitors or activators prior to pull down. To confirm the presence of GTPases in the cell protein extract, a 5% input control were also runned in the gels. Gels were transferred to nitrocellulose membranes and antibodies against Cdc42, RAC1 and RhoA (included in the kit) were incubated overnight with gentle agitation. Proteins were analyzed by chemiluminescence using the ECL Western blotting detection reagent and films (both from GE Healthcare).

Chicken embryo *in vivo* tumorigenesis and angiogenic assay

The chicken embryo chorioallantoic membrane (CAM) model was used to evaluate the angiogenic response and growth capability of Mock and MST3Gal IV cells (n = 13 for each group). According to the European Directive 2010/63/EU, ethical approval is not required for experiments using embryonic chicken. Correspondingly, the Portuguese law on animal welfare does not restrict the use of chicken eggs. Briefly, fertilized chick (*Gallus gallus*) eggs obtained from commercial sources were incubated horizontally at 37.8°C in a humidified atmosphere and referred to embryonic day (E). On E3 a square window was opened in the shell after removal of 1.5–2 mL of albumin to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and the eggs returned to the incubator. The window in the egg shell does not interfere in any way with the normal development of the chick embryo. Cells, re-suspended in 10 μL of complete medium (1×10^6 cells per embryo), were placed on top of E10 growing CAM into a 3 mm silicon ring under sterile conditions. The eggs were re-sealed and returned to the incubator for an additional 3 days. At this point the embryos are at E 13, thus still in the first 2/3 of development. The embryos were euthanized by adding 2 mL of fixative in the top of the CAM which is a very efficient and fast method. After removing the ring, the CAM was excised from the embryos, photographed *ex ovo* under a stereoscope, at 20x magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (less than 20 μm diameter) growing radial towards the ring area was counted in a blind fashion manner. The area of CAM tumors was determined using the Cell A (Olympus) software.

Immunohistochemistry analysis and tumor invasive phenotype

Excided CAMs were fixed in 10% neutral-buffered formalin, paraffin-embedded for slide sections and stained with hematoxilin-eosin for histological examination. Slides with clear view of the CAM tumors were also processed for cytokeratin, SLe^x and p-Met immunohistochemical detection in order to characterize the phenotype of CAM tumors. Briefly, sections were dewaxed, rehydrate and the endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 minutes. Then, sections were incubated with normal rabbit or swine serum diluted 1:5 in PBS containing 10% BSA for 30 minutes followed by incubation with the monoclonal antibodies overnight at 4°C. Incubation with both biotinylated rabbit anti-mouse and swine anti-rabbit secondary antibodies (DAKO) was done during 30 minutes at room temperature followed by avidin/biotin complex detection (Vectastain). Staining was performed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma) containing 0.02% hydrogen peroxide and counter staining of the nucleus was done with Mayer's hematoxylin. Monoclonal antibodies used were KM93 1:60, p-Met 1:100 and cytokeratins AE1/AE3 1:300, and for both antigen retrieval was achieved with citrate buffer pH:6.0. Evaluation of tumour invasion was performed in a blind fashion way by two independent observers. The semi-quantitative evaluation took into consideration the quantity of human AE1/AE3 labeled cells present in the CAM mesenchyme.

Statistical analysis

Statistical analysis was performed using Graph Pad program. ANOVA tests were used to calculate significance in an interval of 95% confidence level. All statistics were compared with Mock group and values of $p<0.05$ were considered to be statistically significant.

Results

Induction of SLe^x by overexpression of ST3GAL4 in gastric carcinoma cells

To evaluate the role of ST3GAL3 and ST3GAL4 sialyltransferases in the synthesis of SLe^x structures, the previously established MKN45 cell line model stably transfected with full length of either ST3GAL3 (MST3Gal III), ST3GAL4 (MST3Gal IV) genes, or an empty vector as control (Mock) were used [22]. The evaluation of the expression levels of ST3GAL3 and ST3GAL4 genes by Real Time-PCR (Figure 1A), showed approximately 4 fold increase of ST3GAL3 gene in MST3Gal III cells in comparison with Mock and MST3Gal IV cells, and a 160 fold increase of ST3GAL4 gene expression in MST3Gal IV cells in comparison with Mock and MST3Gal III cells.

The biosynthesis of SLe^x antigen was further assessed by immunofluorescence and by Western blot analysis of total cell lysates and secreted proteins (secretome). Immunofluorescence results showed expression of SLe^x in MST3Gal IV cells when compared with Mock and MST3Gal III cell lines (Figure 1B). Consistently, Western blot results demonstrated the expression of SLe^x in MST3Gal IV cells, both in total cell lysates as previously described [22] and secreted proteins (Figure 1C). No expression of SLe^x was detected in total protein extracts or secreted proteins from Mock and MST3Gal III cells.

Since only MST3Gal IV transfected cells were able to produce SLe^x antigen, further experiments were performed using the MST3Gal IV and Mock cells.

In vitro biological behavior of SLe^x expressing cells - MST3Gal IV

Cell growth, invasive capacity and adhesion properties of cells transfected with the ST3Gal IV gene were evaluated in order to characterize these cellular phenotypes, and also to address the biological role of SLe^x expression. MST3Gal IV cells showed no statistical differences when compared to Mock cells in terms of BrdU incorporation (Figure 2A), suggesting that the expression of ST3Gal IV sialyltransferase and of SLe^x do not affect the proliferation rate of these cells.

Cell invasion was analyzed by counting the number of invasive cells on Transwell Matrigel invasion chambers. This analysis revealed that cells overexpressing ST3Gal IV sialyltransferase presented 3 fold increased ability to invade *in vitro*, when compared with Mock control cells (Figure 2B). This result evidence the importance of SLe^x expression for the invasive phenotype of MST3Gal IV cells.

In addition, the adhesion to extracellular matrix proteins was also evaluated by seeding cells in plates pre-coated with collagen type IV, fibronectin or vitronectin. Interestingly, SLe^x expressing cells (MST3Gal IV) present an increase capacity to adhere to collagen IV and to vitronectin when compared with Mock control cells (Figure 2C). In contrast, no statistical differences were found in the adhesion capacity of these cells to fibronectin.

In vivo evaluation of angiogenesis, tumor growth and invasion capacity of MST3Gal IV cell line using chicken embryo chorioallantoic membrane model

Transfected cells were inoculated into the chicken embryo chorioallantoic membrane (CAM) and different parameters were evaluated after 3 days of inoculation, specifically, the angiogenenic response, tumor size and tumor cell invasive capacity (Table 1). The angiogenic potential was assessed by counting the number of vessels with less than 20 μm diameter growing radially towards the inoculation area. The results show no statistical differences in vessel number between Mock control cells and MST3Gal IV indicating that ST3Gal IV and SLe^x expression do not influence the angiogenic response. Tumor size was assessed by measuring the area (mm^2) of the tumor in the different groups. The results show no statistical differences in tumor size arising from the different cell lines, indicating no influence of ST3Gal IV and SLe^x expression in tumor growth potential.

For the evaluation of tumor cell invasive capacity, CAMs were excised from the embryos, fixed with formalin and paraffin-embedded. Invasion of inoculated cells was evaluated in sections of CAM tumors immunostained for human citokeratins. The results show an increased invasive capacity of MST3Gal IV cells inoculated in CAM in comparison to Mock cells (Table 1). To assess if cells invading the CAM expressed SLe^x antigens, CAM sections were immunostained for SLe^x. The results show that MST3Gal IV invasive cells expressed SLe^x antigens, contrary to the observed in Mock control cells (Figure 3).

Increased activation of c-Met receptor in SLe^x expressing cells-MST3GalIV

To evaluate the possible effects of SLe^x expression on the activation of cell surface receptors and on the induction of the cancer cell invasive phenotype, a receptor tyrosine kinase array was performed using total cell lysates from Mock and MST3Gal IV cells. The results show consistently that MST3Gal IV cells induce increased activation of hepatocyte growth factor receptor (HGFR/c-Met) (Figure 4A). The increased level of c-Met receptor tyrosine phosphorylation (p-Met) was further evaluated by

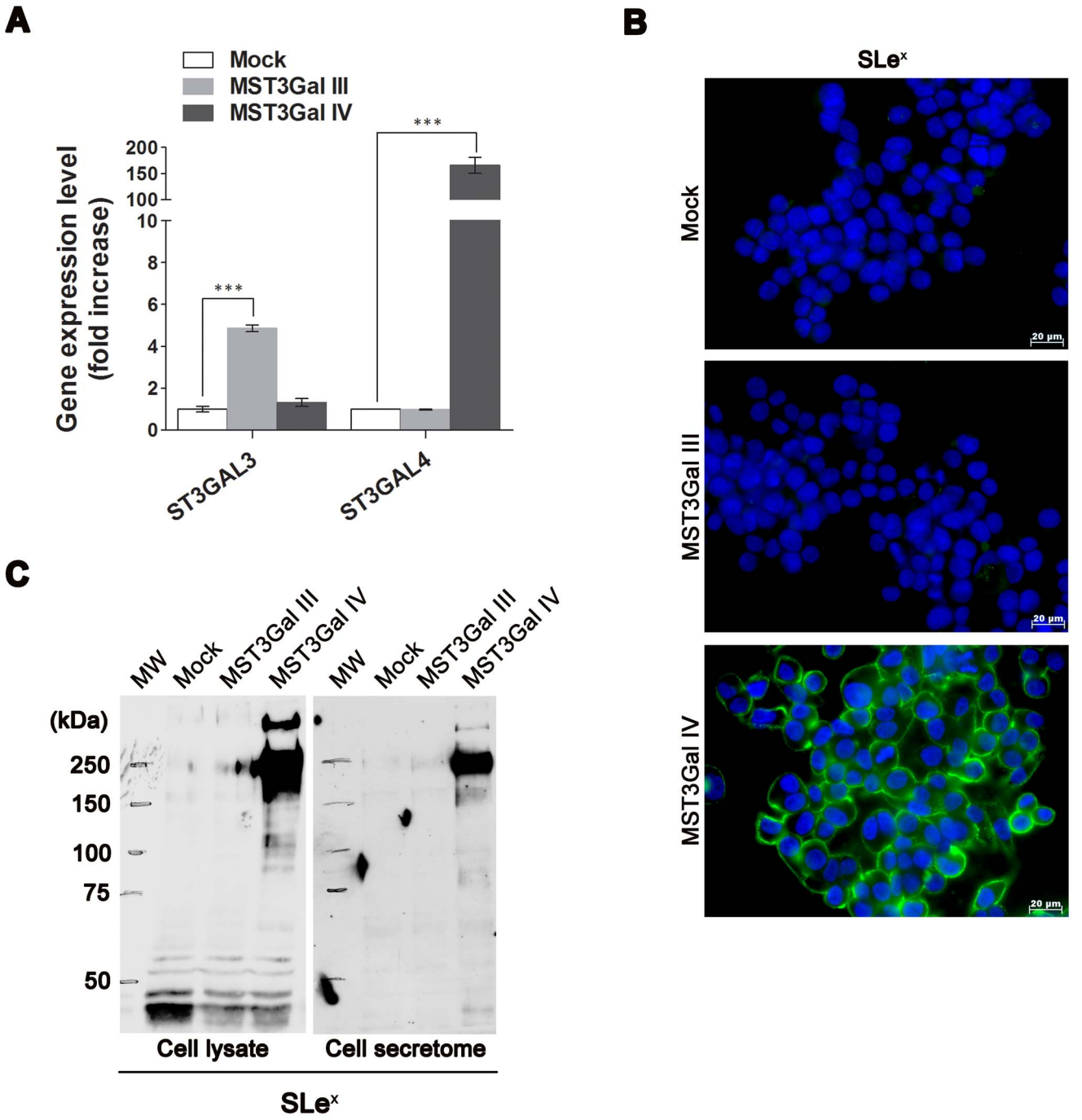

Figure 1. Induction of SLe^x expression by ST3Gal IV transfection in gastric carcinoma cells. A – Relative quantification of ST3GAL3 and ST3GAL4 mRNA expression in MOCK, MST3Gal III and MST3Gal IV transfected cells showing a significant overexpression of ST3GAL3 gene in MST3Gal III cells and ST3GAL4 in MST3Gal IV cells when comparing gene expression levels in mock cells; ***$p<0.001$ Mock versus MST3Gal III for ST3GAL3 gene and ***$p<0.001$ MST3Gal IV versus Mock for ST3GAL4 gene. Results are presented as means ± SD. **B** – Immunofluorescence detection of SLe^x expression in Mock, MST3Gal III and MST3Gal IV cells evidencing the presence of SLe^x in MST3Gal IV (magnification 200x); **C** – Western blot detection of SLe^X in proteins from total cell lysate and secreted proteins from Mock, MST3Gal III and MST3Gal IV cells. SLe^x expression was observed on cell lysates and secretome from ST3GAL4 transfected cells (MST3Gal IV).
doi:10.1371/journal.pone.0066737.g001

Western blot, and the results confirmed that phosphorylation of c-Met is increased in MST3Gal IV cells, with no differences in total c-Met protein expression levels (Figure 4B).

To assess if the CAM invading cells are expressing the activated c-Met, CAM sections were immunostained for phospho c-Met, demonstrating that MST3Gal IV invasive cells are indeed expressing activated c-Met (Figure 4C).

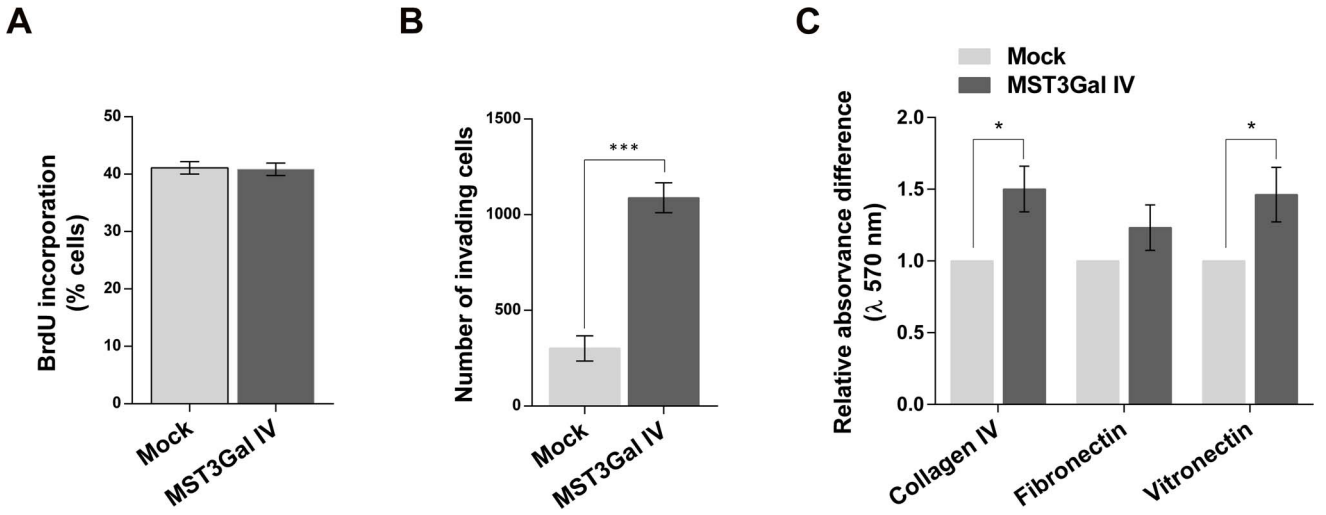

Figure 2. SLe^x overexpression induces cell invasion and increases matrix-cell adhesion *in vitro*. **A** - BrdU proliferation assay in transfected cells. After 50% confluence, cells were incubated for 30 min with BrdU reagent and fluorescent labeled for proliferative index measurement. No differences were found between the different cell lines. **B** - Cell invasion assay on Matrigel chambers. Cells were seeded on Matrigel-coated filters inserted into two-compartment chambers and invading capacity was measured by counting the number of cells that invade, through the Matrigel-coated filter, 6 hour after incubation. MST3Gal IV cells demonstrated an invasive phenotype presenting a significant increased number of invasive cells when compared with MOCK cells (***$p < 0.001$). **C** - Cell adhesion to ECM proteins. Adhesion potential of cells was assessed by incubating cells 30 minutes in pre-coated plates with collagen IV, fibronectin and vitronectin. Results demonstrate an increased adhesion of SLe^x expressing cells to collagen IV (*$p < 0.05$ MST3Gal IV versus Mock) and vitronectin (*$p < 0.05$ MST3Gal IV versus Mock) matrix proteins. No statistical difference was found in the adhesion capacity of cells to fibronectin. Results are presented as means ± SD.
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Evaluation of downstream effectors of c-Met activation

c-Met activation relies on stereotypical signaling modulators common to many RTKs [28,29]. To evaluate possible downstream effectors of c-Met activation, we analyzed the activation of Src, FAK, STAT3, AKT and ERK, proteins involved in different c-Met downstream pathways. Our results show that MST3Gal IV cells present increased activation of Src and FAK proteins which are known to be involved in cell motility and invasion (Figure 5A, B). The small GTPases of the Rho family, such as Rac1, Cdc42, and RhoA were also evaluated as possible downstream modulators of c-Met activation by pull-down of activated GTPases. Our results demonstrate that in MST3Gal IV cells, the expression of sialyltransferase IV and SLe^x induce activation of Rac1, Cdc42 and RhoA (Figure 5C).

Table 1. Parameters evaluated in the CAM model: angiogenesis, tumor growth and invasion potential of cells.

	MOCK	MST3Gal IV	p-value
Angiogenesis *(vessel number ± SEM)*	20.15±4.06	19.31±4.05	n.s.
Tumour growth *(total area mm ^{2}± SEM)*	4.13±1.45	4.29±1.24	n.s.
Cell invasion on CAM *(% cases)*	1/7 14.3%	5/7 71.43%	* 0.0308

n.s.(non-significant) p≥0.05.
*p<0.05.
SEM Standard Error of the Mean.
doi:10.1371/journal.pone.0066737.t001

Inhibition of invasion in SLe^x expressing cells using c-Met and Src activation inhibitors

In order to confirm the biological role of c-Met and Src activation in the invasive capacity of SLe^x expressing cells, inhibition of phosphorylation of c-Met, Src and both in combination were performed. The inhibition was tested using different concentration of each inhibitor, and different time-points of incubation (data not shown). Longer incubations with 0.1 μM of PHA-665752 c-Met inhibitor (24h and 48h) led to decrease in cell proliferation and cell death (data not shown), therefore a 10h incubation time-point, showing no alteration in cell proliferation, was used for the evaluation of cell invasion. Src inhibition occurred after 10h of incubation with 20 μM of PP2 and no differences in cell death and proliferation was observed after longer incubation periods (data not shown). The activation status of c-Met and Src was assessed by Western blot analysis, and results confirmed the decreased in activation of both proteins after 10 h incubation with the inhibitors (Figure 6A).

Given the observation that SLe^x expressing cells present increased cell invasive capacity resulting from the activation of c-Met and Src, invasion of cells was evaluated after c-Met and Src inhibition. The results confirmed the increased invasion of SLe^x expressing cells in DMSO control treatment, and demonstrated the abolishment of this invasion capacity upon inhibition of c-Met, Src and both proteins in combination (Figure 6B). Moreover, the results showed that inhibition of Src or both Src and c-Met in combination were more effective in precluding cell invasion (Figure 6B).

Discussion

Aberrant glycosylation has been described for many years as a hallmark of cancer, and many of the resulting altered glycosyl epitopes are tumor associated antigens [30,31]. These cancer-related antigens are caused by disease-specific alterations in the

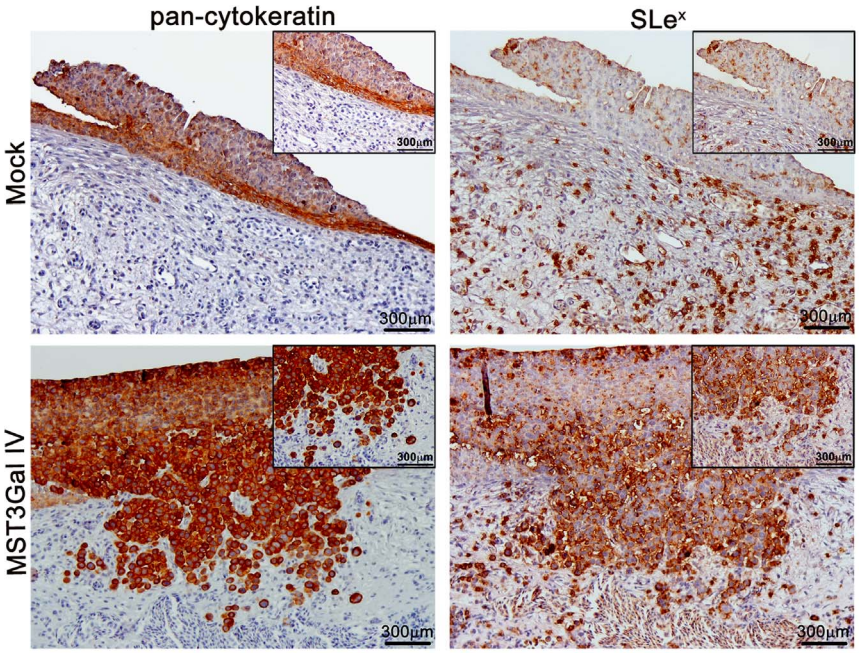

Figure 3. SLe^x expressing cells present an invasive phenotype *in vivo* chicken embryo chorioallantoic membrane (CAM) model. Transfected cells were inoculated in CAM and different parameters were evaluated after 3 days of inoculation. Invasive capacity of inoculated cells was evaluated by immunolabeling CAM tumors with human cytokeratin and SLe^x to assess the presence of human epithelial cells expressing SLe^x. Human cytokeratins immunostaining was used to prove the presence of inoculated human gastric carcinoma cells. SLe^x expression and the invasive capacity of cells were match up to cytokeratins expression. Immunostained tissues evidence the presence of SLe^x structures in CAM tumors from MST3Gal IV cells that, in constrast with Mock cells, invaded the CAM tissue.
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glycan synthesis pathway such as changes in the Golgi and Endoplasmic Reticulum compartments, mutations in enzymes or chaperons, altered expression of enzymes and biochemical competition, and even variations in sugar donor availability [for a review see 1]. A common alteration is the abnormal expression of sialyltransferases, responsible for adding sialic acids residues to cell surface molecules and to secreted proteins, and which have been involved in the oncogenic transformation, as well as in invasion and metastasis [30,31,32]. Sialic acids are typically attached to the outermost ends of glycoproteins and glycolipids that can mediate and modulate a wide variety of physiological and pathological processes [33].

The SLe^x antigen is a sialylated glycan structure which expression has been associated with cancer progression and aggressiveness as well as poor overall patient survival [4,5,6,7,8,9,10,11,12]. The expression of SLe^x in cancer results from the altered expression of sialyltransferases, that adds the sialic acid in a α2,3 linkage to Galactose residues on type-II chains [13].

In this study, we have characterized the role ST3Gal IV sialyltransferase in the synthesis of SLe^x antigen. Expression analyzes of SLe^x in stably transfected gastric carcinoma cells by immunofluorescence and Western blot confirmed that ST3Gal IV leads to the biosynthesis of SLe^x. Moreover, our results indicate that SLe^x antigen is expressed on proteins from total cell lysates as well as on secreted proteins from MST3Gal IV cells. These results confirm previous observations that described the importance of ST3Gal IV in the synthesis of SLe^x, the glycan ligand of selectins [18,19]. In addition, our results are in agreement with recent reports showing an increased mRNA level of ST3Gal IV and α2,3 sialic acid residues expression in gastric cancer tissues [23].

The carbohydrate SLe^x functions as a ligand for cell adhesion molecules of the selectin family, usually expressed on vascular endothelial cells. The expression of SLe^x on cancer cells is known to facilitate tumor cell spreading by mediating tumor-endothelial cell interactions [34,35]. The SLe^x antigen is known to be important in selectin interactions participating in the adhesion of cancer cells to vascular endothelium thus contributing to hematogenous metastasis [36]. These previous observations further support that SLe^x antigen plays a functional role in malignant cancer cell behavior. Noteworthy, the crosstalk between cancer cells and host mechanisms like cell-cell adhesion and cell-matrix adhesion interactions, tumor cell growth and motility are known to be important in modulating the process of cancer cell invasion. In the present study we performed a comprehensive evaluation of the biological role of SLe^x in gastric cancer cells using *in vitro* and *in vivo* models. The *in vitro* analysis showed that SLe^x expressing cells display a similar proliferative rate when compared with Mock transfected cells. However, SLe^x expressing cells demonstrated a higher capacity to invade *in vitro* in Matrigel chambers, demonstrating the active role of this sialylated glycan structure in tumor cell motility and invasion. Concomitant to this invasive capacity, SLe^x expressing cells evidenced higher capacity to adhere to collagen IV and vitronectin extracellular matrix proteins. These findings highlight the importance of this sialylated glycan in the malignant invasive phenotype. Furthermore, this invasive phenotype was also confirmed *in vivo* where cells transfected with ST3Gal IV and expressing SLe^x antigen presented increased capacity to invade the chorioallantoic membrane of the chicken embryo. Our results are in keeping with studies that associate SLe^x expressing tumors with more aggressive phenotypes [8,37,38]. In the gastric carcinoma context it has also been described that SLe^x antigen expression correlates with liver metastasis [7]. The modulation of cancer cell biological behavior by sialylated glycans has been previously described in

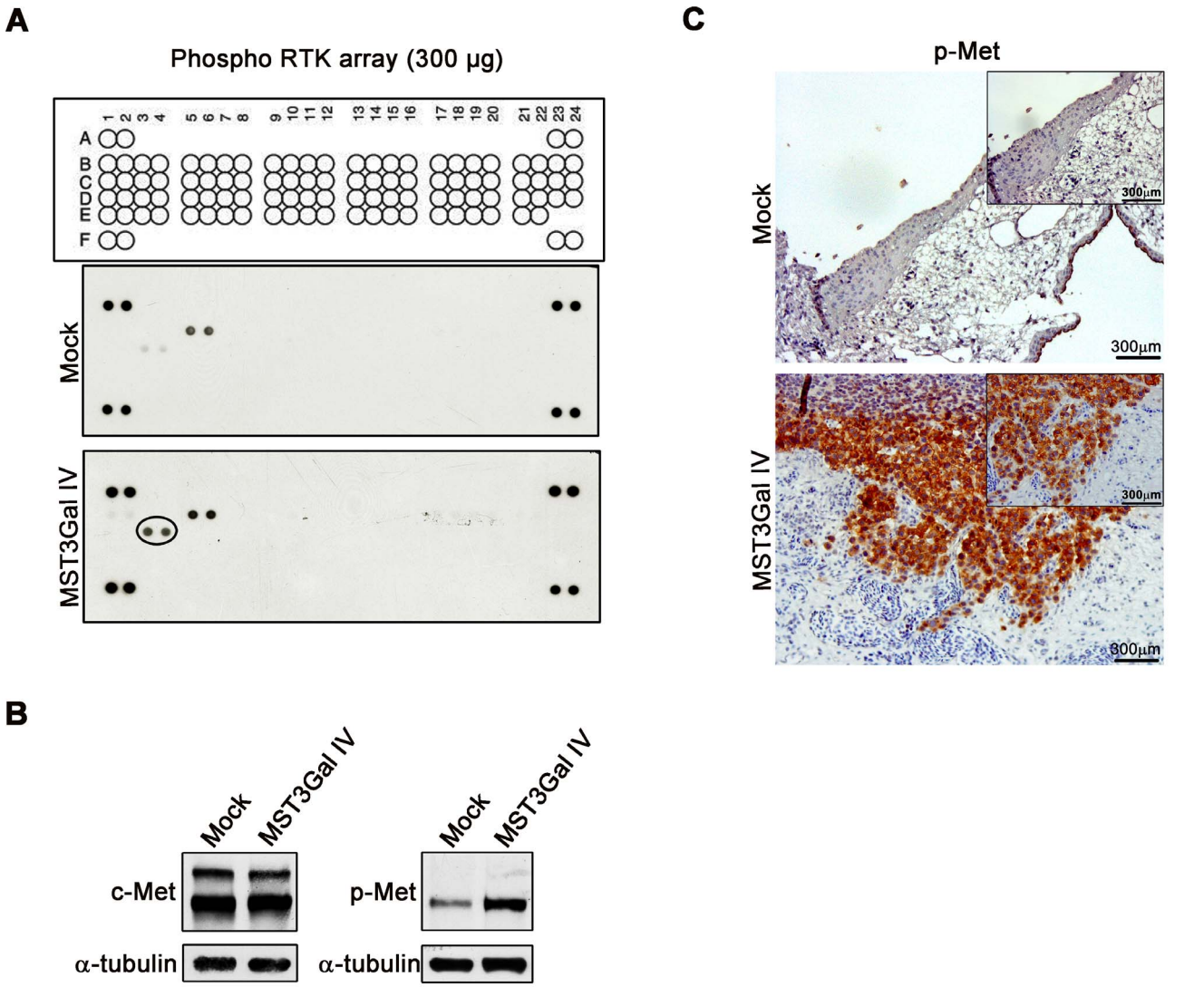

Figure 4. Tyrosine kinase receptors activation evaluation in gastric carcinoma cells; increased c-Met in MST3GAL IV cells. A – phospho-RTK array of transfected Mock and MST3Gal IV cells. Total cell lysates were collected and 300 µg of total protein were incubated into a phospho-RTK membrane array. The array shows an increased activation of c-Met (HGFR) in MST3Gal IV cells. Activated receptors were matched according to the phospho-RTK array coordinates indicated in the material and methods section. c3, c4 correspond to c-Met ; **B** – Analysis of c-Met activation in MOCK and MST3Gal IV cells by Western blot; Cell lysates were analyzed by Western blot with antibodies directed against human c-Met and the phosphorylated tyrosine residues 1234/1235 of the kinase domain to confirm the activation of c-Met in MST3GAL IV cells. The results show an increase expression of phosphorylated c-Met (p-Met) in MST3Gal IV cell line with no differences in c-Met total protein levels in both cell lines. Anti-tubulin antibody was used to assess loading. **C** – Expression of phosphorylated c-Met was assessed in CAM tissues. The evaluation of c-Met activation in CAM tumors show that MST3Gal IV/SLe^x expressing cells present positive staining for phospho c-Met and that the resulting invading cells are also positive for the phosphorylated form of this receptor.
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human pancreatic cells. In this pancreatic model the restoration of α1,2 fucosyltransferase activity, a enzymatic competitor of ST3Gal transferases, reduces the expression of Sialyl Lewis antigens and decreases the adhesive and metastatic properties of these cells [39].

In addition, previous reports have shown that increased cellular sialylation leads to receptor and signaling pathways activation and that the hypersialylation contributes to cancer progression and increased cell motility [40,41]. Moreover, it has been described that TNF-α can induce SLe^x and 6-sulfo-SLe^x expression in human cancer cells, by increasing the expression of ST3GAL4 [21]. This mechanism has also been shown to be mediated by neutrophils expressing TNF-α leading to cancer cells invasiveness and metastasis [42].

In order to clarify the potential implication of ST3Gal IV and of its product SLe^x in the biological behavior of gastric cancer cells, we evaluated the expression of activated tyrosine kinase receptors and downstream modulators involved in cancer cell invasion. The tyrosine kinase receptor array allowed the identification of a constitutive activation of c-Met in SLe^x expressing cells. The activation of tyrosine receptors, directly or indirectly by glycan antigens has previously been observed in other cancer cell models. Singh and colleagues described that the Thomsen-Friedenreich antigen (T antigen) present in CD44v6 promotes the activation of c-Met and MAPK signaling leading to cancer cell proliferation [43]. Furthermore, activation of c-Met receptor has been described in a breast cancer cell model that overexpress

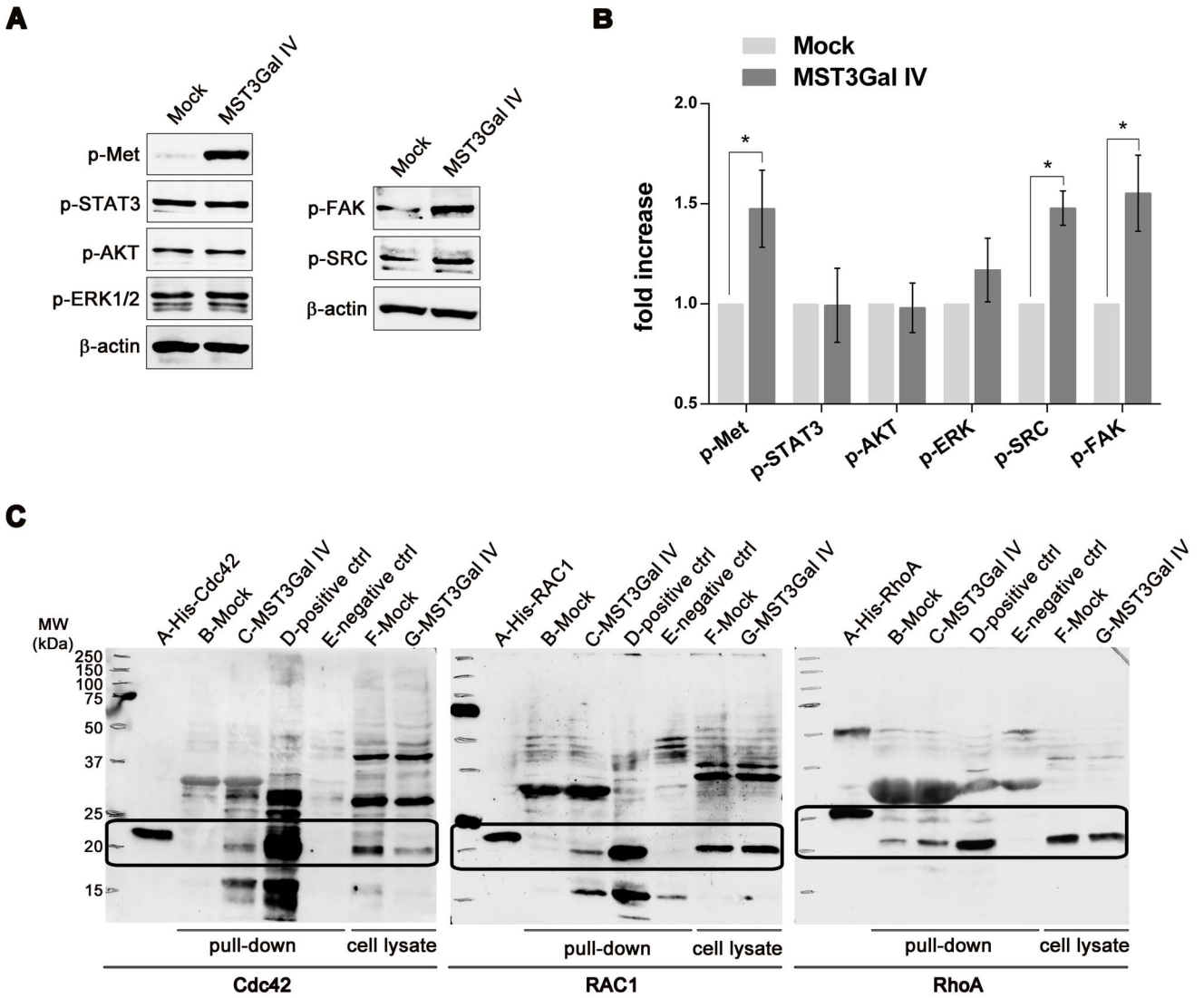

Figure 5. Evaluation of downstream effectors of c-Met activation. A – Increased levels of p-FAK and p-Src proteins in MST3Gal IV cells. The contribution of other effector proteins, such as AKT, ERK, FAK and Src was evaluated by Western blot for their phosphorylated forms in Mock and MST3Gal IV cell lines, and expression of β-actin protein was used as protein loading control. Results show increased levels of phophorylated FAK and Src supporting their involvement as downstream effectors of phosphorylated c-Met (p-Met). **B** – Analysis of 5 independent Western blot of c-Met, STAT3, AKT, ERK, FAK and Src phosphorylated forms in MOCK and MST3 Gal IV cells showing statistically significant increased levels of p-FAK and p-Src, concomitantly with increased phosphorylated c-Met. Results are presented as means ± SD. **C** - Evaluation of Cdc42, Rac1 and RhoA GTPases as potential modulators of c-Met activation by pull-down assay of their activated forms. Western blot analysis of pull-down proteins evidence an increased activation of Cdc42, Rac1 and RhoA in MST3Gal IV cell line. A-GTPase WB protein positive control (His-Cdc42, His-Rac1 and His-RhoA); B-Mock total cell protein pull down; C-MST3Gal IV total cell protein pull down; D-Mock total cell protein pull down with previous GTPases activation (pull down positive control); E-Mock total cell protein pull down with previous GTPases inhibitors (pull down negative control); F-Mock total cell protein input; G-MST3Gal IV total cell protein input. Highlighted areas represent regions of interest regarding the specific protein migration.
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glycosyltransferases and this activation has been implicated in proliferation and invasion of cancer cells [25,26,27]. The MKN45 cell line model has been reported to have a high level of expression and dependence on c-Met [44] and therefore modulation of cellular glycosylation can have implications in this c-Met dependent cells.

c-Met overexpression has been considered a hallmark of cancer, playing a role in many tumors and in metastatic progression [45]. In gastric cancer, c-Met expression alterations have been reported, such as the Tpr/Met rearrangement [46,47] and c-Met copy number amplification [48], as well as increased c-Met activation [49,50]. We evaluated the c-Met activation in a series of gastric carcinoma tissues (data not shown). The variability in tissue sample processing is known to lead to loss of protein phosphorylation which precluded the detection of phosphorylated c-Met in these samples. However, sections analyzed from the chicken chorioallantoic membrane tumors, derived from either Mock or SLe^x expressing gastric carcinoma cells, confirmed phosphorylated c-Met positive staining in SLe^x cancer invading cells. This result further supports the hypothesis that SLe^x expressing cells exhibit invasive capacity through the activation of c-Met.

The activation of c-Met is well known to induce docking sites for proteins that mediate downstream signaling leading to the activation of the mitogen-activated protein kinase (MAPK),

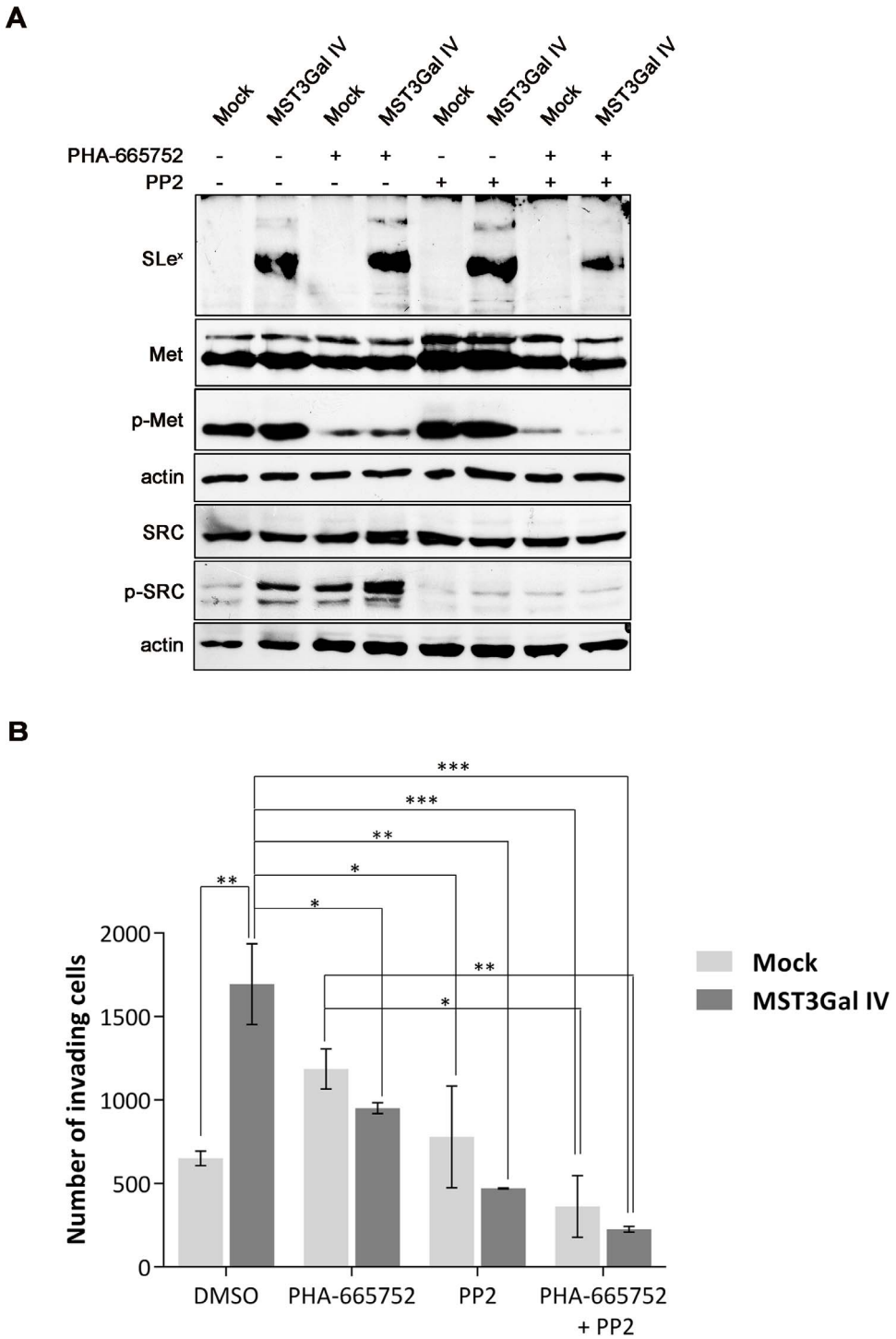

Figure 6. Inhibition of invasion in SLex expressing cells by targeting c-Met and Src activation. A - Evaluation by WB of the activation of c-Met and Src in the MST3Gal IV and Mock cells with or without the presence of inhibitors of c-Met (PHA-665752) and Src (PP2). **B -** Cell invasion assay on Matrigel chambers after inhibition of c-Met and Src activation. Cells were seeded on Matrigel-coated filters after incubation with inhibitors for 10 hours. Invading capacity was measured by counting the number of cells that invade, through the Matrigel-coated filter, after 6 hours. MST3Gal IV cells demonstrated an invasive phenotype presenting a significant increased number of invasive cells when compared with MOCK cells. Inhibition of c-Met, Src, and both in combination led to abolishment of cell invasion capability of the MST3Gal IV cells (*$p<0.05$; **$p<0.01$; ***$p < 0.001$).
doi:10.1371/journal.pone.0066737.g006

phosphatidylinositol 3-kinase (PI3K)-AKT, v-src oncogene homolog (Src), signal transducer and activator of transcription (STAT), which are signaling pathways that are involved in increased cell growth, scattering, motility, invasion, protection from apoptosis, branching morphogenesis, and angiogenesis [28,29]. Taking that into consideration, we evaluated the downstream effectors of c-Met activation and found that FAK and Src proteins showed increased activation in cells expressing ST3Gal IV. In combina-

tion with our invasion assays results (*in vitro* and *in vivo*), these results strongly suggest that c-Met activation mediates tumor cell motility and invasion, also in gastric cancer cells. These results are in agreement with previous studies that associate Src-FAK signaling pathway with the metastization process [51,52,53,54]. Furthermore, our results show that inhibition of c-Met and Src could preclude the increased invasion observed in SLe^x expressing cells supporting the importance of this glycosylation alteration in the activation of this invasive related pathways.

Oncogenic transformation is often associated with changes in organization of the cytoskeleton, which can influence cell migration, adhesion and invasion. The c-Met activation can cause changes in gene expression of cell-cycle regulators (Cdk6, p27), extracellular matrix proteinases (such as matrix metalloproteinases and urokinase plasminogen activator), and in alterations of cytoskeleton functions that control migration, invasion and proliferation [55]. The cytoskeleton is composed of a complex and organized network of various fibrous proteins within the cytoplasm, playing an essential structural and regulatory role in the maintenance of cell structure and strength, in cell division, proliferation, motility, invasion and also in signaling functions [56,57,58]. The activation of tyrosine kinase receptors can modify the phosphorylation status of cytoskeleton regulatory and structural proteins. Signaling pathways initiated by the activation of cell surface receptors can promote distinct membrane protrusions by converging onto the Rho family of GTPases [59,60]. Rho proteins are small (21-25 kDa) molecules that share structural homology and become activated only when bound to GTP. One of the best characterized Rho GTPase family members is RhoA regulating the formation of stress fibers and focal adhesion assembly, while Rac1 and Cdc 42 are mainly involved in membrane ruffling and formation of filopodia, respectively [61]. Estimation of GTPases activation is frequently a molecular marker in the evaluation of cytoskeleton alterations during cell migration [62,63,64]. Here we showed the activation of Rho GTPases, specifically RhoA, Rac1 and Cdc42. These results further supports the evidence that SLe^x expression leads to cytoskeleton protein alterations in cancer cells, underlying the observed increased cell motility and invasion of these cells. Our findings are in keeping with previous reports showing the importance of RhoA, Rac1 and Cdc42 in cancer progression [65], and also the crosstalk between these GTPases and other signaling pathways like Src-FAK in the migratory phenotype of cancer cells [66]. Our present findings support the hypothesis that increased expression of SLe^x on the surface of malignant cells plays an important role in tumor invasion and metastasis. Overall, our study showed that tumor cell invasion is induced by SLe^x expression on gastric cancer cells through the activation of c-Met in association with downstream signaling effectors Src, FAK and RhoA GTPases activation (Figure 7).

These results open new avenues to design intervention strategies that target ST3Gal IV/SLe^x in cancer cells as well as the

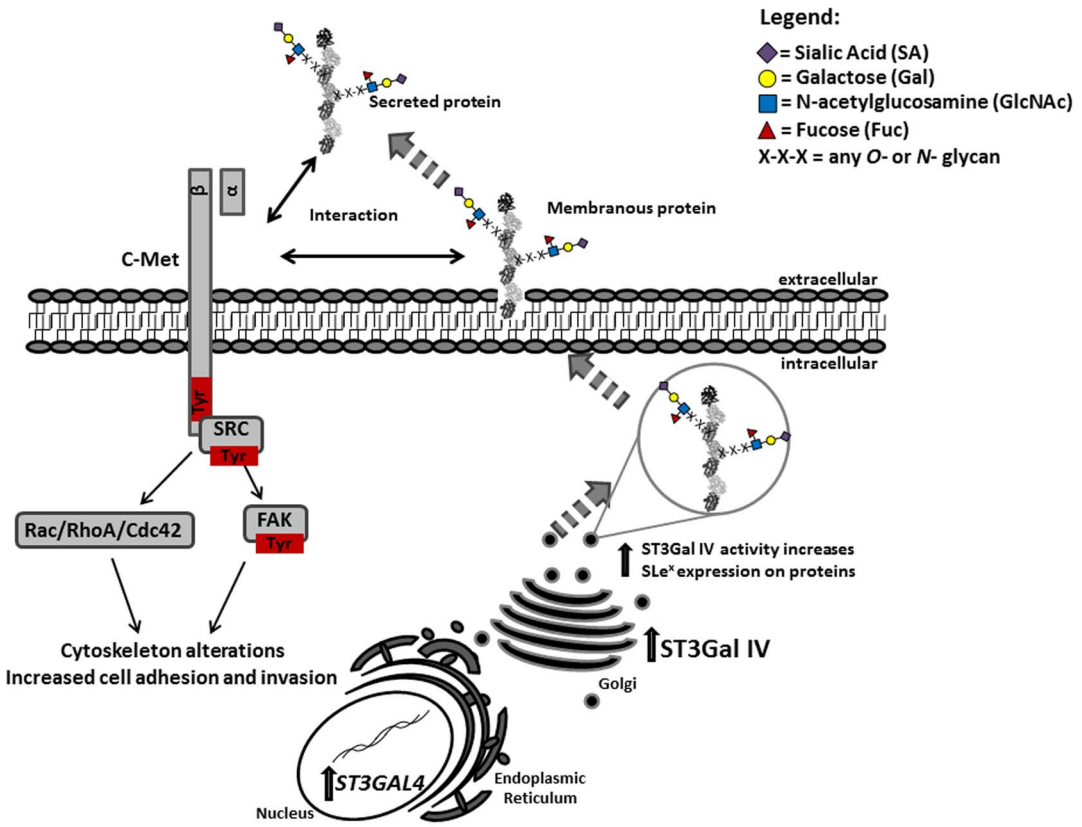

Figure 7. Schematic representation of the alterations induced by increased expression of SLe^x and activation of c-Met. Increased transcription of ST3GAL 4 leads to increased expression of the ST3Gal IV enzyme in the Golgi apparatus of the cells. This enzyme will glycosylate type-2 terminal oligosaccharide chains leading to the presence of SLe^x in glycoproteins targeted for the membrane or to be secreted by the cells. The expression of SLe^x in membrane-associated and secreted proteins can promote the interactions between these proteins and c-Met leading to its activation. c-Met activation leads to downstream signaling activation target proteins Src, FAK and Rho GTPAses leading to a modified cell-matrix adhesion and an increased cell invasion.
doi:10.1371/journal.pone.0066737.g007

inhibition of c-Met and Src in order to improve gastric cancer treatment by targeting invasion and metastasis.

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Author Contributions

Conceived and designed the experiments: CG HO MTP MJO CAR. Performed the experiments: CG MTP. Analyzed the data: CG HO MTP DC MJO CAR. Contributed reagents/materials/analysis tools: CG HO MTP DC MJO CAR. Wrote the paper: CG HO CAR.

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