DEPARTAMENT OF

LIFE SCIENCES

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BSc in Biochemistry

Exploring the crosstalk between Sialyl-Tn (STn) expression and Pancreatic ductal adenocarcinoma (PDAC) progression

Master in Molecular Genetics and Biomedicine

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"It is not the strongest of the species that survive,

nor the most intelligent, but the ones most responsive to change."

– Charles Darwin

Abstract

Pancreatic cancer (PC) is the sixth leading cause of cancer-related deaths due to the lack of effective treatment and its intrinsic aggressiveness. Pancreatic ductal adenocarcinoma (PDAC) represents 85-90% of PC cases, and these tumors are normally detected in advanced stages further contributing to the high mortality of these cancers. In pancreas carcinogenesis, there are three well-known PDAC precursors: Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN).

Glycosylation is a post-translational modification crucial to many biological processes, which become aberrant in neoplastic cells, allowing them to take over many hallmarks of cancer. Particularly, aberrant sialylation results in the formation of cancer specific glycans, including truncated O-GalNAc glycans. Sialyl-Tn (STn) is one of these truncated O-GalNAc glycans being expressed in more than 80% of human epithelial cancers, including PDAC, and nearly absent in healthy tissues. STn expression is mainly attributed to the overexpression of ST6GalNAc I sialyltransferase being commonly associated with poor prognosis contributing to cancer progression and invasion. However, STn profile in a meaningful cohort in different PDAC development stages and its impact on survival in cancer remains yet to be clarified. Therefore, in this work, we evaluated the STn expression during PDAC progression in a meaningful cohort of patients. Additionally, we validated an *in vitro* model of PDAC that overexpresses STn to enable future experiments on this topic, and finally we revised information on the survival impact of STn in different cancer types.

To accomplished this, we examined 185 tissue samples from patients by immunohistochemistry, where we identified variable STn expression across the different PDAC development stages while absent in normal pancreas. Despite heterogeneity, STn mainly occurs in later events of PDAC development, and seems to have a role in PDAC progression. Additionally, we also evaluated if neoadjuvant chemotherapy would have any impact on STn expression, but no significant correlation was observed. Then, the *in vitro* model was validated, by analyzing PANC-1 cells overexpressing or not ST6GalNAc I by western blot and flow cytometry. We observed that cells overexpressing ST6GalNAc I showed STn expression unlike the ones not overexpressing ST6GalNAc I, validating the model and confirming the role of this enzyme in STn expression. Finally, we performed a literature search on the survival impact of STn and identified that STn+ cases tend to have worse outcomes, yet the impact of STn varies between the different cancer types. Interestingly, no PDAC cohort was found, highlighting the need to assess the survival impact of STn in these patients.

Keywords: Pancreatic ductal adenocarcinoma, PDAC precursor lesions, sialyl-Tn, immunohistochemistry, survival.

Resumo

O cancro de pâncreas é a sexta maior causa de morte por cancro devido à falta de tratamento eficaz e da sua agressividade intrínseca. O adenocarcinoma ductal pancreático (PDAC) representa 85-90% dos casos sendo normalmente detetado já em estágios avançados o que contribui para a sua elevada mortalidade. Na carcinogénese do pancreas, existem três percursores bem conhecidos: a neoplasia intraepitelial pancreática (PanIN), neoplasia mucinosa papilar intraductal (IPMN) e a neoplasia quística mucinosa (MCN).

A glicosilação é uma modificação pós-traducional crucial para muitos processos biológicos, que quando aberrante permite que as células adquiram características tumorais. Em particular, a sialilação aberrante, resulta na formação de glicanos específicos de cancro, incluindo glicanos O-GalNAc truncados. O sialyl-Tn (STn) é um destes O-glicanos truncados, expresso em mais de 80% dos cancros epiteliais, incluindo PDAC, e quase ausente em tecidos saudáveis. A expressão de STn deve-se principalmente à sobre expressão da ST6GalNAc I estando geralmente associado a um mau prognóstico, contribuindo para a progressão e invasão tumoral. No entanto, a confirmação do perfil do STn num coorte significativo na progressão de PDAC e o seu impacto na sobrevivência no cancro continuam por clarificar. Assim, neste trabalho, avaliou-se a expressão do STn na progressão do PDAC a partir de um coorte significativo de doentes. De seguida, validou-se um modelo *in vitro* de PDAC que sobre expressa o STn permititindo futuros estudos neste tópico e, finalmente reviu-se o que está descrito sobre o impacto do STn na sobrevivência em diferentes tipos de cancro.

Para isto, foram examinadas 185 amostras de tecido de doentes por imunohistoquímica onde foi identificada uma expressão variável de STn nos diferentes estágios do desenvolvimento do PDAC, estando ausente no pâncreas normal. Apesar da heterogeneidade, o STn ocorre maioritariamente em eventos tardios do desenvolvimento tumoral e parece ter um papel na sua progressão. Verificou-se também se a quimioterapia neoadjuvante teria algum impacto na expressão de STn, no entanto, não foram obtidas correlações significativas. O modelo *in vitro* foi validado pela análise da linha celular PANC-1, que sobre expressava ou não a enzima ST6GalNac I, por western blot e citometria de fluxo. Observou-se que as células que sobre expressavam a enzima, tinham expressão de STn ao contrário das que não a sobre expressavam. Isto não só validou o modelo como confirmou o papel desta enzima na expressão do STn. Por fim, a pesquisa de literatura revelou que os casos STn+ tendem para piores resultados, no entanto, isto varia conforme o tipo de cancro. Curiosamente, não foi encontrado um coorte de PDAC, elucidando a necessidade de estudar o impacto do STn na sobrevivência destes doentes.

Palavras-Chave: Adenocarcinoma ductal do pâncreas, lesões percursoras de PDAC, sialyl-Tn, imunohistoquímica, sobrevivência.

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Abbreviations

A

AJCC - American Joint Committee on Cancer

APCs - Antigen-presenting cells

APC-Cy7 - (Allophycocyanin)-Cy7

Asn - Asparagine

B

C

CD8+ cells - cytotoxic T cells

CI - Confidence interval

COSMC - C1GaLT1 Specific Molecular Chaperone 1

CS - Cumulative survival

CSS - Cancer-specific survival

C1GalT1 - core 1 β1,3-galactosyltransferase or T synthase

C3GnT - core 3 β1,3-N-acetylglucosamine transferase

D

DAB - 3,3′-diaminobenzidine tetrahydrochloride

DAMPs - danger-associated molecular patterns

DCs - Dendritic cells

DMEM - Dulbecco's Modified Eagle Medium

DTT - DL-Dithiothreitol

E

EDTA - Ethylenediaminetetraacetic acid

ER - Endoplasmic reticulum

F

FBS - Fetal bovine sérum

FFPE - formalin-fixed paraffin-embeded

FITC - Fluorescein isothiocyanate

G

Gal - Galactose

GalNAc - *N*-acetylgalactosamine

GALNTs - GalNAc transferases

GlcNAc - *N*-acetylglucosamine

H

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HG - High-grade

HIF-1α - Hypoxia-inducible factor-1α

HRP - horseradish peroxidase

HS - Horse serum

H_2O_2 - Hydrogen peroxide

I

IHC - Immunohistochemistry

IL - Interleukin

IPMN - Intraductal papillary mucinous neoplasm

J

K

L

LG - Low-grade

M

MCN - Mucinous cystic neoplasm

MHC - Major histocompatibility complex

N

Neu5Ac - *N*-acetylneuraminic acid

NK - Natural killer cells

O

OR - Odds ratio

OS - Overall survival

P

PanIN - Pancreatic intraepithelial neoplasia

PAMPs - Pathogen-associated molecular patterns

PBS - Phosphate-buffered saline

PC - Pancreatic cancer

PDAC - Pancreatic ductal adenocarcinoma

PFA - Paraformaldehyde

PRRs - Pattern recognition receptors

Post-CT - Post-chemotherapy

pTNM - Pathological TNM

Q

R

RPMI - Roswell Park Memorial Institute

RT - Room temperature

S

SDS - Sodium dodecyl sulfate

Ser - Serine

Sias - Sialic acids

Siglecs - Sialic acid binding immunoglobulins (Ig)-like lectins

STn - Sialyl-Tn

STs - Sialyltransferases

ST6GalNac I - α-N-acetylgalactosaminide α-2,6-sialyltransferase I

T

T antigen - Thomsen–Friedenreich antigen

TBS - Tris-buffered saline

TBS – T - Tris-buffered saline with tween

TCR - T cell receptor

Thr - Threonine

TNF-α - Tumor necrosis factor

Tn antigen - Thomsen-nouveau antigen

U

V

W

WT - Wild-type

X

Y

yPTNM - Post-therapy TNM

Z

1. Introduction

1.1 Cancer

Cancer is a general term for a group of diseases that can affect any region of an organism's body[1]. In 2022, nearly 10 million deaths given to cancer were reported, being classified as one of the leading causes of death worldwide[2]. The most common cancers are lung (2.48 million cases), female breast (2.31 million cases), colorectal (1.93 million cases) and prostate (1.47 million cases)[2]. The development of cancer can be influenced by several factors, such as genetic and environmental factors (such as radiation), virus infections and even the individual lifestyle (tobacco, alcohol, diet) being only 5-10% of the cases hereditary[1,3,4].

The process by which normal cells transform into cancer cells is referred to as carcinogenesis. This process is characterized by several genetic and molecular alterations that can accumulate over time, such as mutations in proto-oncogenes, in tumor suppressor genes, resulting in their inactivation, and/or in genes involved in deoxyribonucleic acid (DNA) repair mechanisms[3–5]. Consequently, the result is an uncontrolled and abnormal growth of cells[1,3]. Additionally, cancer cells can go through a process called metastasis, where they are able to enter the bloodstream and lymphatics and spread to other parts of the body[3].

1.1.1 Hallmarks of cancer

There is a set of features acquired by cells during tumor development that distinguishes cancer from normal cells, known as the hallmarks of cancer[6].

Currently, eight hallmarks and two enabling characteristics, which contribute to cancer hallmarks acquisition, can be described along with other emerging hallmarks and enabling characteristics that are recently being considered (**Figure 1**). Cancer cells have the capacity to sustain proliferative signaling, evade growth suppressor factors, resist cell death and acquire an infinite capacity of replication[6]. This abnormal and uncontrolled cell growth has to be sustained by deregulating cell metabolism which confers an advantage for cancer cells by providing building blocks for rapid proliferation and supporting the frequent deficiency in nutrients[6,7]. However, for the tumor to expand, the cells still need to have access to more oxygen and nutrients. Therefore, cancer cells are able to induce angiogenesis which also confers the cells the opportunity to invade and metastasize[3]. The evasion of the immune system, which will be further detailed, is another cancer hallmark, along with two enabling characteristics that are genome instability and inflammation promoted by the tumor[6].

Nowadays, the capacity of cells to dedifferentiate back to progenitor-like cell states or to transdifferentiate into other types and the action of senescent cells are classified as emerging hallmarks[6]. As enabling characteristics, are being considered, non-mutational alterations of the epigenome and interactions with the microbiome[6]. The identification of these hallmarks is important to categorize the mechanisms behind the development of cancer and, consequently, might help identify possible treatments.

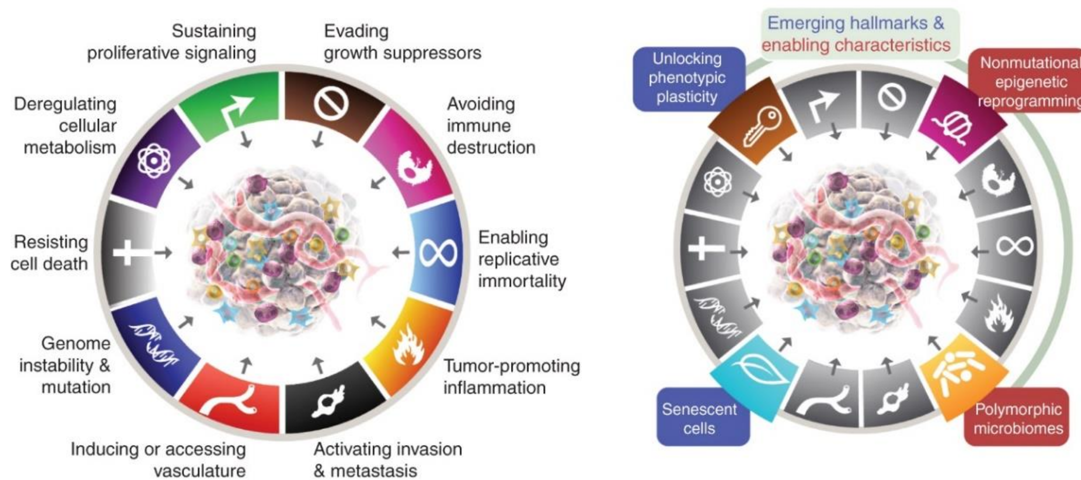


Figure 1 - The hallmarks of cancer. Nowadays, there are 8 hallmarks and 2 enabling characteristics. More recently, two other hallmarks and enabling characteristics are being considered[6].

1.1.2 Cancer and the immune system

The immune system is able to prevent neoplasia and regulate the abnormal growth of altered cells[8]. This is given to its ability to eliminate viral infections that could induce tumors, other pathogens and by preventing an inflammatory microenvironment that could promote tumor development[8,9]. However, sometimes tumors can evade the immune system. The next sections will elucidate the main mechanisms of the immune response and how cancer can evade them.

1.1.2.1 Overview of the immune system

The immune system comprises an interactive network between several cells, their respective secreted molecules and mechanisms that protect us from pathogens, toxins and cancer cells through the recognition of unsafe "non-self" antigens distinguishing from "self" antigens. The immune response can be divided into two types according to the speed and specificity of the reaction: innate and adaptive immunity[10,11].

The innate immune response refers to the first line of defense, able to recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) that proceed in the same way having no immunologic memory[10,11]. This type of immune response comprises physical and chemical barriers, phagocytic cells such as macrophages and

neutrophils, antigen-presenting cells (APCs) such as dendritic cells (DCs), natural killer cells (NK) and several inflammation mediators[10,12].

These cells recognize PAMPs and DAMPs through the pattern recognition receptors (PRRs) presented on their surface[10,12,13]. When these patterns are recognized, signal transduction pathways are activated, initiating host defense responses leading to the release of cytokines and other factors[13]. The key inflammatory cytokines released, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF-α), promote the recruitment and activation of immune cells for the clearance of pathogens[10]. In addition, PRRs stimulation also leads to the maturation of APCs to present antigens and, together with the cytokine expression, triggers the adaptive immunity[13].

The adaptive immune response, in contrast, is an antigen-dependent mechanism that triggers specific effector responses through T and B cells[12]. Compared to innate immunity, this type of response requires a prolonged time to generate a specific response upon antigen exposure. This is related to the fact that it requires the expansion of antigen specific clones that become effector cells. Then, some of these generate memory cells, therefore, upon a second exposure to the same antigen the specific immune response is more efficient[10–12].

B cells have their own antigen-binding receptor; therefore, they can recognize the antigens directly. When this recognition occurs, B cells undergo proliferation and differentiation into antibody-secreting plasma cells, that produce large amounts of antibodies to enter the circulation, or into memory B cells[10]. Thus, B cells play a vital role in humoral or antibody-mediated immune response[12].

T cells, on the other hand, are only capable of recognizing small and processed antigens that are presented by other cells through the major histocompatibility complex (MHC) molecules, also known as human leukocyte antigen[12]. The mechanism begins with the antigen being presented to the T cell receptor (TCR) so the T cells can be activated. This presentation, as mentioned, is driven through the MHC molecules that can be divided into two classes: the MHC class I and class II[11,12]. The MHC class I is expressed by all nucleated cells, whereas the MHC class II is only expressed by APCs, such as DCs, macrophages and B cells. The MHC class I is recognized by a cluster of differentiated T cells, the cytotoxic T cells (CD8+) and the MHC class II is recognized by another cluster, known as helper T cells (CD4+)[11,14,15]. These cells play a role in the cell-mediated immune response[10,12].

Together, both innate and adaptive responses play an important role in immune surveillance by distinguishing “self” antigens from “non-self” antigens.

1.1.2.2 Cancer evasion of the immune system

Tumors can develop even in the presence of a functioning immune response. Therefore, the concept of immunoediting has emerged, and it comprises 3 phases: elimination, equilibrium, and escape[9,16].

The elimination phase, also known as tumor immune surveillance, is when the immune system recognizes cancer cells through tumor-associated antigens or stress-induced molecules in order to eliminate them[9]. However, some cells can escape this process. Herein a phase of equilibrium is reached, where cancer cells are not destroyed but are also unable to progress. After some time, cancer cells can undergo several mechanisms, due to their high genetic instability, to induce immune tolerance when they grow and metastasize[9,16–18].

Some of those strategies are loss of MHC class I molecules, the expression of non-immunogenic tumor-associated antigens and the development of an immune suppressive tumor environment by expressing immunoregulatory molecules[8,19].

Additionally, cancer cells are able to use the immune system to their advantage, such as promoting the development of new blood vessels, which helps the tumor to grow and to invade nearby tissues[8].

The mechanisms of cancer evasion depend on the microenvironment that surrounds the tumor, where there is an interplay between several cell types, cellular components and signaling pathways that together support the development of the tumor[19].

1.2 Pancreatic cancer (PC)

PC is the sixth leading cause of cancer-related deaths worldwide being expected to become, in some regions, the second during the next few years[2,20,21]. The patients diagnosed with PC have an extremely low 5-year survival rate since no efficient target treatment exists, with surgery being the most successful clinical treatment[20–22]. Along with the lack of effective treatment and intrinsic aggressiveness, the fact that these tumors are normally detected in advanced stages contributes to their high mortality. This is related to the fact that in the early stages the symptoms are nonspecific and progress slowly over time[22,23]. About 90% of PC cases are sporadic, while 10% are inherited[22]. There are several complex and multifactorial risk factors[24]. Among the non-variable risk factors are age, some comorbidities, family history and genetic susceptibility while the variable ones are the consumption of alcohol, diet, tobacco, obesity, etc[21,24].

The majority of PC derives from the exocrine pancreas, originating in the ducts where there is an abnormal growth of the cells. This abnormal growth may lead to the development of the

pancreatic ductal adenocarcinoma (PDAC)[25]. PDAC represents 85-90% of all pancreatic neoplasms being characterized by an immunosuppressive microenvironment[23,26].

1.2.1 PDAC treatment

As mentioned, surgery is currently the most effective treatment for PDAC. However, since this disease is often diagnosed in advanced stages, only some patients are suitable for the surgical approach[27,28].
Therefore, the treatment chosen is influenced by the stage of the disease and the performance status of the patient, categorized by the Eastern Cooperative Oncology Group status[27].

The standard procedure for individuals with treatable disease and good performance is surgery and then adjuvant chemotherapy. However, for other patients, neoadjuvant treatment is an option, where the chemotherapy administration is prior to surgery[27]. Furthermore, chemotherapy can also be combined with radiation therapy (chemoradiotherapy) still, the benefit of this type of treatment on patient survival has not yet been proven[29,30].

1.2.2 Precursors lesions of PDAC

Some non-invasive precursor lesions are associated with the development of PDAC, such as pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN)[22–24].

PanINs are the most common microscopic pre-malignant lesions, that can be classified in different grades, based on the cellular and architectural changes, such as, PanIN-1A, PanIN-1B, PanIN-2 and PanIN-3[23,31,32]. They are classified in low-grade (LG), which includes PanIN-1 and 2, and high grade (HG) which corresponds to PanIN-3. PanIN-1A shows minimal cellular changes and therefore is the earliest stage of PanIN characterized by flat lesions in the ductal epithelium[31,32]. PanIN-1B is a stage with a slight increase in cellular atypia, with papillary architecture, but identical to PanIN-1A[31,32]. The next stage is PanIN-2 with mostly papillary architecture, where nuclear abnormalities might be observed[31,32]. PanIN-3, also known as carcinoma *in situ,* is characterized by luminal necrosis, dystrophic goblet cells, prominent nucleoli and loss of polarity[31,32].

IPMNs and MCNs are macroscopically pancreatic cystic lesions[23,32]. Both exhibit a progressive morphological spectrum with epithelium lesions that can present mild/LG dysplasia (adenoma), moderate dysplasia (borderline) or severe/HG dysplasia (carcinoma *in situ*)[32].

IPMNs are diagnosed in at least 20% of all samples from pancreatectomy resections[23]. The prognosis is related to the location, nature (non-invasive, minimally invasive or invasive),

subtype of the IPMN-associated carcinoma and epithelial subtype[23,33]. For non-invasive IPMNs, the 5-year survival rate after resection is high, around 90-100%[23].

IPMNs involve the main pancreatic duct or its major branches being distinguished into four epithelial subtypes[31,32]. The subtypes are gastric, intestinal, pancreato-biliar and oncocytic and can be differentiated by their immunohistochemical patterns of mucin glycoproteins[23,32,34]. The gastric subtype is normally characterized by LG dysplasia being negative for MUC1 and MUC2 and positive for MUC5AC. The intestinal subtype is normally characterized by moderate/HG dysplasia and negative for MUC1 and positive for MUC2 and MUC5AC. The pancreato-biliar subtype normally presents HG dysplasia, being negative for MUC2 and positive for MUC1 and MUC5AC. The oncocytic subtype also commonly presents HG dysplasia, being positive for MUC5AC and sometimes also positive for MUC1 and/or MUC2[23,34,35]. All subtypes, except the intestinal, are positive for MUC6[23,35].

MUC1 can be found on the luminal surface of acinar cells in the normal pancreas. MUC2 is a marker of intestinal differentiation, not expressed in normal pancreas. MUC5AC is expressed by surface mucus cells of the stomach not being expressed in the normal pancreas. However, all IPMN subtypes and PanINs consistently express this mucin. MUC6 is expressed by gastric pyloric and duodenal Brunner's glands. Occasionally, it is also expressed by small intralobular ducts in the pancreas[35].

MCNs are very rare, being only diagnosed in 10% of resected pancreatic cystic lesions and occurring almost only in women[23]. These pre-malignant lesions are characterized by an ovarian type stroma surrounding the cyst and can present LG and HG dysplasia[31,32].

1.2.3 TNM staging classification

The TNM staging classification, where T stands for tumor, N for nodes, and M for metastases, is based on the cancer anatomy, according to the American Joint Committee on Cancer (AJCC) (**Table 1**). Each of the letters are related to specific categories, which are associated with a number[36]. The T, N and M categories are given for specific groups, such as pathological (pTNM) and post-neoadjuvant therapy (ypTNM). The pTNM staging classification is based on the pathological assessment of surgical specimens obtained after surgery prior to any treatment. On the other hand, the ypTNM staging classification of surgical specimens is applicable when surgery is done after neoadjuvant therapy[36].

Table 1 - TNM designations associated with the stage of disease for PC[36].

Stage	TNM
0	Tis – carcinoma *in situ* (PanIN-3, HG IPMN or MCN) N0 – No regional lymph node metastases M0 – No distant metastasis
IA	T1 – Tumor size ≤ 2 cm N0 – No regional lymph node metastases M0 – No distant metastasis
IB	T2 – Tumor size between 2 – 4 cm N0 – No regional lymph node metastases M0 – No distant metastasis
IIA	T3 – Tumor size > 4 cm N0 – No regional lymph node metastases M0 – No distant metastasis
IIB	T1 – Tumor size ≤ 2 cm or T2 - Tumor size between 2 – 4 cm or T3 – Tumor size > 4 cm N1 – Metastasis in one to three regional lymph nodes M0 – No distant metastasis
III	T1 – Tumor size ≤ 2 cm or T2 - Tumor size between 2 – 4 cm or T3 – Tumor size > 4 cm N2 – Metastasis in four or more regional lymph nodes M0 – No distant metastasis **OR** T4 – Tumor involves celiac axis, superior mesenteric artery and/or hepatic artery Any N M0 – No distant metastasis
IV	Any T Any N M1 – Distant metastasis

1.2.4 Molecular alterations in PDAC

The most frequent genetic alteration linked to PDAC progression is point activating mutations in *KRAS* gene[25,37]. This genetic change can also be found expressed in both LG and HG PanINs and in approximately 95% of PDACs. The inactivation of *p53,* a tumor suppressor gene, is also found in HG PanINs and in 75% of PDACs[37–39]. However, PDAC is also associated with other genetic alterations in *p16, BRCA2* genes and in transforming growth factor (TGF)-β pathway components[38]. Additionally, altered glycosylation patterns are also associated with PDAC, including altered mucin expression[40].

1.3 Glycosylation

Glycosylation is an enzymatic process that generates carbohydrate associated post-translational modifications where carbohydrates (glycans) are commonly attached to proteins (glycoproteins) or lipids (glycolipids)[41–43]. These modifications can occur in almost all known mammalian proteins and comprehend a coordinated action of a group of enzymes such as glycosyltransferases and glycosidases, organelles, and other factors[43]. This post-translational modification is crucial in many biological processes, such as protein synthesis and degradation, immune responses, cell-cell interactions and signal transduction[42].

In protein glycosylation, this linkage is made in a predetermined way in certain amino acids. Depending on the linkage site, protein glycosylation can be classified into these two main types: N-glycosylation or O-glycosylation (**Figure 2**)[43].

N-glycosylation occurs in two phases and in two compartments, the endoplasmic reticulum (ER) and the Golgi complex[44,45]. The first phase is the linkage of an N-linked glycan to an asparagine (Asn) residue, through a nitrogen atom, of the polypeptide chain in the ER[44,45]. In the second phase, the polypeptide is transported to the Golgi complex, where trimming, processing, and maturation occurs[45].

The O-glycosylation is the attachment of glycans to proteins through an oxygen atom of a hydroxyl group of a serine (Ser) or threonine (Thr) residue of the polypeptide chain. The most common type of O-glycosylation is the mucin-type O-glycosylation, that takes place at the Golgi complex[42,46,47]. This mechanism is mediated by glycosyltransferases, where each transfers a monosaccharide from a donor nucleotide-sugar[47,48]. Therefore, both competition for the same substrates and location of these distinct enzymes influence the O-glycosylation patterns expressed in each cell[49].

The first step is the transfer of a single monosaccharide, *N*-acetylgalactosamine (GalNAc) from uridine 5'-diphospho-GalNAc, by the action of GalNAc transferases (GALNTs) to Ser/Thr

residues in the protein. This structure forms the core that can be extended to form more complex and elongated structures[42,46,47].

Both N-glycosylation and O-glycosylation mechanisms can be further modified by sialylation or fucosylation among other modifications[50].

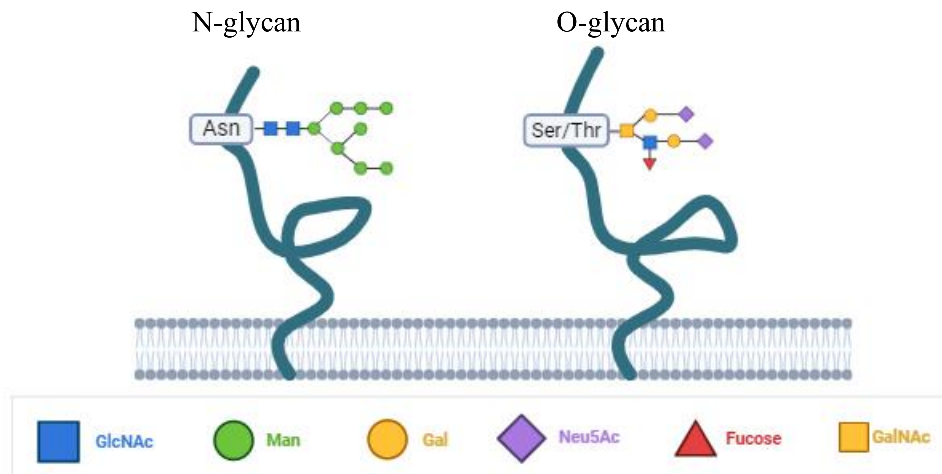


Figure 2 - N-glycans vs O-glycans. N-glycans are linked via N-linkage to Asn (left) and the O-glycans are linked via O-linkage to Ser or Thr (right). *N*-acetylglucosamine (GlcNAc), mannose (Man), galactose (Gal), *N*-acetylgalactosamine (GalNAc). Created by BioRender.

1.3.1 Mucin-type O-glycosylation

Mucin-type O-glycosylation is important to regulate protein stability, being also essential for cell and tissue growth, development and differentiation[42].

As previously mentioned, the Ser/Thr-GalNAc structure is the simplest mucin O-glycan. This antigenic structure is also known as Thomsen-nouveau (Tn) antigen, and it can be elongated into different core structures, being the most common the core 1 O-GalNAc glycan that is found in many mucins[47]. This structure, which forms the core of many other elongated forms, is also antigenic and known as Thomsen–Friedenreich (TF or T) antigen[47].

Tn antigen is converted to T antigen by the addition of Gal via the action of the enzyme T synthase or core 1 β1,3-galactosyltransferase (C1GalT1); however, it requires a specific chaperone known as C1GalT1 Specific Molecular Chaperone 1 (COSMC) to be fully active in the Golgi[42]. Another way of extension, but less common, of the Tn antigen is through the core 3 β1,3-N-acetylglucosamine transferase (C3GnT) that forms the core 3 structure. These core structures, as explained above, can be further elongated and capped by different terminal residues, often sialic acids (Sias) residues by the action of sialyltransferases (STs)[42,51]. The

premature addition of Sia to the Tn antigen can lead to the formation of its sialylated form (**Figure 3**), as we will further elucidate.

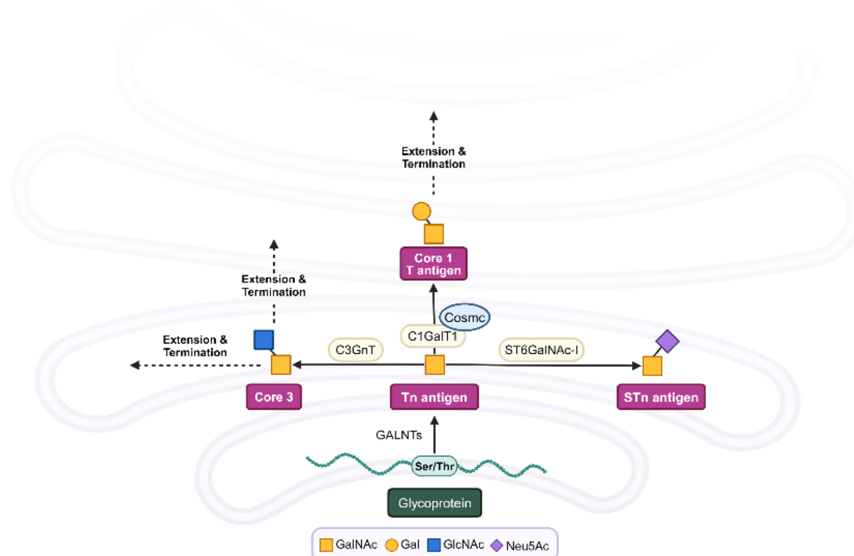


Figure 3 - Biosynthesis of mucin-type O-glycans. Their biosynthesis is a sequential process that takes place in the Golgi apparatus. The process is initiated by the addition of GalNAc via GALNTs to the oxygen atom of the hydroxyl group of Ser/Thr, resulting in the formation of the Tn antigen. The Tn antigen can be further extended by the addition of Gal via C1GalT1 facilitated by the chaperone COSMC or by the addition of GlcNAc via C3GnT. Both core 1 and 3 can undergo further extension. Aberrations in the O-glycans synthesis commonly lead to truncated structures. The premature addition of Sia to the Tn antigen, mainly due to overexpression of ST6GalNAc I, results in the formation of Sialyl-Tn (STn) antigen. Glycan structures are represented according to the Symbol Nomenclature for Glycans (SNFG) format (https://www.ncbi.nlm.nih.gov/glycans/snfg.html). The catalytic activity of each enzymatic step is represented by arrows. Created by BioRender.

There are many glycoproteins bearing mucin-type O-glycans[42,50]. One example of that are mucins which represent the class of glycoproteins most heavily O-glycosylated, once they possess a high proportion of threonine and serine residues[47,50].

Mucins are produced by epithelial cells and can be secreted or membrane-bound. They cover the epithelial surfaces like the gastrointestinal, genitourinary, and respiratory tracts where they protect against external stress and infections and play an important role in self-recognition by the immune system[47,50]. The secreted mucin family is composed by MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC12 and MUC19 while the membrane-bound family comprises MUC1, MUC3A, MUC3B, MUC4, MUCH-13, MUC15-17, MUC20, MUC21, and MUC22[42]. The mucin type O-glycans are known to control the chemical, physical, and biological properties of mucins. They promote the binding of water and salts since O-GalNac

glycans are hydrophilic and normally negatively charged contributing to the viscosity and adhesiveness of mucus[47].

1.3.2 Sias and its role in immunomodulation

Sias are a nonulosonic acid subclass of nine-carbon backbone monosaccharides[51,52]. In humans, *N*-acetylneuraminic acid (Neu5Ac) is the most abundant form of Sias[51]. Usually, they are the terminal residue of glycoconjugates (glycoproteins and glycolipids) at the cell surface or secreted glycoproteins[52,53]. The process by which Sias are added via STs is designated sialylation[47,54].

Therefore, Sias play numerous roles in physiological and pathological mechanisms via interactions of carbohydrate-protein, such as cellular communication and recognition, modulation of the immune system, pathogen infection mediation, and tumor growth[53].

As previously described, the immune system is in constant surveillance mode, recognizing and distinguishing "self" from "non-self" antigens. Given the location of Sias in the membrane surface as a terminal residue of glycoproteins, it is possible to understand that it might be recognized by the immune system as a "self" antigen[52,55,56]. The immune cells recognize Sias by lectins such as the sia binding immunoglobulins (Ig)-like lectins (Siglecs)[50,52]. These regulate the antigen-specific immune responses[50]. Therefore, after binding to Sias residues, Siglecs mainly negatively regulate the immune response[52]. Thus, when cancer cells alter their glycosylation profile with the accumulation of Sias, they are able to escape the immune response[54,57].

1.4 Glycosylation in cancer

Changes in the glycosylation pattern provide cancer cells the ability to take over various developmental processes, such as receptor activation, cell adhesion, angiogenesis and invasion, making it possible for them to infiltrate and disseminate[57].There are several factors that can lead to alterations in the normal glycosylation pathways in cancer cells. The location of glycosyltransferases in the Golgi, their under- or overexpression due to transcription dysregulation, altered chaperone function or altered function of glycosidases, are some of these factors. Other factors can be alterations in the tertiary conformation of the peptide backbone and/or the availability and abundance of the sugar nucleotide donors and cofactors[58].

One of the major cancer-associated glycosylation alterations is the mucin-type O-GalNAc truncation and aberrant sialylation that can lead to truncated structures and their sialylated forms[47,58]. The abnormal sialylation leads to Sias accumulation on the cell surface contributing to the suppression of the immune system, as mentioned[54,57].

Moreover, the immature truncated mucin-type O-glycans, derived from these mechanisms, can directly induce increased proliferation and invasive growth promoting the development of metastasis[49,54,59].

One of the most frequently observed truncated mucin-type O-glycan, in cancer, is the sialyl-Tn (STn)[59].

1.4.1 STn antigen

The STn is a carbohydrate antigen that results from premature sialylation of the Tn antigen. STn is a disaccharide formed by a residue of GalNAc and Neu5Ac linked to the carbon 6 of the GalNAc (**Figure 4**). As mentioned, this aberrant sialylation prevents the elongation typically found in mucin-type O-glycans[49,60].

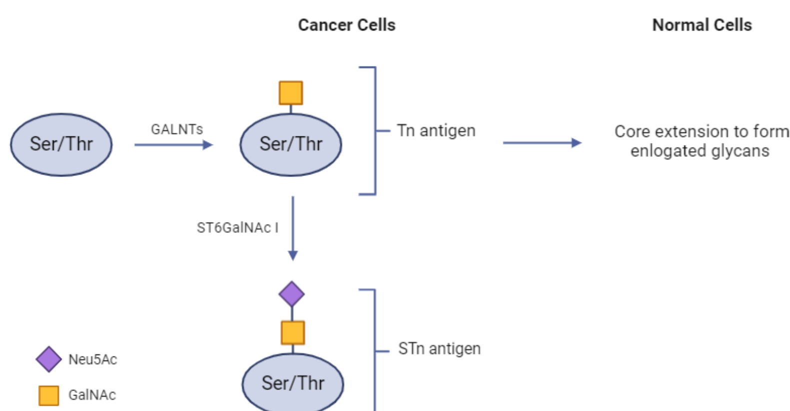


Figure 4 - STn biosynthesis. The process is initiated by the addition of GalNAc via GALNTs to the oxygen atom of the hydroxyl group of Ser/Thr, resulting in the formation of the Tn antigen. Then, the Tn antigen can be further elongated. However, in cancer cells, this process is prematurely stopped by the addition of Neu5Ac via ST6GaLNAc I enzyme leading to the formation of STn antigen. Created by BioRender, adapted from[49].

The STn antigen biosynthesis is mediated by the α-N-acetylgalactosaminide α-2,6-sialyltransferase (ST6GalNAc) I. Therefore, one of the main reasons for the STn expression found in cancer is the overexpression of this enzyme in conjugation with increased synthesis of precursors and a decrease in competitors[49,60,61]. In some cases, hypermethylation, mutations and loss of heterozygosity in the chaperone *COSMC* gene can also lead to STn expression in cancer[59,62]. However, there are other factors that might trigger STn expression. Hypoxia and deregulation of cell metabolism adaptation by cancer cells occurs mainly through hypoxia-inducible factor-1α (HIF-1α), which affects glycosylation[63]. In fact, it has been reported that the expression of some enzymes involved in the first stages of O-glycosylation is altered by hypoxia and HIF-1α seems to favor simple mucin-type sialylated O-glycans expression, including STn[64]. Inflammation resulting from tumor microenvironment also alters glycosylation

in cancer cells[63]. Additionally, mutations in *p53* tumor suppressor gene increased ST6GAlNac I expression in lung cancer cells[65]. The oncogene *ras* also increases sialylation by regulating the sialyltransferase ST6Gal1[66]. But it remains elusive if it affects the expression of other sialyltransferases.

The STn expression in normal tissues is found to be rare or low whereas in cancer tissues it is expressed in more than 80% of human epithelial cancers like colorectal, ovarian and PC, being associated with poor prognosis[60,67].

PDAC is one of the epithelial cancers that is known to overexpress truncated O-glycans, and almost 40% of the 46 PDACs cases correlated with hypermethylation of the *COSMC* gene that was also correlated with loss of C1GalT1 enzyme expression and truncated O-glycans expression[59]. In terms of STn expression, a previous study reported that normal pancreas did not express STn antigen whereas 97% of 30 PC tissues expressed STn, being cytoplasmic expression predominant[68]. In addition, other study also reported absent STn expression in normal pancreas while 67% of 45 primary PDAC were positive together with 71% of 42 liver metastases[69]. Regarding the precursor lesions of PDAC, in some, Tn antigens can be detected but mainly in intracellular compartments, whereas STn seems to appear in more late events of the disease development[69,70]. Therefore, these truncated O-glycans are commonly associated with tumor progression and invasion[59,70,71].

For these reasons, STn was thought to be an excellent tumor marker that might be helpful in diagnosis, prognosis but also as a therapeutic target[49,60].

1.4.1.1 STn immunosuppression driving carcinogenesis

As previously mentioned, glycans play an important role in the modulation of the immune response. One of the major ways of modulating the immune system is by modulating DCs function. While DCs mature when they recognize antigens, process and present them to T cells, its maturation depends on the proper cytokine milieu and signaling pathways[72]. If not properly matured, DCs can induce immune tolerance often identified in the tumor microenvironment[73].

In fact, in bladder cancer, a correlation was found between the overexpression of STn and an immature phenotype of DCs and lower levels of pro-inflammatory cytokines, such as IL-12 and TNF-α[73]. In addition, mucins carrying STn can also inhibit the activity of NK cells[74]. Moreover, STn overexpression was significantly associated with low CD8+ cell infiltration, mainly due to the induction of cyclo-oxygenase-2 enzyme expression. This enzyme is responsible for inflammation processes and has pathological significance in several cancers[75].

In conclusion, STn seems to be associated with immunosuppression suggesting that tumors expressing STn might respond to immunotherapies.

1.4.1.2 STn prognostic value in cancer

Several studies in different types of cancer have reported an association between STn and poor prognosis, through models with altered STn biosynthetic enzymes.

In fact, ST6GalNAc I overexpression, in colorectal cancer, enhances cancer stem phenotypes and in lung cancers, cell migration and metastasis are promoted by MUC5AC decorated with STn[65,76]. Additionally, studies in breast and gastric cancer cell lines confirmed that ST6GalNAc I overexpression increases STn expression being associated with tumor growth and invasiveness[77,78]. Regarding PDAC, *C1GalT1* knockout human cell lines showed increased growth, migration, tumorigenicity and metastasis due to O-glycosylation truncation and subsequent STn expression[79]. *In vitro* studies showed that *COSMC* knockout cell lines, overexpressing Tn/STn antigens have a more invasive phenotype promoting a metastatic tumor behavior[59]. Thus, the consensus link between STn and decreased survival is made conceivable by its suggested relationship with tumor growth and invasiveness. However, this might not be transversal across all types of cancer[60]. Therefore, it is important to analyze what has already been studied about the impact of STn on survival in a wide set of different types of cancers.

1.5 Introduction to the aims of this thesis

PDAC is becoming one of the leading causes of cancer-related deaths[20,21]. The diagnosis commonly happens in late stages of the disease being the treatment options limited[22,23]. Therefore, to overcome this limitation, new biomarkers that may enable early diagnosis and therapeutic targets are needed.

STn is a tumor-associated carbohydrate antigen that is found to be expressed in 80% of human epithelial cancers, including PDAC, being therefore proven to be an excellent target[60,68,69,]. This antigen is also associated with poor prognosis contributing to cancer progression and invasion[60]. However, the STn profile confirmation, in a meaningful cohort in different PDAC development stages, and its impact on survival remains to be clarified.

We aim to better elucidate these questions regarding the STn profile and association with PDAC progression and to gather information on what is known about STn's impact on survival. Therefore, the main objectives of this thesis, which is integrated into the InnO-Glyco project, are focused on 1) Evaluating STn expression in different PDAC development stages, including precursor lesions, untreated and post-chemotherapy (post-CT) PDACs and metastases, from a cohort of 185 tissue samples from Champalimaud Foundation. 2) Validating an *in vitro* model of PDAC that overexpresses STn to enable future experiments on understanding the underlying molecular mechanisms of the role of STn in PDAC. 3) Revising literature regarding what has been studied about the impact of STn on survival in different cancer types.

2. Materials and Methods

2.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate, 10xPhosphate-buffered saline (PBS) solution and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer 1M were obtained from Corning® (NY, USA). 1xRoswell Park Memorial Institute (RPMI) medium 1640 with L-glutamine, trypan blue stain solution (0.4%), 1xtriple select, and pen-strep solution (Penicillin 10,000 units mL-1 and Streptomycin 10,000 µg mL-1) were obtained from Gibco™ (Grand Island, NY, USA). Heat inactivated fetal bovine serum (FBS) was obtained from Biowest (Nuaillé, France). Normal Horse Serum (HS) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) Peroxidase Substrate Kit were purchased from Vector Laboratories (Newark, CA, USA). 10xAntigen retrieval solution pH 6 was obtained from Leica biosystems (Nussloch, Germany). Hydrogen peroxide 35 w% (H_2O_2), xylene, goat anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody and Pierce™ BCA Protein assay kit were obtained from Thermo Fisher Scientific (Watlham, MA, USA). RIPA buffer was obtained from Abcam (Cambridge, UK). 10xTris/Glycine/ Sodium dodecyl sulfate (SDS) Buffer and 4xLaemmli loading buffer were obtained from BioRad (Hercules, CA, USA). The transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol), Paraformaldehyde (PFA) 4%, Ethylenediaminetetraacetic acid (EDTA) and 10xTris-buffered saline (TBS) solution were provided by the Glass wash and media preparation platform from Champalimaud Foundation. Tween20 and DL-Dithiothreitol (DTT) solution were obtained from Sigma-Aldrich (Merck, Darmstadt, Germany). DNase I 10 mg/mL was purchased from Merck (Darmstadt, Germany). IRDye 800CW and 680RD conjugated secondary antibody goat anti-mouse IgG and LI-COR blocking buffer were purchased from LI-COR Biosciences (Lincoln, NE, USA). Fluorescein isothiocyanate (FITC)-conjugated secondary antibody goat anti-mouse IgG was obtained from Southern Biotech (Birmingham, AL, USA). The monoclonal mouse anti-GADPH IgG antibody was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Zombie NIR Fixable Viability Kit was obtained from BioLegend (San Diego, CA, USA). The monoclonal primary mouse anti-STn antibody was the IgG2 version of L2A5 clone from Loureiro et al[80].

2.2 Ethics Statement

This project has the approval of the ethics committee of Champalimaud Foundation for the use of formalin-fixed paraffin-embeded (FFPE) archival blocks. Tissue samples were collected from the archives of the Service of Anatomy Pathology of Champalimaud Foundation, classified by a pathologist and the presence of consent signed by the patient was verified. All the human samples and their respective clinical data were received after being pseudonymized through the Champalimaud Foundation Biobank. Clinical information from the patient, such as age at the time of the diagnosis; gender and tumor stage were also obtained in a pseudonymized manner for clinicopathological correlations.

2.3 Immunohistochemistry (IHC)

For IHC analysis, FFPE blocks of a cohort of 185 tissues samples from 177 patients, were sectioned in 4 µm slides to evaluate STn expression. From the 185 slides, 207 different lesions were identified and individually evaluated.

Therefore, the cohort included precursor lesions of PDAC with different dysplasia grades, more specifically PanINs and IPMNs, untreated PDACs, post-CT PDACs and metastases in different organs, namely in lung, liver, peritoneum and diaphragm (**Table 2**).

Table 2 - Number of cases from the cohort of each PDAC development stage.

<table>
<tr><td rowspan="2">PanINs</td><td>LG</td><td colspan="3">3</td></tr>
<tr><td>HG</td><td colspan="3">1</td></tr>
<tr><td rowspan="8">IPMNs</td><td rowspan="4">LG</td><td rowspan="4">37</td><td>Gastric-type</td><td>16</td></tr>
<tr><td>Intestinal-type</td><td>13</td></tr>
<tr><td>Pancreato-biliar-type</td><td>8</td></tr>
<tr><td>Oncocytic-type</td><td>0</td></tr>
<tr><td rowspan="4">HG</td><td rowspan="4">19</td><td>Gastric-type</td><td>4</td></tr>
<tr><td>Intestinal-type</td><td>4</td></tr>
<tr><td>Pancreato-biliar-type</td><td>10</td></tr>
<tr><td>Oncocytic-type</td><td>1</td></tr>
<tr><td colspan="2">PDACs</td><td colspan="3">93</td></tr>
<tr><td colspan="2">post-CT PDACs</td><td colspan="3">41</td></tr>
<tr><td rowspan="4">Metastases</td><td>Liver</td><td rowspan="4">16</td><td colspan="2">5</td></tr>
<tr><td>Lung</td><td colspan="2">4</td></tr>
<tr><td>Peritoneum</td><td colspan="2">6</td></tr>
<tr><td>Diaphragm</td><td colspan="2">1</td></tr>
</table>

The IHC technique used is based in the antibody-antigen binding visualization by using a secondary antibody conjugated to an enzyme, in this case to a HRP, and a colored chromogen

such as DAB. This chromogen produces an insoluble brown precipitate at sites of peroxidase activity[81].

To remove the paraffin from the sections, a deparaffinization protocol was conducted. In this procedure the slides were placed in xylene and then hydrated in a series of graded alcohols: 100%, 95% and 70% ethanol. After this step, the slides were placed in dH_2O and washed in 1xPBS. For blocking endogenous peroxidase enzyme activity, slides were incubated with 1xPBS: H_2O_2 in a proportion of 25:1 for 10 minutes.

The following step was the antigen unmasking, where slides were placed in antigen retrieval solution 1x (pH 6) for 30 minutes using a steamer to boil. Then, slides were washed in 1xPBS, and we proceeded to the blockage of unspecific bounds with 10% HS for 30 minutes. Next, slides were incubated with the correspondent primary antibody diluted in 10% HS: anti-STn mouse antibody (1:1000) overnight at 4ºC.

On the next day, the slides were washed with 1xPBS and incubated with the secondary antibody goat anti-mouse IgG HRP conjugated (1:400) diluted in 10% HS for 1 hour at room temperature (RT). Then, the slides were washed again with 1xPBS and exposed to DAB Peroxidase Substrate Kit for 10 minutes. The counterstaining was made using hematoxylin staining. The slides were then dehydrated using a series of graded alcohols: 70%, 95% and 100% ethanol and xylene. Finally, the slides were mounted with mounting medium and scanned with Philips Ultra-Fast Scanner 1.6 (Philips, Amsterdam, NL).

For positive control, HG bladder cancer tissues, that were known to overexpress STn, were, used.

2.3.1 STn expression quantification

The STn expression quantification was made using the H-score, taking into consideration only the luminal and cytoplasmic staining intensity, within a range of 0-3 categories (being 0 no staining and 3 the strong intensity), excluding the expression in the secreted mucus. In each intensity category a percentage of tumor that presents that staining was given.

Then, the H-score is calculated by the following equation, giving a result within a range of 0-300:

H-score = (% of negative tumor for the staining x 0) + (% of positively stained tumor luminal and cytoplasmic at weak intensity category x 1) + (% of positively stained tumor luminal and cytoplasmic at intermediate intensity category x 2) + (% of positively stained tumor luminal and cytoplasmic at strong intensity category x 3)

This evaluation was made by Ana Soares and the pathologist Dr. Mireia Castillo (Champalimaud Foundation). Every time that there was a disagreement, the slides were reviewed, and an agreement was reached.

2.3.2 Clinicopathological Data

The clinicopathological data from the patients included, in this study, characteristics such as the mean and range of age at the time of the diagnosis, gender as well as the AJCC staging group of the untreated and post-CT PDACs cases (**Table 3**).

Table 3 - Clinicopathological data from the 177 patients whose tissue samples were analyzed. This table contains the mean and range of the age at the time of diagnosis and gender. It also contains the stage of the disease from untreated and post-CT PDACs. NA stands for not assessed.

Total patients	177
Age at time of diagnosis (years)	
Mean	67.3
Range	37 - 90
Gender	
Female	92 (51.98%)
Male	85 (48.02%)
AJCC staging group (pTNM)	
Untreated PDACs	93
IA	7 (7.53%)
IB	15 (16.13%)
IIA	3 (3.23%)
IIB	36 (38.71%)
III	32 (34.41%)
IV	0 (0.00%)
AJCC staging group (ypTNM)	
Post-CT PDACs	41
0	2 (4.88%)
IA	3 (7.32%)
IB	4 (9.76%)
IIA	1 (2.44%)
IIB	12 (29.27%)
III	4 (9.76%)
IV	9 (21.95%)
NA	6 (14.63%)

2.4 Cell culture

The cell lines used were PANC-1 human and Pan02 mouse cell lines, that are *in vitro* models of PDAC and were initially kindly provided by Dr. Bruno Costa Silva (Champalimaud Foundation) and then transduced with the sialyltransferase *ST6GALNAC I* gene, to overexpress STn, as described[82] (by Molecular and Transgenic Tools (MTT) Platform Champalimaud Research). The cells overexpressing STn were named PANC-1 STn+ and Pan02 STn+. To determine any non-specific effect caused by this transduction process, a control of mock transfected PANC-1 wild-type (WT) cells (PANC-1 Mock) and Pan02 WT cells (Pan02 Mock) were used.

PANC-1 cell lines were cultured in DMEM 4.5 g/L glucose with L-glutamine and sodium pyruvate, supplemented with 10% FBS and 1% PenStrep. Pan02 cell lines were cultured with complete RPMI medium supplemented with 10% FBS and 1% PenStrep. Both cell lines were incubated at 37ºC, 5% CO_2 in T175 culture flasks (Thermo Fisher Scientific).

The cells were passed when verified a confluency of 80%, being detached with 1xtriple select for 5 minutes at 37ºC and were then centrifuged for 5 minutes at 330 *g* (Sorvall, ST40R centrifuge, Thermo Fisher Scientific). The respective pellets were resuspended or used for further experiments, and the supernatant used to access mycoplasma test. The cell culture and following experiments were made by Ana Soares with contribution from Catarina Pereira.

2.5 Western Blot

Western Blot is a technique often used to separate and identify proteins, in this case in cell lysates[83].

Cell lysates were prepared, from the cell lines' pellets mentioned above, using RIPA buffer + 1x cocktail of inhibitors (protease and phosphatase inhibitors) for 30 minutes at 4ºC with constant agitation. Then, the lysates were centrifuged for 20 minutes at 13800 *g* (Sorvall Legend Micro 21R, Thermo Fisher Scientific), and the supernatant stored for protein quantification. Protein quantification was measured by the BCA protein colorimetric assay.

The protein samples, after the quantification, were prepared with 4xLaemmli loading buffer with DTT to load 47 μg of protein. Then, the samples were boiled at 95ºC for 5 minutes. After the sample preparation, they were applied in a gel for electrophoretic protein separation (Mini-PROTEAN, BioRad). The samples run at 80V for 90 minutes in running buffer (1xTris/Glycine/SDS Buffer). After the protein separation, samples were electrotransfered at 400 mA for 1 hour at 4ºC to nitrocellulose membranes (Amersham Protran Premium 0.45 μm, cytiva, Germany), in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). Then the membranes were blocked with Li-Cor blocking buffer for 1 hour at RT with gentle shaking.

After the blocking step, the membranes were incubated with the primary antibody mouse anti-STn (1:1000) diluted in blocking buffer overnight at 4ºC. Membranes were washed three times, 5 minutes each, with Tris-buffered saline with tween (TBS-T) 0,1% solution and incubated with the IRDye 800CW conjugated secondary antibody goat anti-mouse IgG (1:5000) diluted in blocking buffer for 1 hour at RT, protected from the light. Finally, the membranes are washed again with TBS-T 0,1% and with a final wash of 1xTBS, to remove the residual Tween20, and scanned in Odyssey (LI-COR Biosciences, Lincoln, NE, USA).

GAPDH was used as housekeeping protein, by incubating membranes with mouse anti-GAPDH antibody (1:1000) overnight at 4ºC, followed by the IRDye 680RD conjugated secondary antibody goat anti-mouse IgG (1:5000) for 1 hour at RT.

2.6 Cell surface staining

5 x 10^5 PANC-1 STn+ and PANC-1 Mock cells were collected per condition: unstained, only secondary antibody and stained (anti-STn antibody + secondary antibody). Before cell staining, a viability dye protocol was conducted using Zombie NIR Fixable Viability Kit to assess live vs. dead status of cells. Zombie NIR is a non-permeant to live cells amine reactive fluorescent dye. The Zombie NIR dye, previously reconstituted, was diluted in 1xPBS at 1:2000. Then, cells were resuspended in Zombie NIR solution and incubated for 20 minutes at RT in the dark. Afterwards, cells were washed one time with FACS buffer (2% FBS, 4% EDTA, 5% HEPES buffer 1M and 1% DNaseI 10mg/mL), and the antibody staining procedure was performed protected from light.

Cells were washed three times with 1xPBS and centrifuged at 13800 *g* for 5 minutes at 4ºC (Sorvall Legend Micro 21R, Thermo Fisher Scientific). Then, for the stained condition, the cell pellets were incubated with the primary mouse anti-STn antibody (1:300) for 30 minutes at 4ºC. The cells collected for the unstained and only secondary condition were incubated with 1xPBS instead.

Afterwards, cells were washed again with 1xPBS and centrifuged, under the same conditions, and incubated with the FITC-conjugated secondary antibody goat anti-mouse IgG (1:400) for 20 minutes at 4ºC. The cells from the unstained condition were incubated again with 1xPBS.

After the incubation, cells were washed with 1xPBS and fixed with 4% PFA for flow cytometry analysis.

2.7 Flow cytometry analysis

Flow cytometry is a technique used to count and/or distinguished cells from different populations[84], being also used to analyze the expression of cell surface molecules, such as STn.

At least 1 x 10^4 events, for each sample, were acquired using LSRFortessa X-20 Cytometer. (BD-GenCell Biosystems, Limerick, Ireland). Data was analyzed, using FlowJo software version 10.0.5 (TreeStar, San Carlos, CA, USA), after cell gating and doublet exclusion to ensure single cells were counted (**Figure 5**).

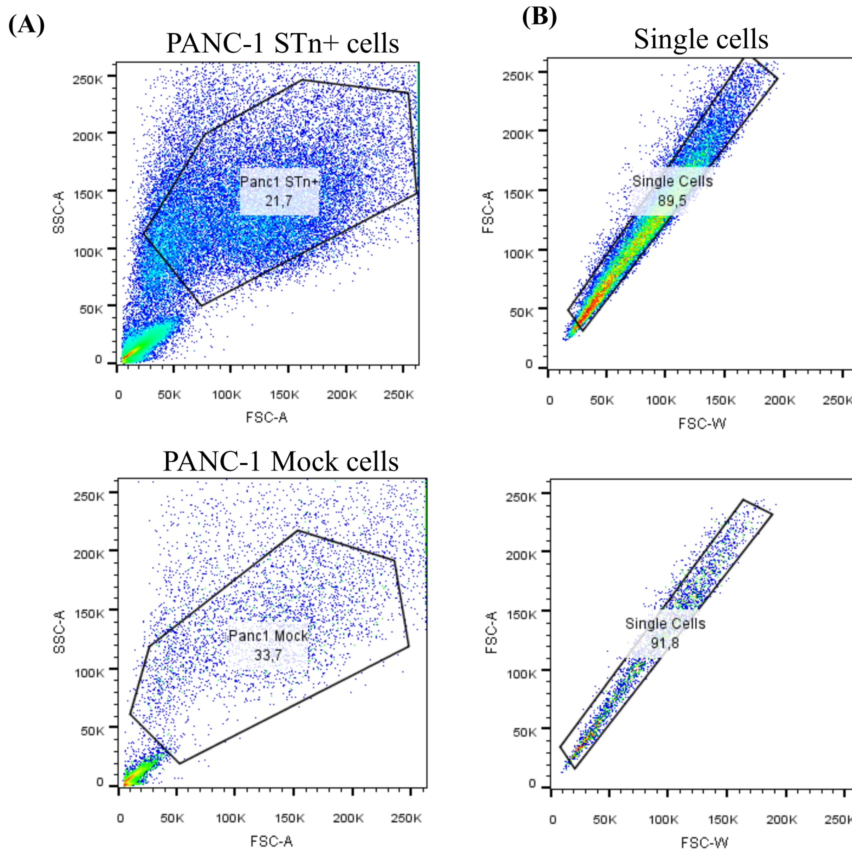


Figure 5 - PANC-1 STn+ and Mock cell lines population gating strategy. (A) Viable PANC-1 STn+ and Mock cells were gated from debris. **(B)** Single cells gating for doublet exclusion.

2.8 Literature Search

2.8.1 Data collection, selection and analysis

A comprehensive analysis was conducted to determine the survival rate of STn in several cancer types using the PubMed/MEDLINE database. The search strategy included a combination of established keywords which resulted in the query: ("sialyl-Tn" OR "sialosyl-Tn" OR "sialyl Tn" OR "sialosyl Tn" OR "Sialyl Thomsen nouveau" OR "Sialyl Thomsen-nouveau" OR "Sialyl-Thomsen nouveau" OR "Sialyl-Thomsen-nouveau" OR "STn") AND ("Cancer" OR "Tumor" OR "Tumour") AND "Survival". Articles were firstly screened for the titles and abstracts to select potential studies for further assessment of eligibility. The PRISMA flow diagram (**Figure 6**) schematizes the study selection process, including the number of articles excluded, the

exclusion criteria, and the number of articles included. The literature search was performed by Ana Soares, Rita Lourenço and Miguel Dias.

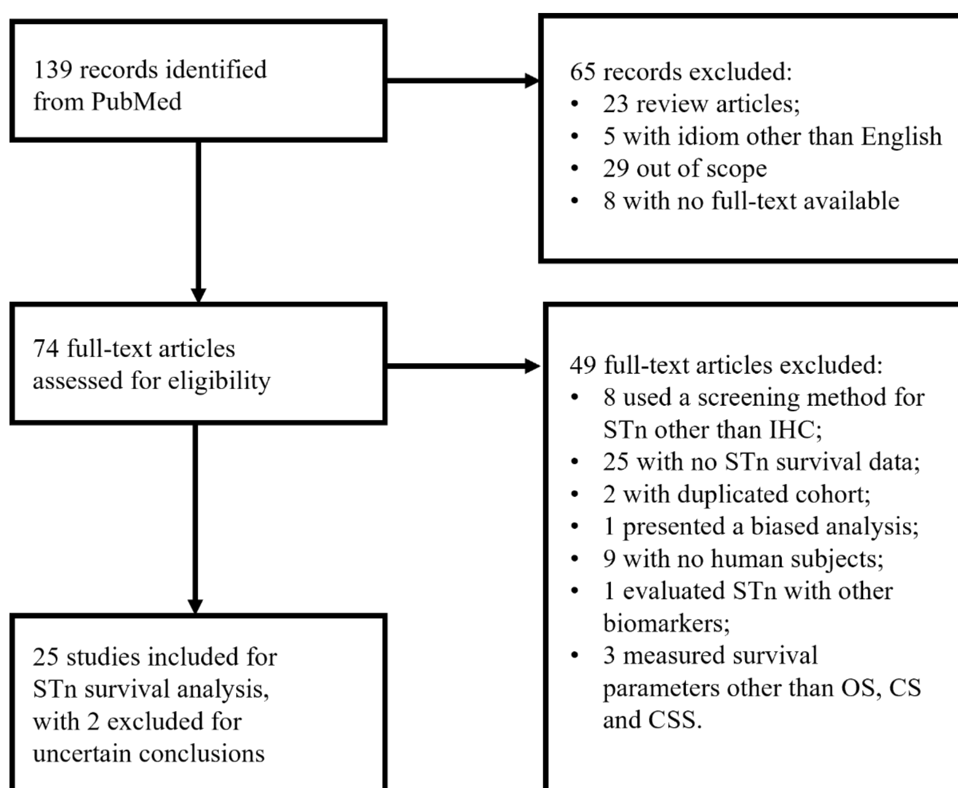


Figure 6 - PRISMA flow diagram illustrating the study selection process for the literature search on overall survival (OS), cumulative survival (CS), and cancer-specific survival (CSS) associated with STn expression. Schematic illustration of the methodology used from the article selection from the database search until the inclusion of the final articles, including the number of articles excluded and exclusion criteria.

Data pertaining to the cancer type, number of positive and high STn expression (STn+, for simplification) cases and low and negative STn expression (STn-, for simplification) cases, and 5-year survival for each subpopulation were extracted from each article. When necessary, survival data from Kaplan-Meier plots were extracted using GetData Graph Digitizer 2.26.

To better validate the articles' classification according to the impact of STn, an odds ratio (OR) was calculated for each one. OR was represented by the expected number of deaths and survivals, among STn+ and STn- cases, assessed and weighted among the total cases in each study. These expected numbers were calculated considering the total cases and the probability of a patient surviving after 5 years extrapolated from the survival analysis. An OR greater than 1 would lean towards an association between STn and "death" as a direct outcome, implying a

poorer survival in cancer, given the presence of STn in that study. If the study reported no deaths or survivals for one of the subpopulations, a value of 0.5 was added to all the numbers of that study, allowing the OR calculation.

2.9 Statistical Analysis

The data analysis of STn expression by IHC and the OR representation was performed using GraphPad Prism 8.0.2 (GraphPad Software Inc, San Diego, CA, USA). The STn expression by IHC results were represented using median with 95% confidence interval (CI). Mann-Whitney test was applied to compare two independent groups to check if there was a significant difference. Kruskal-Wallis test was applied to check if there were significant difference between more than two independent groups. This test was followed by Dunn's test to check which groups were significantly different from each other. A p-value greater than 0.05 was considered statistically non-significant. A p-value < 0.05 is statistically significant (*), p-value < 0.01 (**) is very statistically significant and p-value < 0.001 (***) or p-value < 0.0001 (****) are extremely significant.

3. Results and Discussion

3.1 Establishment of STn staining intensities

For the assessment of STn expression quantification in the tissues, using the H-score, an establishment of staining intensities was made. For luminal and cytoplasmic staining, it was established what was considered as negative (0), weak intensity (+1), intermediate intensity (+2) and strong intensity (+3) (**Figure 7**).

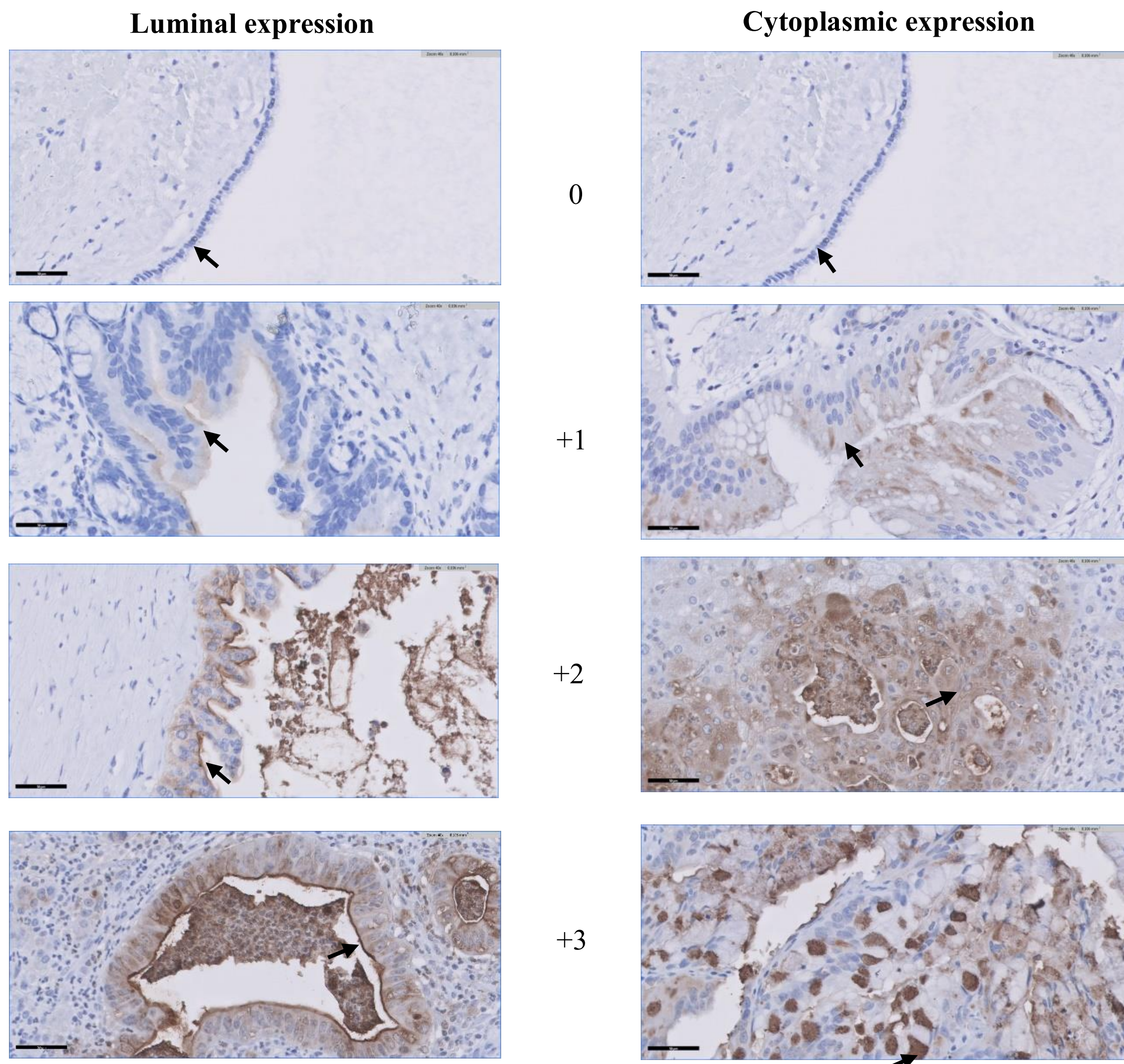


Figure 7 - Establishment of STn staining intensities for luminal and cytoplasmic expression. Representative images of stained slides to represent the different expression intensities established for negative (0), weak (+1), intermediate (+2) and strong (+3). On the left, luminal expression is represented whereas on the right, cytoplasmic expression is represented. Scale bars correspond to 50 µm.

3.2 STn expression correlation with PDAC progression in a collection of human tissue samples

To evaluate the STn expression correlation with PDAC progression, a collection of human tissue samples including the different PDAC development stages was stained.

The results showed that STn is absent in the normal pancreas (**Figure 8A** and **8B**), which corroborates the findings in other studies[68,69,85]. On the other hand, the goblet cells from the normal duodenum, when present, always stain positive for STn in the cytoplasm (**Figure 8C**), as also reported in the literature[68,85].

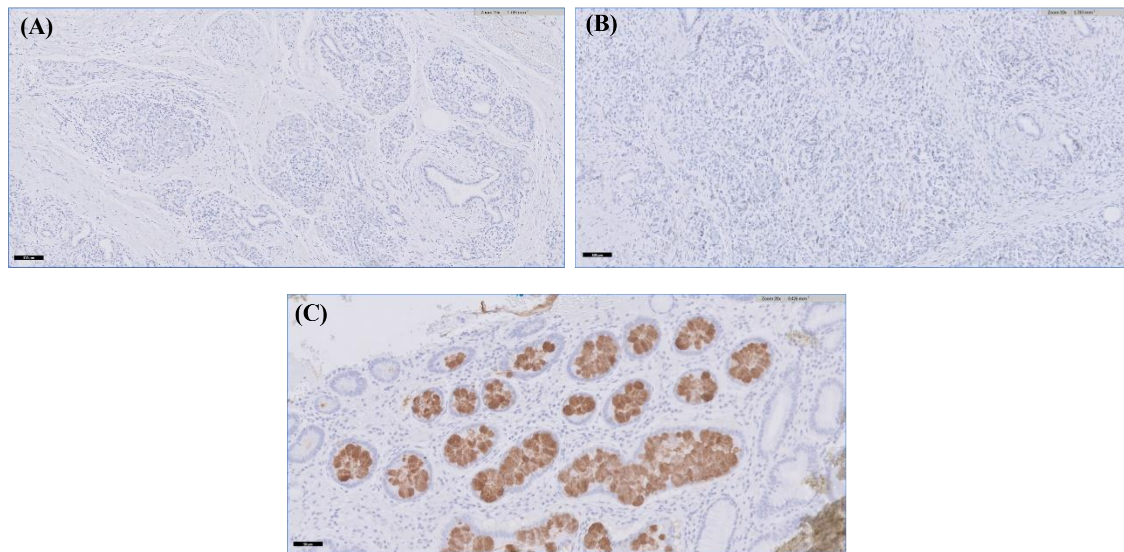


Figure 8 - STn expression in healthy pancreatic and duodenal tissues. **(A)**, **(B)** STn antigen is absent in the normal pancreas. **(C)** Goblet cells from the normal duodenum present stain positive for STn. Scale bars correspond to 50 and 100 µm.

In the stained lesions, STn was expressed in several locations, such as the cytoplasm, the luminal surface and the mucus. However, the mucus expression was excluded, since we were not interested in the secreted residues.

3.2.1 STn expression in PDAC precursor lesions

To evaluate STn expression in PDAC progression, it is important to start by studying its expression in the initial lesions. Therefore, both PanINs and IPMNs lesions were stained.

For the PanINs, a higher expression of STn in HG PanIN (PanIN-3) is observed whereas for LG PanINs (PanIN-1A and PanIN-1B) the STn expression is almost absent (**Figure 9**). No PanIN-2 lesion was included in our set. A study also reported a notable increase in STn expression in PanIN-3 cases[86]. Controversially, other study reported almost no STn expression for HG PanINs[69].

As there was only one lesion of PanIN-3 in this cohort, for further conclusions, more cases would be needed.

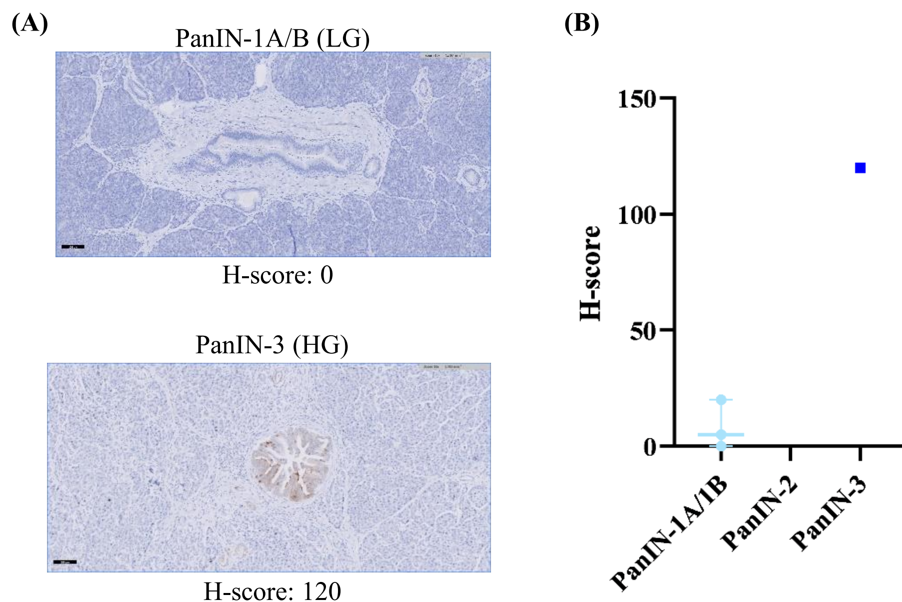


Figure 9 - STn expression in LG PanINs and HG PanIN. **(A)** Representative images of the stained slides that presented LG and HG PanINs and respective H-scores. Scale bars correspond to 100 µm. **(B)** Scatter-plot representation of the PanINs' H-score values (median with 95% CI). No statistical analysis was performed given the number of lesions identified (PanIN-3: n = 1).

Regarding the IPMNs, it is possible to observe a heterogeneity in the expression of STn between the LG and HG IPMNs with a slight increase for the last group (**Figure 10**).

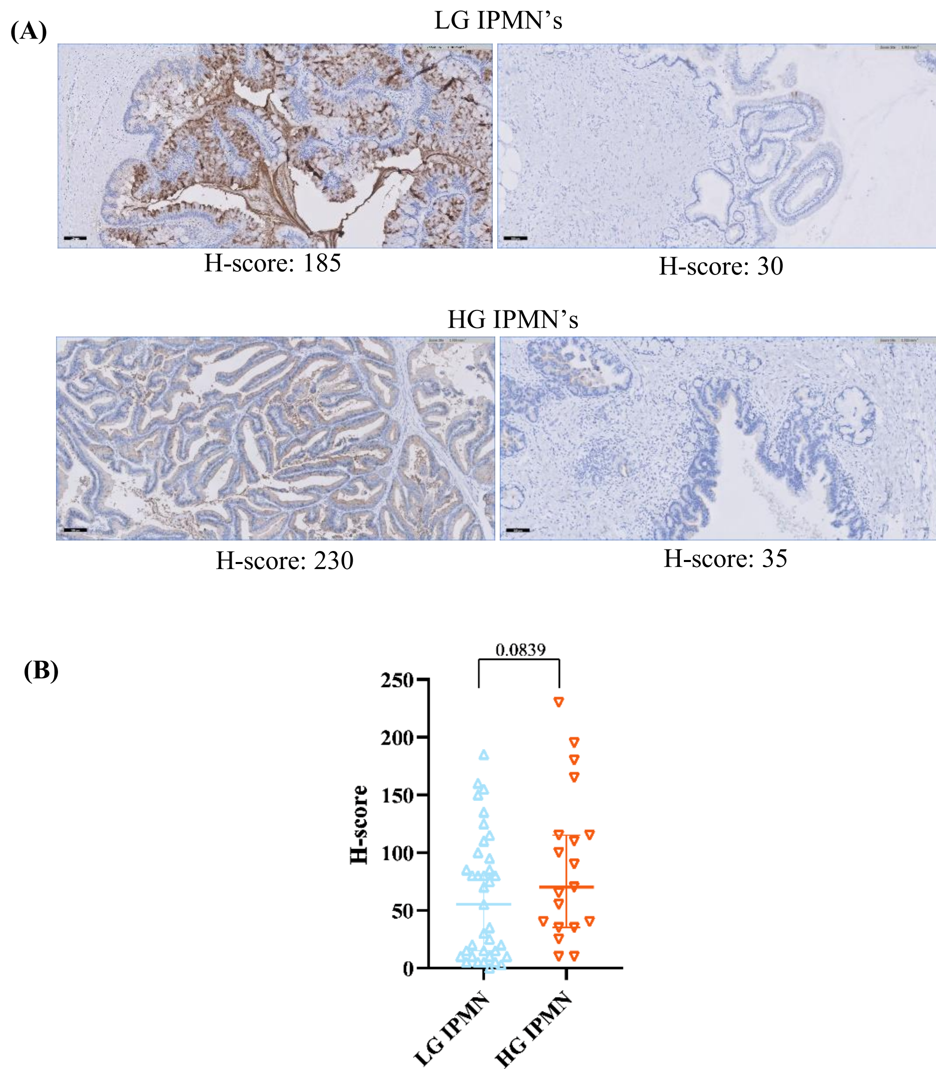


Figure 10 - STn expression in LG and HG IPMNs. (A) Representative images of the stained slides that presented LG and HG IPMNs and respective H-scores. Scale bars correspond to 100 µm. **(B)** Scatter-plot representation of the IPMNs' H-score values (median with 95% CI). P-value presented was obtained by Mann-Whitney test between LG IPMN and HG IPMN.

A study that analyzed STn expression in nine cases of IPMNs, reported 78% of the cases as positive. The expression of the positive cases was focal and showed heterogeneity in the different cases[87].

As these lesions can be classified in different types, regarding their mucin pattern, which are STn carriers, these results suggest that aberrant glycosylation might occur in the mucins[87].

Therefore, the different types were evaluated for the STn expression to verify if there was a specific type more related to STn expression. The LG IPMNs cases were mostly of the gastric subtype and the HG IPMNs mostly of the pancreato-biliar subtypes, as expected[34,88].

Most of the gastric-type IPMNs, both LG and HG dysplasia, presented low STn expression. No statistical difference was obtained between these two groups (**Figure 11**). These IPMN types, are characterized by expressing MUC5AC and MUC6[23,34].

(A) LG IPMN's

H-score: 15 H-score: 10

HG IPMN's

H-score: 35 H-score: 25

(B)

Figure 11 - STn expression in gastric-type LG and HG IPMNs. **(A)** Representative images of the stained slides that presented gastric-type LG and HG IPMNs and respective H-scores. Scale bars correspond to 100 µm. **(B)** Scatter-plot representation of the gastric-type IPMNs' H-score values (median with 95% CI).

For the intestinal-type LG and HG IPMNs, STn expression was more heterogeneous, and no significant difference was observed (**Figure 12**). However, the median of the HG IPMNs slightly increases compared to the LG. These IPMNs are characterized by the expression of MUC2 and MUC5AC[23,34]. Additionally, the presence of goblet cells is a specific characteristic of the intestinal type which, for most of them, stained positive for STn.

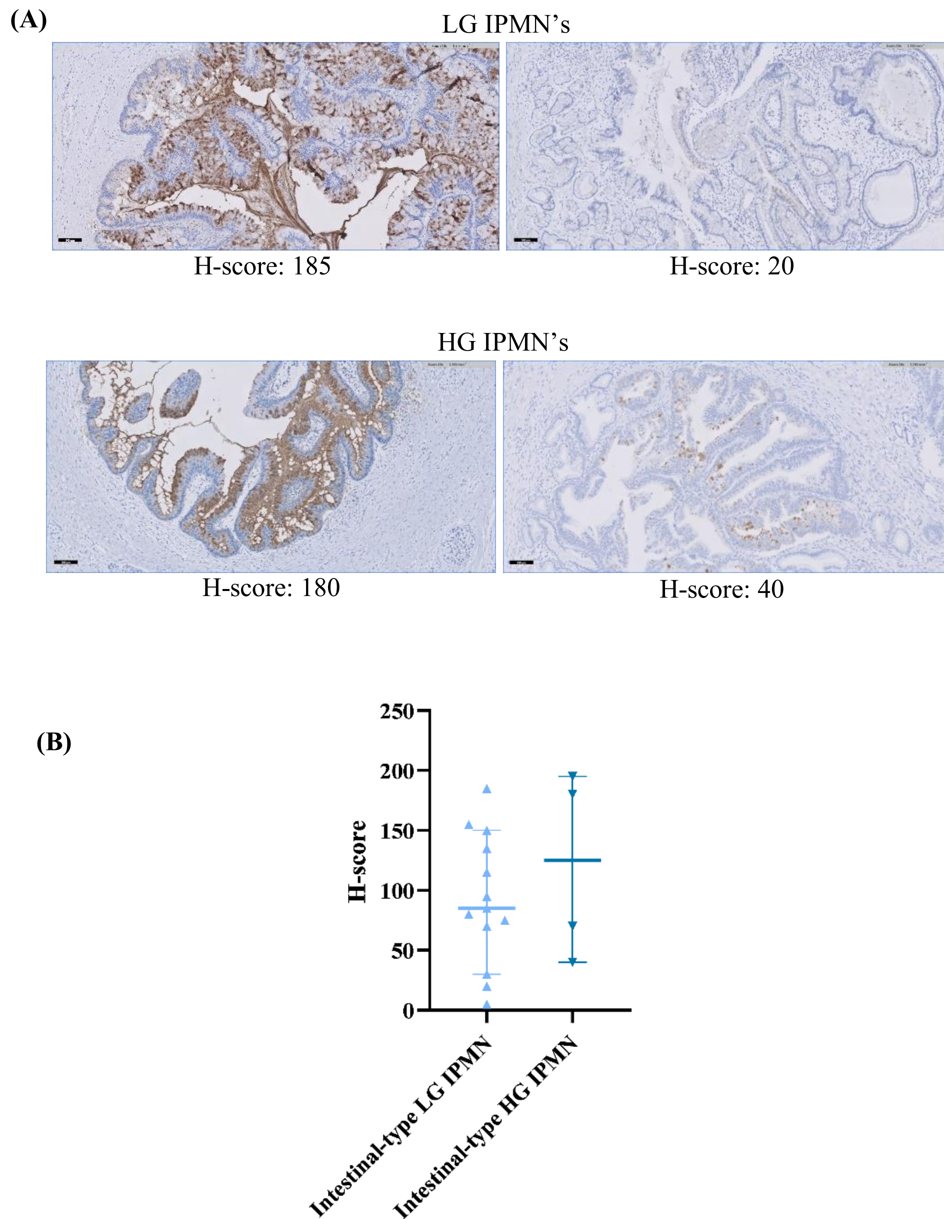


Figure 12 - STn expression in intestinal-type LG and HG IPMNs. **(A)** Representative images of the stained slides that presented intestinal-type LG and HG IPMNs and respective H-scores. Scale bars correspond to 100 µm. **(B)** Scatter-plot representation of the intestinal-type IPMNs' H-score values (median with 95% CI).

The pancreato-biliar-type HG IPMNs, presented a significant increased STn expression when compared to the LG (**Figure 13**). This IPMN type is characterized by the expression of MUC1, MUC5AC and MUC6[23,34].

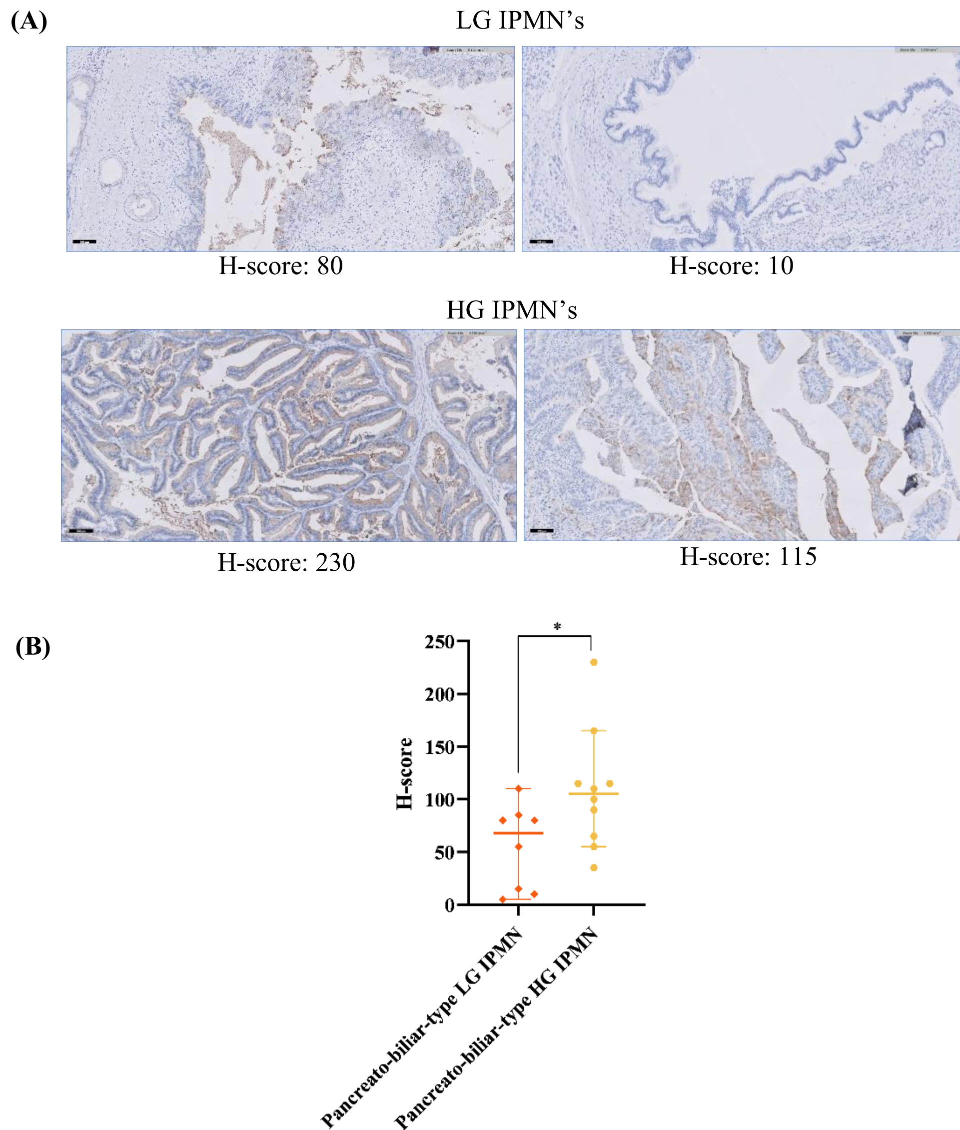


Figure 13 - STn expression in pancreato-biliar-type LG and HG IPMNs. **(A)** Representative images of the stained slides that presented pancreato-biliar-type LG and HG IPMNs and respective H-scores. Scale bars correspond to 100 μm. **(B)** Scatter-plot representation of the pancreato-biliar-type IPMNs’ H-score values (median with 95% CI). Significant difference at $*p < 0.05$.

For the oncocytic-type, only one was identified. This lesion presented HG dysplasia and showed low STn expression (**Figure 14**). As this IPMN type is more likely to present in younger people, less cases are described explaining the reason why only one was identified[88]. This IPMN type, expresses MUC5AC and MUC6 and sometimes expresses also MUC1 and/or MUC2 focally[23,35].

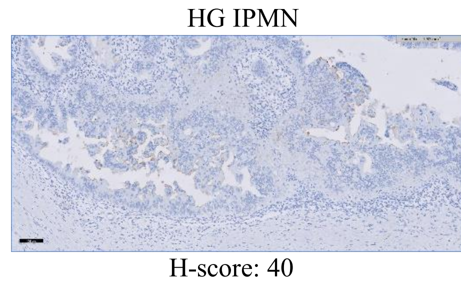


Figure 14 - STn expression in oncocytic-type HG IPMN. Image of the stained slide with oncocytic-type HG IPMN and the respective H-score. Scale bar corresponds to 100 µm.

When comparing the different types of IPMN, it is possible to observe that pancreato-biliar-type HG IPMN is the one that presents significant differences compared to gastric-type LG IPMN. Additionally, it is possible to conclude that both intestinal and pancreaticobiliary-type IPMNs both LG and HG seem to be more correlated with higher STn expression than the others (**Figure 15**).

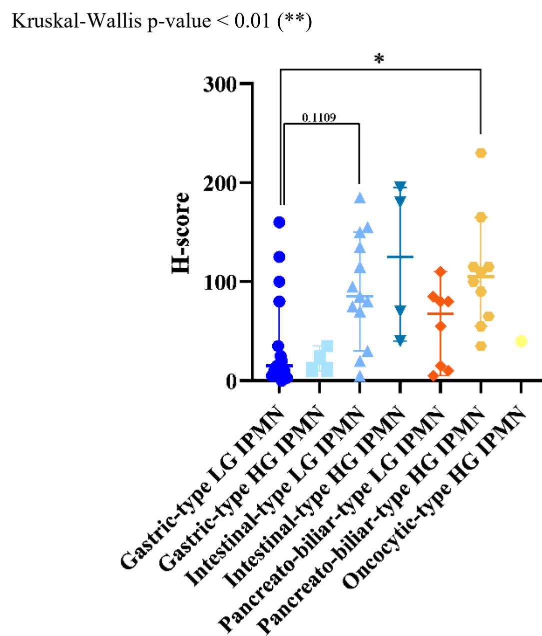
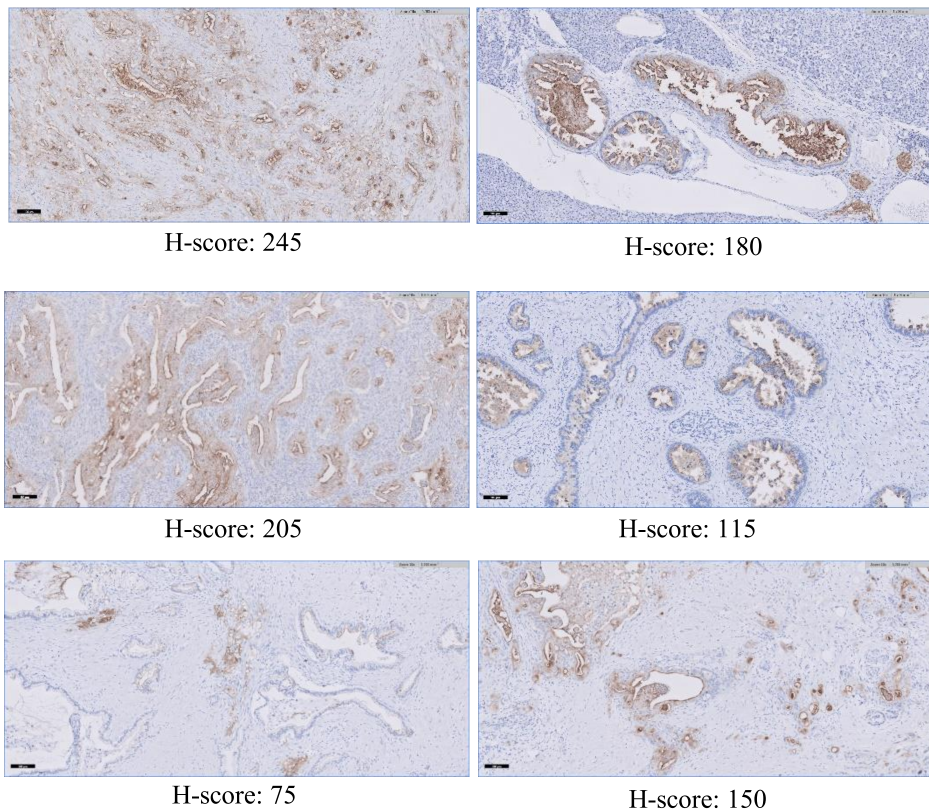


Figure 15 - STn expression in all LG and HG IPMNs types. Scatter-plot representation of the all the IPMNs types' H-score values (median with 95% CI). To check for significant differences between the groups, a Kruskal-Wallis test was performed. Significant difference at $^{**}p < 0.01$. Then, to check for which groups were significantly different from each other, a Dunn's test was performed. Significant difference at $^{*}p < 0.05$. P-value presented was obtained by the Dunn´s test. The oncocytic-type HG IPMN (n = 1) was not considered for the statistical analysis.

These results might be explained by the mucin pattern of each lesion type. A literature search regarding STn carriers described in different cancer types, was previously conducted by our group (Appendix 1). This revealed that MUC1 and MUC2 are well described STn carriers for several types of cancers being MUC5AC and MUC6 also carriers but less described[61,65,78,89–97]. The MUC1 was already identified as a STn carrier in PC cell lines[89]. Therefore, as the intestinal-type is characterized by expressing MUC2 and the pancreato-biliar-type by expressing MUC1, both mucins that are already well described by being STn carries, this could explain the STn expression pattern found in these lesions.

3.2.2 STn expression in PDAC untreated cases

Almost all PDAC untreated cases were positive for STn expression, with only one case being completely negative. Despite the heterogeneity in the staining intensities for the different cases, the majority revealed high STn expression (**Figure 16**). Several studies, also reported, that the majority of PDAC cases stain positive for STn being the expression also heterogeneous[68,69,85,86].



(B)

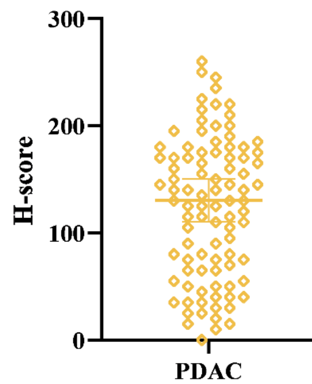


Figure 16 - STn expression in PDAC untreated cases. **(A)** Representative images of the stained PDAC untreated slides and respective H-scores. Scale bars correspond to 100 µm. **(B)** Scatter-plot representation of the PDACs H-score values (median with 95% CI).

To assess if this heterogenous STn expression was related with the different cancer stages of this disease, according to AJCC staging (pTNM), they were individually evaluated. However, no significant differences were obtained for the different PDAC stages being the expression of STn still highly heterogeneous (**Figure 17**). Besides, there were no cases of stage IV, probably since these patients are not commonly suited for surgical approach[27].

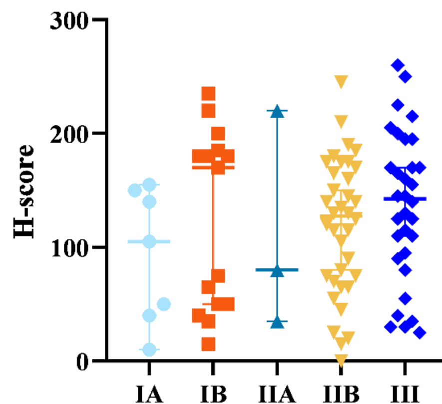


Figure 17 - STn expression in the different PDAC cancer stages. Scatter-plot representation of the all the different PDAC stages, according to AJCC, H-score values (median with 95% CI).

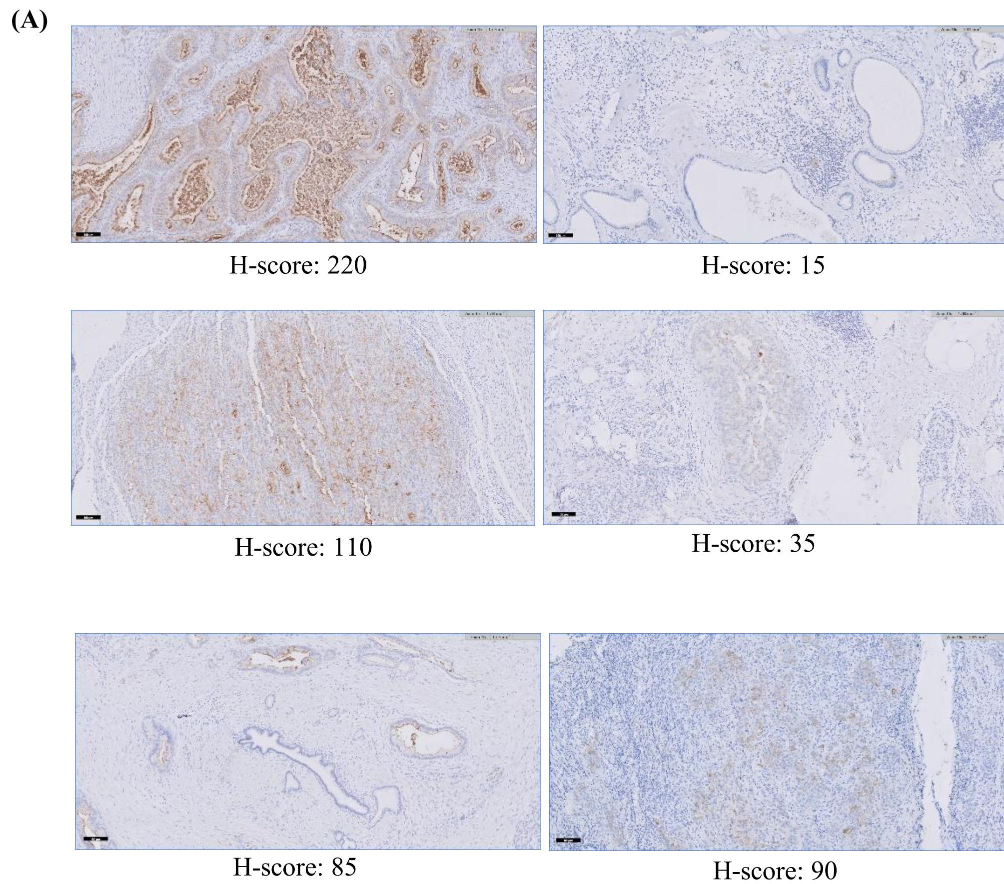
3.2.3 STn expression in PDAC post-CT cases

To evaluate if neoadjuvant chemotherapy would have any impact on STn expression, tissue samples from PDAC post-CT patients were stained. Then, they were compared with the untreated PDACs. From the 41 PDAC post-CT cases, 38 were evaluated for STn expression. This was given to the fact that 2 cases had no tumor present after the treatment and in another case the tumor could not be identified on the slide.

Even though some cases remain with high STn expression, in the majority of the post-CT cases, it is possible to observe a slight decrease in STn expression. However, no statistical difference was observed (**Figure 18**), maintaining the question whether neoadjuvant chemotherapy has any impact on STn expression. Other study reported a slight decrease in STn expression, for patients that underwent neoadjuvant treatment, and no significant difference was observed when compared to the untreated prior to surgery cases, leading them to conclude that these treatments did not influence the expression of STn. However, they consider both neoadjuvant chemotherapy and chemoradiotherapy[85].

Recently, it was reported that neoadjuvant chemotherapy in PDAC patients impacted the tumor stroma and the neoplastic cell populations composition. Additionally, it was also reported that different chemotherapies may influence differently different neoplastic cell phenotypes[98].

As the neoadjuvant chemotherapy of PDAC post-CT patients from our cohort is known to be heterogeneous, the impact on STn expression may vary according to the type of treatment used. Therefore, all these factors could explain the decrease in STn expression as well as why some cases still present high STn expression. Future analysis of finding correlations between STn expression and the different neoadjuvant chemotherapy treatments used in these patients will clarify this hypothesis.



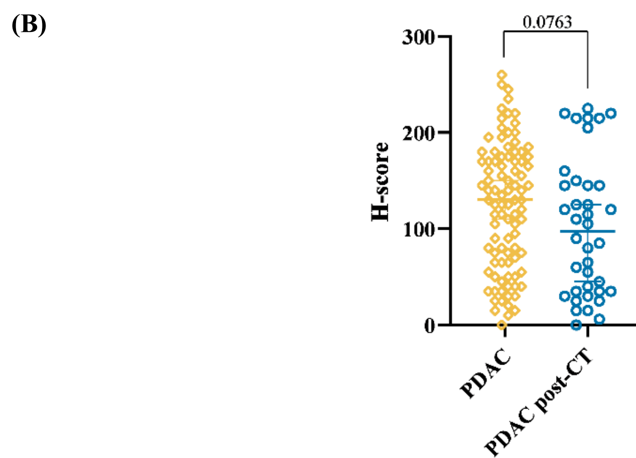


Figure 18 - STn expression in untreaded PDAC and post-CT cases. **(A)** Representative images of the PDAC post-CT cases and respective H-scores. Scale bars correspond to 100 µm. **(B)** Scatter-plot representation of both PDAC and PDAC post-CT H-score values (median with 95% CI). P-value presented was obtained by Mann-Whitney test between PDAC and PDAC post-CT.

Then, the different stages of the PDAC post-CT cases, according to AJCC staging (ypTNM) were also evaluated for STn expression, to see if any correlation could be made. In this group, for some cases, the information about the stage of the disease was not assessed. The results showed that no significant difference was obtained between the different stages (**Figure 19**).

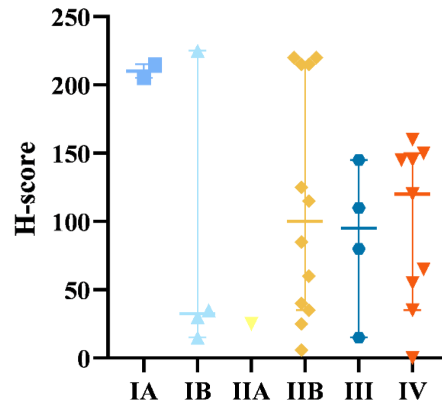
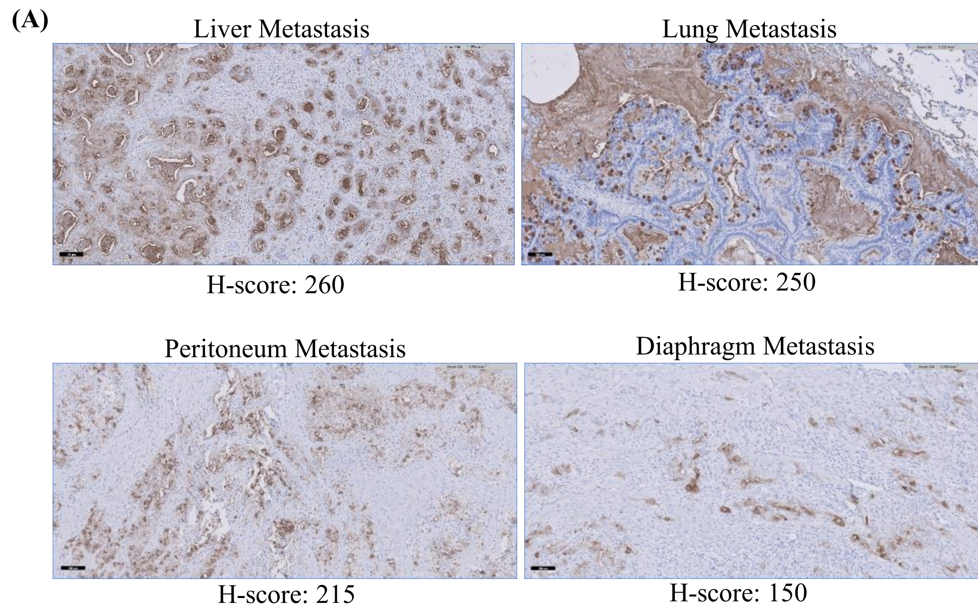


Figure 19 - STn expression in the different PDAC post-CT cancer stages. Scatter-plot representation of the all the different PDAC post-CT stages, according to AJCC, H-score values (median with 95% CI). The IIA stages group was not considered for statistical analysis (n = 1).

3.2.4 STn expression in PDAC metastases

As the last stage of PDAC development, we have the metastatic PDAC. Hence, we stained some of the most common organs to which the PDAC metastasizes to see their STn expression pattern. The metastases, in general, revealed high STn expression, presenting the highest median value. In comparison with the untreated primary tumors, it is possible to observe that STn expression slightly increases in the metastases (**Figure 20**). Previous studies also reported increased expression of STn namely in liver and lung metastasis comparing to primary tumors[69,99].

(A)



(B)

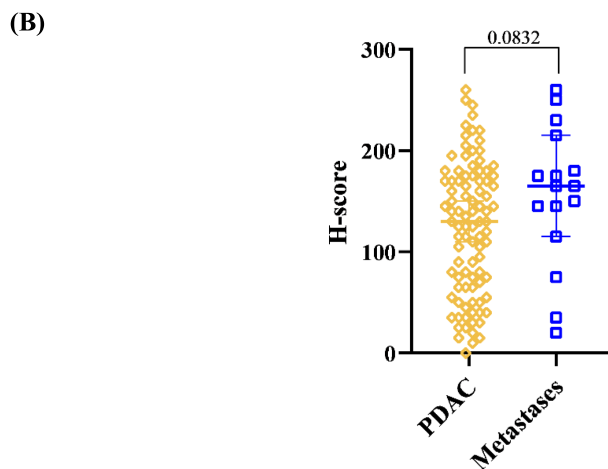
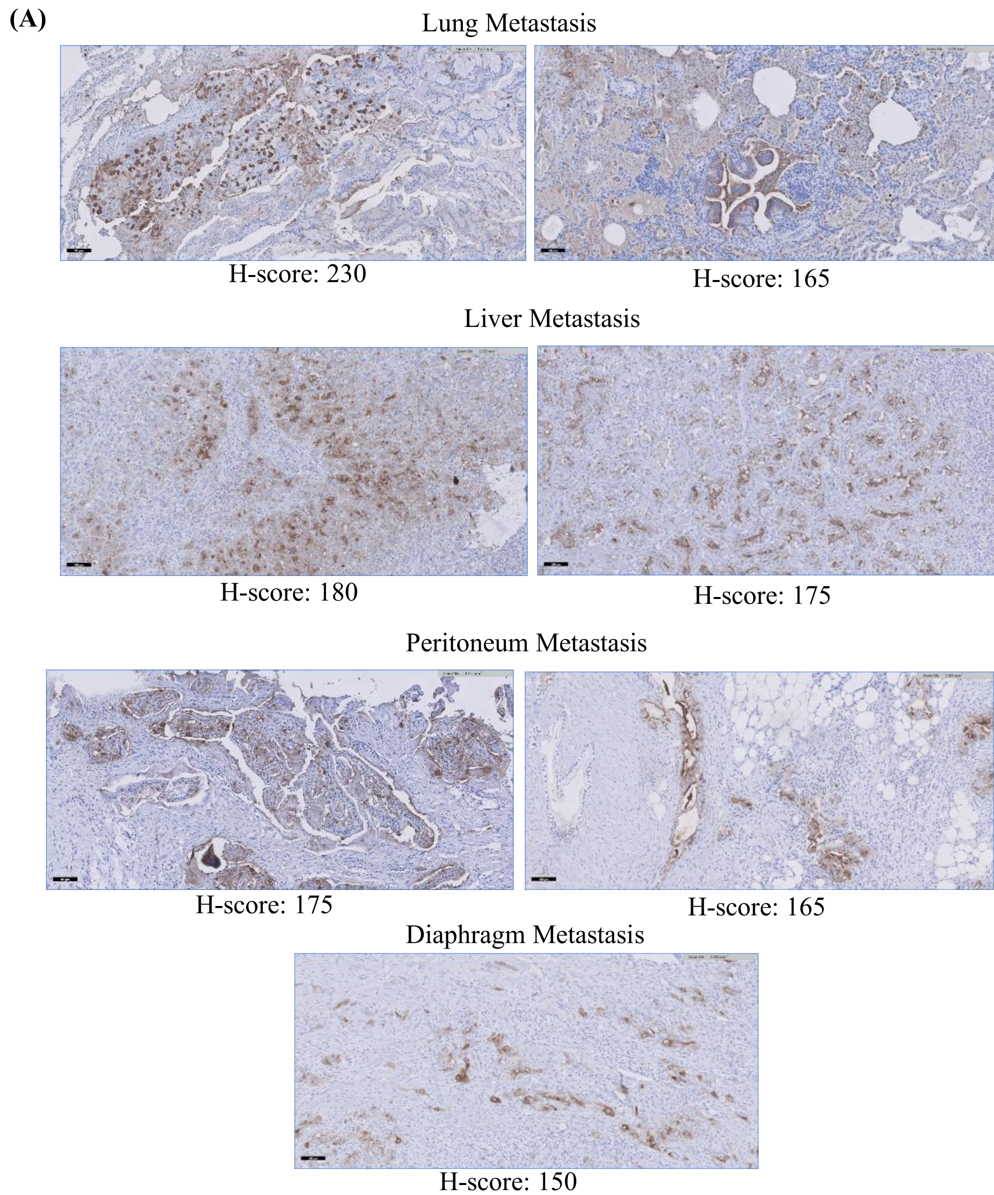


Figure 20 - STn expression in untreaded PDAC and metastases. **(A)** Representative images of the metastases and respective H-scores. Scale bars correspond to 100 µm. **(B)** Scatter-plot representation of both PDAC and metastases H-score values (median with 95% CI). P-value presented was obtained by Mann-Whitney test between PDAC and metastases.

Individually, the lung and liver metastases, with exception for one liver metastasis that was almost negative, all had high STn expression. For the peritoneum and diaphragm metastases, STn expression slightly decreased. However, no significant difference is observed among the different organ's metastasis for STn expression (**Figure 21**).



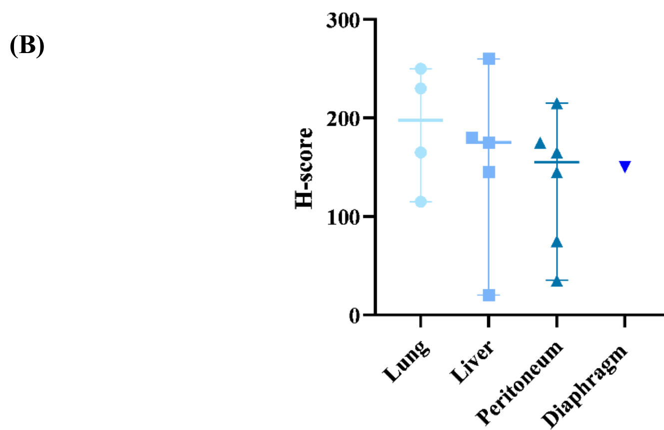


Figure 21 - STn expression in the different metastasis per organ. **(A)** Representative images of the metastases and respective H-scores. Scale bars correspond to 100 µm. **(B)** Scatter-plot representation of all the metastases H-score values per organ (median with 95% CI). The diaphragm metastasis was not considered for statistical analysis (n = 1).

3.2.5 STn expression during PDAC development

Overall, STn expression tends to increase as the cancer develops, suggesting a role for STn in PDAC progression. The heterogeneity of STn expression within each PDAC development stage may be due to some cancer cell subsets that may be differently expressing STn, which might be explained by the fact that the glycosylation patterns often varies from cell to cell, depending on several factors, as previously explained[49,58,63].The considerable significant difference between the LG IPMNs and both PDACs untreated and metastases cases, together with the significant difference between the HG IPMNs and metastases cases, emphasizes that STn antigen expression mainly occurs in later events during the PDAC development (**Figure 22**) as previously described by the literature[69,86]. Given the low number of PanIN lesions identified, they were not considered for this analysis.

Kruskal-Wallis p-value < 0.0001 (****)

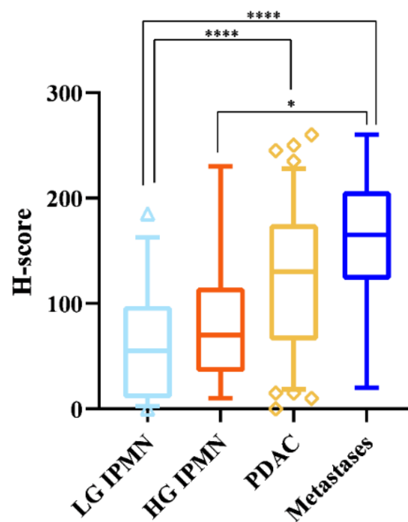


Figure 22 - STn expression during PDAC development. The H-score values are represented by a box & whiskers with 5-95 percentile. To check for significant differences between the groups, a Kruskal-Wallis test was performed. Significant difference at ****p < 0.0001. Then, to check for which groups were significantly different from each other, a Dunn's test was performed. Significant difference at *p < 0.05, **** p < 0.0001.

3.3 *In vitro* model validation

To perform future studies that might elucidate the underlying molecular mechanisms of the role of STn in PDAC progression, an *in vitro* model validation of PDAC cell lines overexpressing STn at their surface is needed.

Herein, PANC-1 human cell line, was previously modified to overexpress STn, generating PANC-1 STn+ cell line. The validation of this model is needed to guarantee and evaluate the percentage of cells that are indeed expressing STn, in comparison with PANC-1 Mock cell line. This validation was made using western blot followed by flow cytometry.

PANC-1 STn+ cell line is expressing STn whereas the PANC-1 Mock are not expressing (**Figure 23A**), as expected. MUC1 STn IgGA was used as positive control for STn expression. These results confirm that the enzyme ST6GalNAc I has an important role in STn expression. Additionally, the molecular weight of the band that appears in the PANC-1 STn cell line is approximately the same of the MUC1 protein. Previous studies already revealed that STn decorates MUC1 in PC cell lines, namely in PANC-1 cells[89]. Therefore, we speculate that this protein decorated with STn in the PANC-1 cell line might be the MUC1.

Pan02 mouse cell line was also previously modified and, therefore, the validation by western blot was also performed. However, no STn expression was found in Pan02 STn+ cell line (**Figure 23B**). This might be given to the fact that the human gene that codes for ST6GalNAc I was not inserted in the genome of these cells. Sequencing will be needed to check if this gene is inserted in the genome.

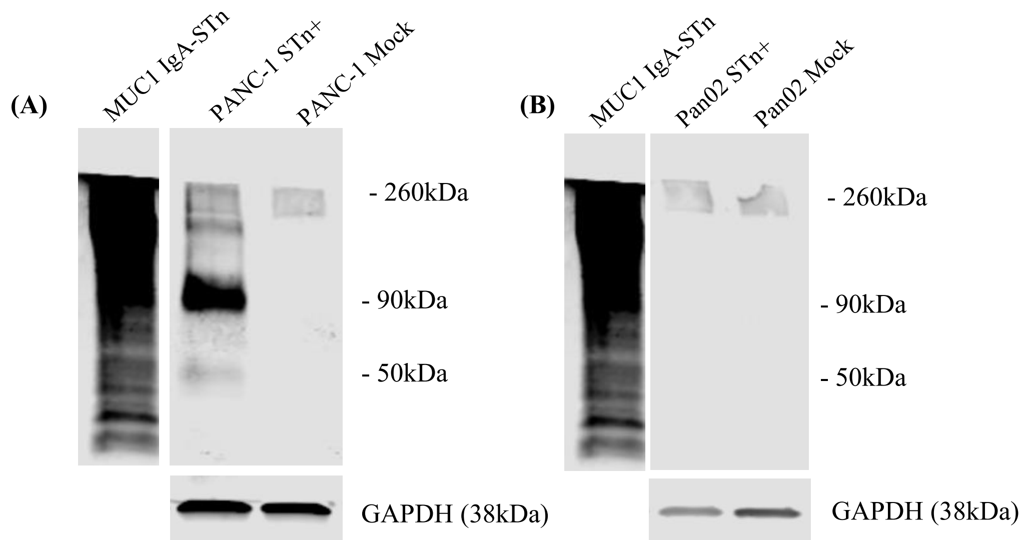


Figure 23 - ***In vitro*** **model validation by western blot analysis**. **(A)** PANC-1 STn+ and PANC-1 Mock cell line model validation by western blot. **(B)** Pan02 STn+ and Pan02 Mock cell line model were not validated by western blot. MUC1 IgA-STn was used as a positive control for STn expression.

Following the western blot, to check for the STn surface expression in PANC-1 STn+ cell lines, a cell surface staining protocol for flow cytometry analysis was conducted. To assess live vs dead status cells, a viability dye was also used in conjugation with the anti-STn antibody (**Figure 24A**). Then, only the live cells (Allophycocyanin (APC)-Cy7-A -) were selected to check STn positive cells (FITC-A+). The results showed that approximately 74.4% of PANC-1 STn+ cells are expressing STn in their surface. As expected, the PANC-1 Mock cells are not expressing STn (**Figure 24B**).

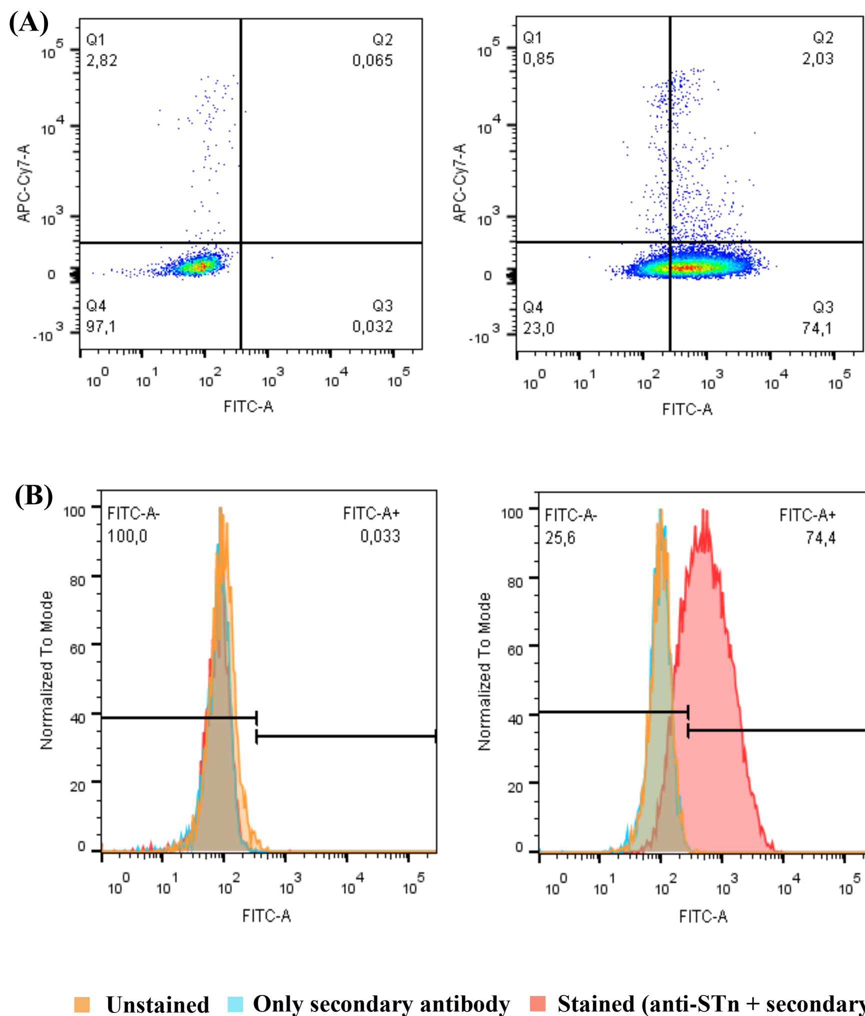


Figure 24 - *In vitro* model validation by flow cytometry analysis. **(A)** Assessment of live (APC-Cy7-A -) vs dead (APC-Cy7-A +) cells in conjugation with STn- (FITC-A-) and STn+ populations (FITC-A+) in stained PANC-1 Mock (left) and PANC-1 STn+ (right) cells. **(B)** Assessment of STn+ population (FITC-A+) to validate the PANC-1 STn+ cell line model (right) in comparison with the PANC-1 Mock cell line model (left) that does not express STn.

These results, validate the *in vitro* cellular model, PANC-1 STn+ cell line. Therefore, *in vitro* future studies regarding the role of STn in PDAC can be performed.

3.4 Literature search: STn survival analysis in different cancer types

To analyze what is already known about the impact of STn on survival in different types of cancer, a literature search of STn survival analysis was conducted, as previously described.

From our screening, 139 articles were identified, from which 25 articles were selected and categorized according to the impact of STn expression on patient survival. The categories were "Better" if the STn expression was correlated with increased survival, "Worse" if it was correlated with poor survival and "non-significant impact" if a correlation between STn and survival could not be established. When an article did not fit into these categories, it was considered "Uncertain". When the study carried out the STn evaluation and survival analysis in part of the total cases, only that part was considered in our analysis.

From our analysis results of the selected 25 articles, we observed that none categorized the STn expression as "Better", while 14 studies categorized STn+ cases as "Worse"[100–113]. In the remaining studies, 10 retrieved "non-significant Impact"[105,114–122] and 2 were "Uncertain"[123,124] and therefore excluded from further analysis. Werther and collaborators evaluated the impact of STn on survival in four different cohorts from Japan, Chile, Brazil, and the USA. We considered the 4 cohorts individually since they had different conclusions regarding STn's impact on survival. The cohorts from Chile and the USA are included individually in the group of articles that found STn+ to be associated with "Worse" survival, while in the cohorts from Japan and Brazil, there was no association, so they were categorized as having "non-significant impact" on survival[105]. Leivonen and collaborators evaluated both 5 and 10-year survival, having identified STn with a worse impact at 5-year than at 10-year survival[110]. As Terasawa and collaborators evaluated STn expression using two different antibodies, obtaining varying numbers of positive cases, we considered only the cases detected by the antibody that gave the highest number[117].

Additional scrutiny of our analysis unveiled disparities between the dimensions of the patient cohort ranging from 30 to 408 patients and encompassing different types of cancer, namely gastric, colorectal, breast, lung, bladder, cervical, bile duct, ovary, ampullary and sinonasal cancers.

The selected 23 articles included a total of 3642 histological tissues that were evaluated by IHC for STn expression. 1780 were STn+ and 1862 cases were STn- (**Table 4**). For the different cancer types, the percentage of STn+ cases varied from 35.1% to 84.2%. The ampullary cancer, sinonasal and colorectal cohorts presented higher percentages (above 70%) of STn+ cases, whereas the lung and breast cancer cohorts exhibited lower percentages (below 50%).

Table 4 - Description of the total number of patients with survival data obtained for each cancer type. The table shows the number of STn+ and STn- cases per cancer type from all 23 articles included in this study.

Cancer types	All cases	STn+ cases	STn- cases	% of STn+ cases per cohort
Gastric	1222	589	633	48.2
Breast	1042	366	676	35.1
Colorectal	519	370	149	71.3
Lung	408	170	238	41.7
Bladder	176	113	63	64.2
Cervical	83	48	35	57.8
Bile Duct	67	36	31	53.7
Ovarian	57	35	22	61.4
Ampullary	38	32	6	84.2
Sinonasal	30	21	9	70.0
All	3642	1780	1862	48.9

Among the STn+ cases, 52% (926/1780) were related to worse survival. Particularly, of the STn+ cases identified as having worse impact in digestive system cancers (colorectal, gastric, ampullary and bile duct cancers), 66.3% corresponded to gastric cancer. Although most authors report a worse impact of STn in this type of cancer[100,102,105,106,109,113] some authors also report a non-significant impact on patient survival[105,122].

In other cancers, such as bladder and sinonasal cancers, the prognosis was worse in all STn+ cases[101,104,108]. However, there is some inconsistency regarding breast, colorectal and ovarian cancer, with some studies reporting that STn+ cases have worse survival[103,107,110–112], while others found non-significant impact[115,118,120,121]. On the other hand, the survival of lung, cervical, ampullary and bile duct cancer patients exhibited no discernible association with STn[114,116,117,119] (**Figure 25**).

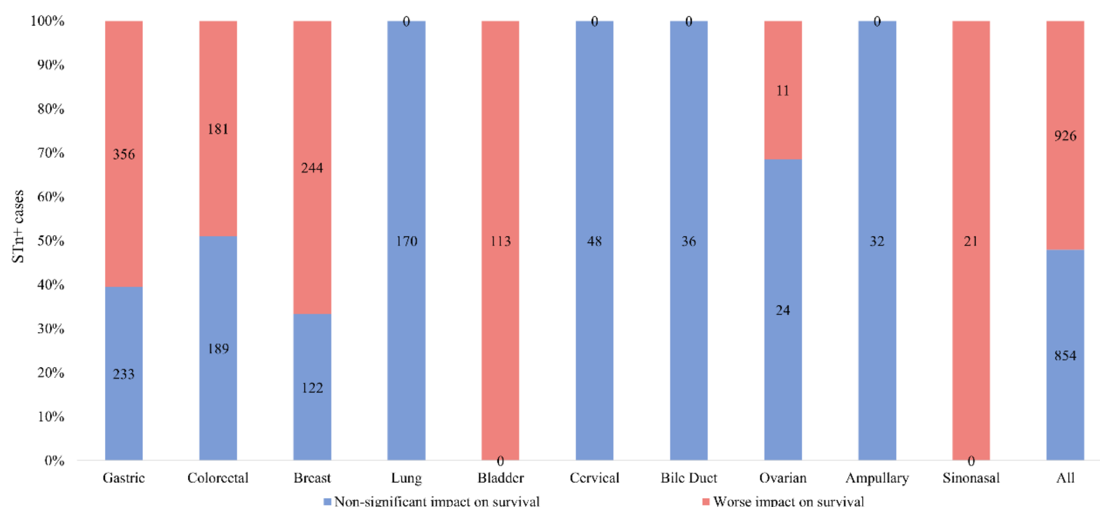


Figure 25 - Distribution of STn+ cases by cancer type cohorts for their impact on survival. These values were obtained from each article cohort and evaluated for each type of cancer, where the impact on survival was verified. The STn expression was associated with either a non-significant or worse impact on survival, but no better survival was found in the literature investigated.

Even though no cohort refers to STn expression as a better prognosis, a direct correlation with survival could not be made, given these conflicting results for the different cancer types. Therefore, the expected number of deaths or survivals at 5 years for each study cohort were calculated considering the survival analysis extrapolated from the Kaplan-Meier plots. This analysis confirmed a higher number of expected deaths for the STn+ cases, considering all cancer types (**Table 5**). Mortality was determined by the number of expected deaths in both STn+ and STn- populations.

Table 5 - Description of expected deaths and survivals for STn+ and STn- expression populations. Description of the probability of survival and corresponding OR and 95% CI per study cohort. Mortality was calculated and is represented per cancer-type cohort. The expected deaths and survival numbers are rounded to zero decimal places, but the corrected value was used for both OR and mortality calculations.

		STn+ cases				STn- cases					
Study	Cancer type	Total Cases	Probability of surviving after 5-years (%)	Expected deaths/ survivals	Mortality (%)	Total Cases	Probability of surviving after 5-years (%)	Expected deaths/ survivals	Mortality (%)	OR	95% CI
Yamada et al 1995[109]	Gastric	27	31	19/8	66.47	26	63.75	9/17	49.54	3.914	[1.25; 12.27]
Werther et al.1996 (Japan)[105]		107	55.8	47/60		106	63.6	39/67		1.384	[0.80; 2.40]
Werther et al.1996 (Brazil)[105]		31	40.75	18/13		9	43	5/4		1.097	[0.24; 4.92]
Werther et al. 1996 (USA)[105]		21	12.6	18/3		10	50.2	5/5		6.992	[1.17; 41.8]
Werther et al.1996 (Chile)[105]		15	36.65	10/5		18	70.85	5/13		4.201	[0.97; 18.1]
Victorzon et al.1996[113]		148	32.8	99/49		89	51.5	43/46		2.176	[1.27; 3.73]

Miles et al. 1995[122]		95	21	75/20		44	26.4	32/12		1.349	[0.59; 3.10]
Ma et al.1993[102]		53	47.2	28/25		32	84.4	5/27		6.052	[2.02; 18.1]
Terashima et al 1998[106]		35	36	22/13		176	56	77/99		2.263	[1.07; 4.80]
Kakeji et al 1995[100]		57	4.4	54/3		123	25	92/31		7.242	[1.92; 27.4]
Miles et al 1994[118]	Breast	74	70.5	22/52	45.14	163	79.4	34/129	20.71	1.613	[0.86; 3.02]
Xu et al.2021[103]		159	29.7	112/47		221	69	69/152		5.268	[3.38; 8.21]
Leivonen et al 2001[110]		85	71	25/60		133	85	20/113		2.315	[1.19; 4.51]
Kinney et al 1997[121]		48	85.5	7/41		159	88.7	18/141		1.331	[0.52; 3.41]
Itzkowitz et al. 1990[112]	Colorectal	112	73	30/82	49.56	16	100	0/16	40.52	12.33	[0.72; 212]
Lundin et al 1999[120]		189	52.3	90/99		50	52.3	24/26		1.00	[0.54; 1.87]
Vierbuchen 1995[107]		69	8.7	63/6		83	56	37/46		13.36	[5.20; 34.3]
D'Amico et	Lung	170	60	68/102	40.00	238	63	88/150	37.00	1.135	[0.76;

al.1999[114]											1.70]
Costa et al. 2015[101]	Bladder	57	47	30/27	56.32	39	75	10/29	25.23	3.383	[1.39; 8.26]
Lima et al 2017[104]		56	40.3	33/23		24	74.4	6/18		4.305	[1.49; 12.4]
Terasawa et al. 1996[117]	Cervical	48	81	9/39	19.00	35	80	7/28	20.00	0.938	[0.31; 2.81]
Takao et al.1999[116]	Bile Duct	36	28	26/10	72.00	31	27 (low); 13 (negative)	24/7	78.87	0.689	[0.22; 2.13]
Davidson et al.2000[115]	Ovarian	24	45	13/11	60.66	14	50	7/7	39.82	1.222	[0.33; 4.58]
Ghazizadeh et al.1997[111]		11	27	8/3		8	78	2/6		9.586	[1.13; 81.3]
Kitamura et al 1996[119]	Ampullary	32	37.4	20/12	62.60	6	33	4/2	67.00	0.824	[0.13; 5.22]
Franchi et al. 1996[108]	Sinonasal	21	18	17/4	82.00	9	73	2/7	27.00	12.317	[1.95; 77.96]
Total		1780		965/815	54.23	1862		665/1197	35.69		

The difference in mortality between the STn+ and STn- for all cancer cases was considerably different, with the STn+ group having 54.23% mortality compared to 35.69% in the STn- group. However, when considering each cancer type individually, the biggest difference regarding mortality between the STn+ and STn- was observed for sinonasal cancer, with 82% and 27% mortality for both groups, respectively. This cancer is followed by bladder (STn+ = 56.32%; STn- =25.23%), breast (STn+ = 45.14%; STn- = 20.71%), ovarian (STn+ = 60.66%; STn- = 39.82%) and gastric cancer (STn+ = 66.47%; STn- = 49.54%). The remaining cancers had almost no difference in mortality or very low between the two groups, such as the cervical (STn+ = 19%; STn- = 20%), lung (STn+ = 40%; STn- = 37%), ampullary (STn+ = 62.6%; STn- = 67%), bile duct (STn+ = 72%; STn- = 78.87%) and colorectal cancer (STn+ = 49.56%; STn- = 40.52%). However, one colorectal cancer study reported no deaths for the STn- subpopulation. The heterogeneity in the number of cases for each study cohort makes comparing cancer types difficult. Yet, it is generally accepted that the larger the cohort, the more reliable the difference seen between the two groups tends to be.

To validate the studies classification regarding the STn impact on survival, the OR was then calculated. The articles classified as "worse" presented OR values within a range of 2.176 to 13.36, whereas the articles classified as "non-significant impact" presented OR within a range of 0.689 to 1.613, which validates the studies classification performed (**Figure 26**). The OR values below or above 1 until 1.613 are being considered as "non-significant impact". This is given to the fact that, independently, both populations have no considerable difference in the survival analysis between the probabilities of 5-year survival.

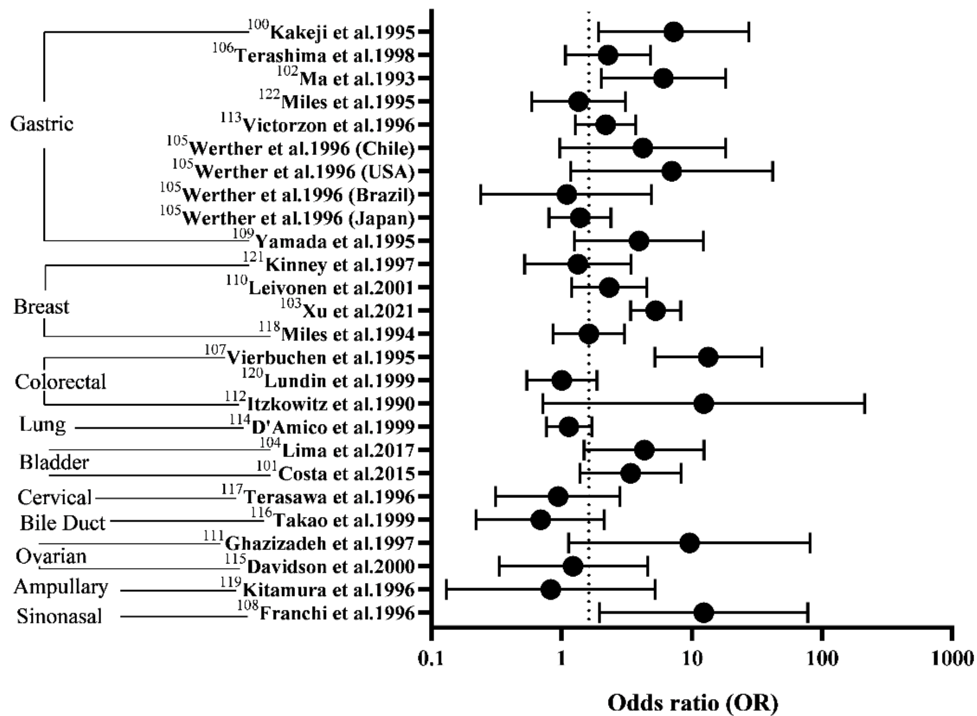


Figure 26 - Forest plot for the validation of each article classification in relation to the impact of STn expression on survival outcome. Articles with OR values below 1.613 (dashed line) are classified as "non-significant impact", and articles with ORs above 1.613 are classified as "worse". ORs per study are depicted in a forest plot with the corresponding 95% CI in a logarithmic scale, obtained using GraphPad Prism 8.0.2.

In conclusion, our overall analysis shows that STn+ cases tend to have worse outcomes. Yet, the impact of STn expression on overall survival depends on the cancer type.

Despite conflicting results in survival analyses across different cancer types, it is clear that STn is associated with poor survival, especially in bladder, sinonasal, gastric and breast cancers. It is however imperative to further elucidate the impact of STn on patient outcomes, through a better standardization of immunohistochemistry methodologies and the expansion of cohort sizes, while exploring additional factors that may influence STn-associated prognoses, such as tumor stage and molecular subtypes. Interestingly, no study regarding STn survival impact on PDAC was found, highlighting the importance of performing a survival analysis in a meaningful patient cohort.

4. Conclusion and Future Perspectives

There has been a growing effort in discovering new biomarkers and new therapeutic targets to develop more effective treatment strategies for PDAC. This cancer is known to overexpress truncated O-glycans, including STn, therefore exploring the role of this disaccharide in PDAC is important. In addition, the impact of STn on cancer survival is not transversal across the distinct cancers making important to clarify what has been studied about the role of STn on survival for different cancer types.

Therefore, the work developed during this dissertation involved understanding the correlation between STn expression and PDAC progression, as well as revising literature about the impact of STn on cancer survival.

The results highlight the STn tumor specificity, since this antigen is nearly absent in normal tissues and show that, despite the heterogeneity, STn mainly occurs in later events of PDAC. Besides, its expression seems to increase during cancer development, especially in metastases, suggesting a role for STn in cancer progression. Regarding neoadjuvant chemotherapy, even though a slight decrease in STn expression is observed, no significant correlation about the influence of this treatment in STn expression could be made.

Then, the *in vitro* model validation of a human PDAC cell line overexpressing STn, was important to enable further experiments that might elucidate the underlying molecular mechanisms of the role of STn in PDAC progression. In addition, this validation also confirmed the role of the enzyme ST6GalNAc I in the STn expression.

Concerning the STn impact on survival, the third part of this project, elucidated that, in general, STn+ cases were more associated with worse survival. Interestingly, it also revealed the need to proceed with survival analysis in a meaningful cohort of PDAC patients to assess the impact of STn in overall survival in these patients.

In fact, our data will enable more correlations with clinical features and gene expression that might be common to other cancer types, including survival data.

Furthermore, further experiments are aimed to validate STn-related immunophenotype from the PDAC patient's cohort and then proceed to an *in vivo* mouse model and finally test anti-STn antibodies' efficacy in overcoming immunosuppression and reducing tumor progression using the *in vitro* model.

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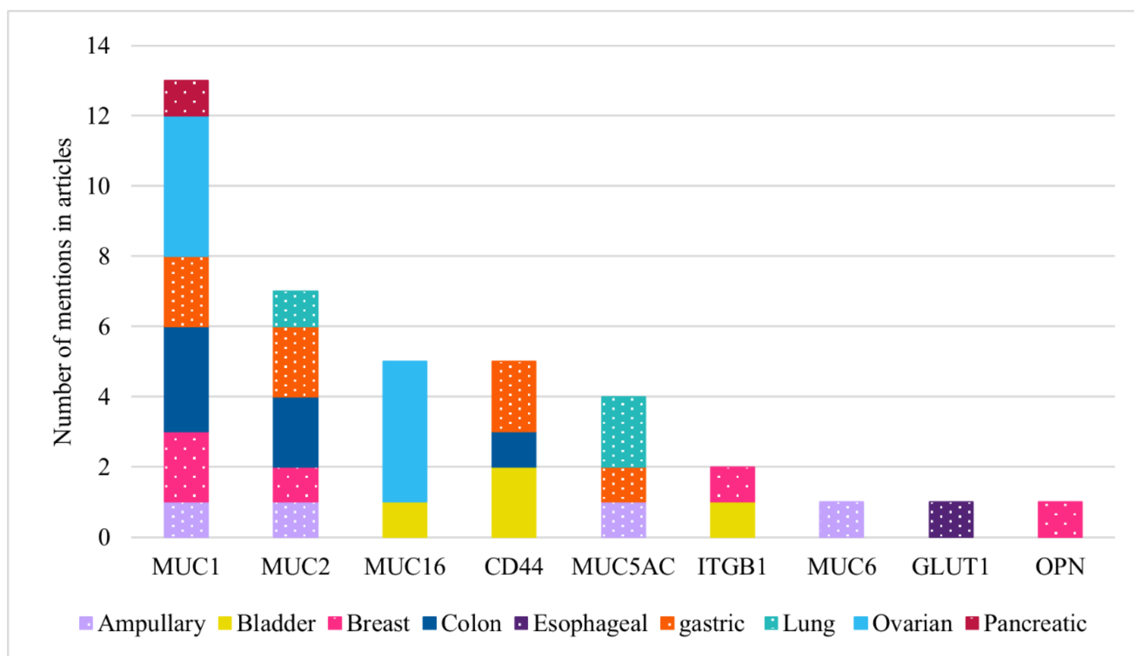
6. Appendix

Appendix 1: Supplementary unpublished results



Appendix Figure 1 - Distribution of cancer type per each protein identified as STn carrier. The plot depicts the number of mentions of each protein reported as an STn carrier in the articles selected and the distribution of each cancer type. A systematic search on PubMed/MEDLINE was performed using the following query: ("Sialyl Tn" OR "Sialosyl Tn" OR "Sialyl-Tn" OR "Sialosyl-Tn" OR "STn") AND ("STn-carrying" OR "Carrier*" OR "Glycoprotein*" OR "Glycoform*") AND ("Cancer*"). Articles were initially screened for titles and abstracts for the selection of potential studies to be evaluated and assessed for eligibility. This process led to the identification of 21 studies on STn protein carriers. The work is currently submitted (Lourenço RA, Soares ACF, Pinto DR, Dias MFD, Corr SC, Ramos NP, Delannoy P, Videira PA. Keeping an Eye on Sialyl Tn Target. In: SPR Carbohydrate Chemistry Vol.47. The Royal Society of Chemistry. 2024.)

2024 Ana Soares EXPLORING THE CROSSTALK BETWEEN SIALYL-Tn (STn) EXPRESSION AND PANCREATIC DUCTAL ADENOCARCINOMA (PDAC) PROGRESSION

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