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Departamento de Química

Elisabete Cristina Nunes Fernandes

Resposta humoral associada a glicosilação no cancro do esófago

Humoral responses against esophageal cancer-associated glycosylation

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica do Professor José Alexandre Ferreira, Investigador de Pós-Doutoramento do Centro de Invesigação do Instituto de Oncologia do Porto (IPO- Porto) e do Departamento de Química da Universidade de Aveiro e Professor Doutor Lúcio Lara Santos do IPO-Porto

dedicatória

Dedico este trabalho a todos que o tornaram possível.

O júri

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palavras-chave

Cancro do esófago, biomarcadores tumorais, glycanas associadas ao cancro, resposta humoral, glicosilação de imunoglubolinas com sialil Lewis (a), autoanticorpos

Resumo

O cancro de esófago (CE) tem um mau prognóstico e uma baixa taxa de sobrevivência. É diagnosticado tardiamente, por endoscopia, um método invasivo que carece de especificidade e sensibilidade. Atualmente, não existem biomarcadores para melhorar a precisão do diagnóstico.

A transformação maligna é acompanhada por alterações no padrão de glicosilação das células, que são utilizadas no diagnóstico não-invasivo, monitorização da doença, bem como terapêutica. Além disso, as proteínas exibindo glicosilação alterada são capazes de induzir respostas humorais. Os auto-anticorpos são uma nova geração de biomarcadores tumorais, com capacidade de amplificar alterações moleculares nos tumores. São ainda mais resistentes a proteólise o que os correspondentes epitopos. No entanto, pouco se sabe sobre a glicosilação em CE e a sua imunogenicidade.

Assim, a primeira parte deste trabalho teve como objetivo identificar padrões de glicosilação no soro de doentes com CE por slot blotting. Foram considerados para este estudo os cinco antigénios tumorais Tn, sTn, T, sLe^a, e sLe^x. Verificou-se que os níveis de Tn, sTn, e do sLe^a estavam aumentados no soro de pacientes com EC, em comparação com um grupo de controlo, comparável em idade e género, sem doença maligna conhecida. A combinação dos antígenos Tn e sTn permitiu uma melhor discriminação entre CE e os controlos (sensibilidade de 93,8% e especificidade de 100%). A expressão destes antigénios nos tecidos de CE correspondentes, estimada por imuno-histoquímica, mostrou uma falta de correlação com as observações feitas no soro. Os dados sugerem que os padrões de glicosilação do soro são maioritariamente influenciados por proteínas não directamente secretadas ou libertadas por células tumorais, apesar de a sua contribuição não poder ser excluída.

A segunda parte do trabalho visou identificar respostas humorais contra proteínas que transportam o sLe^a, um biomarcador associado à glicosilação com maior potencial de migração celular e metástase. A análise do perfil de IgG das amostras apresentou uma expressão aumentada de uma subclasse (IgG1) nos doentes com CE. As IgG1 produzidas *de novo* por indução tumoral demonstraram possuir sLe^a na sua estrutura, contribuindo assim para o aumento dos níveis deste antigénio no soro de doentes com EC. Ainda que os mecanismos biológicos por detrás deste evento não sejam ainda conhecidos, isso poderá permitir melhorar a sensibilidade e especificidade do teste sorológico de sLe^a (teste de CA19-9). Usando uma combinação de técnicas de immunoprecipiações e de Western blotting, foi ainda demonstrado, pela primeira vez, que as proteínas tumorais que transportam sLe^a podem induzir a produção de IgG1. A remoção do ácido siálico confirmou que a expressão de sLe^a era determinante para o reconhecimento de IgG1. Estas observações, bem como a identificação futura de proteínas imunogénicas transportadoras de sLe^a, prmitirão determinar o seu valor clínico e poderão ser um ponto de partida para o desenvolvimento de testes serológicos baseados em autoanticorpos.

Em suma, eviências importantes foram encontradas com este estudo, que permitirão o desenvolvimento de testes serológicos não invasivos para a detecção de CE

keywords

Esophageal cancer; cancer biomarkers, cancer-associated glycans; humoral response, IgG glycosylation, sialyl lewis(a); autoantibodie

abstract

Esophageal cancer (EC) has an extremely poor prognosis and decreased overall survival. It is generally diagnosed at a late stage, by endoscopy, which is invasive and lacks both specificity and sensitivity. At the moment, there are no biomarkers to improve the accuracy of diagnosis.

The modification of cell glycosylation patterns is a recognized hallmark of cancer, explored in non-invasive diagnostic, therapeutic decision, disease monitoring as well as therapeutics. Moreover, abnormally glycosylated proteins have been proven capable of eliciting humoral responses. Autoantibodies are regarded as the new generation of tumor biomarkers, cable to amplifying events occurring in tumors and showing higher stability to proteolysis. Nevertheless, little is known about EC associated glycosylation nor and its immunogenicity.

Based on these considerations, the first part of this work aimed to identify glycosylation patterns in serum associated with EC. Tn, sTn, T, sale, and sLe^x, the most studied tumors-associated carbohydrate antigens, were screened by slot blotting. The levels of Tn, sTn, and sLe^a antigens were found significantly increased in the serum of EC patients, when compared to a control group of matched age and gender, without known malignancy. Moreover, the combination of the Tn and sTn antigens allowed the best discrimination between EC and controls (93.8% sensitivity and 100% specificity). Still, the expression of these antigens in the corresponding EC tissues, estimated by immunohistochemistry, showed a lack of correlation with the observations made in serum. Data suggests that glycosylation patterns of serum are mostly influenced by proteins that are not directly secreted or released from tumor cells, even though their contribution cannot be excluded.

The second part of the work aimed to identify humoral responses against proteins carrying the sLe^a, a glycosylated biomarker associated with increased potential of cellular migration and metastasis. The analysis of the IgG profile of the samples showed increased expression of IgG subclasse 1 (IgG1) in EC patients. *De novo* produced IgG1 were found to carry sLe^a, accounting for the increase in the levels of this glycan in the serum of EC patients. Even though the biological events underlying these observations remain to be clarified, this may allow improving the sensibility and specificity of the serological test for sLe^a (CA19-9 test). Using a combination of immunoprecipiations and western blotting techniques it was further demonstrated, for the first time, that tumor proteins carrying sLe^a could elicit IgG1 production. Furthermore, experiments using desialylated proteins confirmed that the expression of sLe^a expression was determinant for IgG1 recognition.

These observations and the future identification of the immunogenic proteins carrying sLe^a will allow determining the clinical value of this explorative work and guiding the development of autoantibody-based serological tests.

Altogether, important insights have been provided to guide the development of non-invasive serological tests for the detection of EC.

O único lugar onde o sucesso vem antes do trabalho é no dicionário

(Albert Einstein)

Abbreviations

AC	Esophageal adenocarcinoma
β4/3GalTs	β1,-4/3 Galactosyltranferases
β3/4GnTs	β1-3/4 *N*-acetylglucosaminyltransferases
C1GalT	Core 1 β1-3 Galactosyltransferase or T synthase
C2GnT	Core 2 β1-6 *N*-acetylglucosaminyltransferase
C3GnT	Core 3 β1-3 *N*-acetylglucosaminyltransferase
CMP-Neu5Ac	Cytosine monophosphate *N*-acetyl neuraminic acid
Cosmc	Core 1 β1-3galactosyltranferase-specific molecular chaperone
EB	Barrett´s esophagus
EC	Esophageal cancer
ER	Endoplasmic Reticulum
Gal	Galactose
GalNAc	*N*-acetyl-galactosamine
GERD	Gastroesophageal reflux disease
GlcNAc	*N*-acetyl-glucosamine
Neu5Ac	*N*-acetyl-neuraminic acid
ppGalNAc-Ts	Polypeptide *N*-acetylgalactosaminyltransferases
PTM	Post-translational modification
SCC	Squamous cell carcinoma
Ser	Serine
sLe^a	Sialyl Lewis a
sLe^x	Sialyl Lewis x
sT	Sialyl-T antigen
sTn	Sialyl-Tn antigen
sT3Gal	α 2-3 Sialyltransferases galactosamine
sT6GalNAc	α 2-6 Sialyltransferases *N*-acetylgalactosamine
Thr	Threonine
UDP-Gal	Uridine diphosphate - galactosamine
UDP-GalNAc	Uridine diphosphate - *N*-acetylgalactosamine
UDP-GlcNAc	Uridine diphosphate - *N*-acetylglucosamine

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I. Introduction

I. Introduction

This work addresses the clinical value of glycosylation in esophageal cancer envisaging non-invasive diagnosis. It further explores the possibility of having a humoral response triggered by changes in the glycosylation patterns of tumor tissues and its clinical value.

1. Overview on esophageal cancer

Esophageal cancer (EC) is the 8th most common cancer and the most common cause of cancer death in the world [1, 2]. This malignancy, constitutes 7% of all gastrointestinal cancers and it is one of the most aggressive and lethal of all cancers [3, 4]. Their incidence varies with geographic distribution throughout the world, possibly reflecting differences in exposure to specific environmental factors, individual lifestyle, socioeconomic pressures, and diet and nutrition as well as genetic polymorphisms [5-7]. Incidence is more prevalent in the so-called "Asian esophageal cancer belt'', extending from eastern Turkey through the southern former Soviet Union Iraq, Iran, and into western and northern China, Hong Kong, Japan [3, 6]. However, the incidence of esophageal cancer is largely increasing in the Western population, namely in France, Portugal and northern Italy [1, 8]. This suggests that specific environmental factors play a major role in the etiology of this malignancy [3].

Histologically, the EC has two main forms: the esophageal squamous cell carcinoma (SCC) and esophageal adenocarcinoma (AC), each are distinct etiological and specific risk factors [3, 7, 9, 10]. SCC is a multifactorial process associated with a variety of risk factors, that leads to a sequence of histopathological changes involving esophagitis, atrophy, mild to severe dysplasia, carcinoma *in situ* and finally, invasive cancer [3, 5]. Particularly epithelial dysplasia is regarded as the principal precursor lesion of SCC cancer [6]. On the other hand adenocarcinoma is frequently associated with gastroesophageal reflux disease (GERD) [11, 12]. That is known to lead to Barrett´s esophagus (EB), an acquired metaplastic abnormality in which the normal stratified squamous epithelium lining of the esophagus is replaced by an intestinal-like columnar epithelium [13-15] The carcinogenesis cascade after metaplasia, involves progression to low grade dysplasia, high grade dysplasia and finally the adenocarcinoma as demonstrate in figure 1 [8]. More than

90 % of esophageal cancers are either SCC and less than 10 % are AC [16, 17] but the incidence of SCC has been decreasing whereas that of adenocarcinoma has been dramatically increasing, particularly in Western populations [3, 8, 10]. This increase may be due, in part, to increases in the prevalence of obesity and associated gastroesophageal reflux diseases [18].

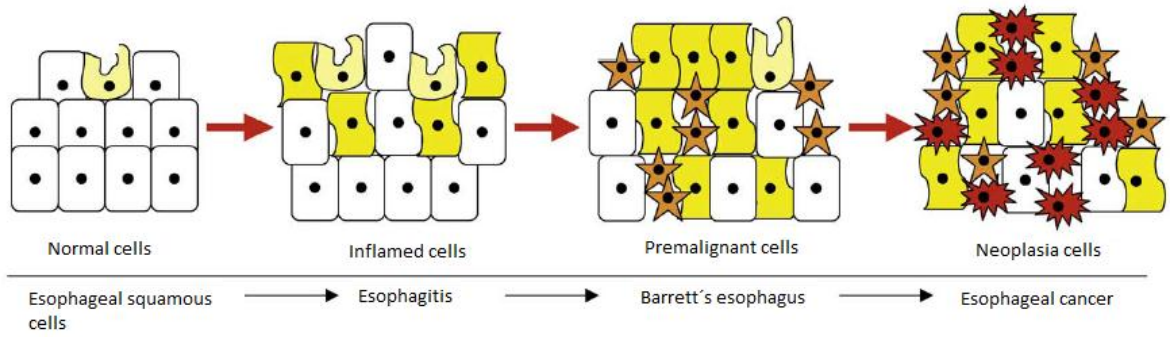

Figure 1. Proposed mechanism of inflammation in the development of metaplasia-dysplasia-adenocarcinoma of the esophagus (adapted from [8]).

The stage of disease at esophageal carcinoma is, so far, considered the most important predictor of survival, but during the early stages of EC, absence of symptoms is very common and the dysphagia is a late symptom that frequently accompanies the advanced stage of the disease [18-20]. Thus, this malignancy has an extremely poor prognosis with an overall survival of 20 % at 3 years [20] and the 5-year recurrence rate was 71 % [21, 22]. In resume, due to late diagnosis, rapid growth and spread, and high rate of recurrence, EC has a poor prognosis [23], and the conventional treatment options frequently include radical esophagectomy [26]. Preoperative and postoperative chemotherapy has been shown to improve survival compared with surgery alone, the addition of concurrent radiotherapy to preoperative chemotherapy improves rates of curative resection, reduces local tumor recurrence, and achieves a significant rate of pathologic complete response [24]. Ongoing research currently involves evaluating new regimens of chemotherapeutic drugs, as well as the incorporation of targeted molecular therapies, making of the molecular characterization of EC a key research field [19].

The diagnostic of EC is done by endoscopy and biopsy [19, 25]. However, endoscopy screening is invasive, costly and lack accuracy [26] since about 20 % of early esophageal cancer lesions are barely visible to the naked eye [19]. Further, this method suffers from poor specificity and sensitivity, which typically results in detection of the

disease only at an advanced stage [25], and consequently reduced overall survival. Individuals presenting histological alterations associated with an increased risk of EC, in particular patients with EB [19].

Molecular and cellular markers for esophageal cancer have not been as extensively investigated as others gastrointestinal tract tumors [17]. However, several *in situ* studies report specific alterations in proteins, genes and metabolic pathways in EC, with some clinical value in diagnosis, prognosis and treatment [12, 17, 25]. Still, only few biomarkers, resumed in Table 1, have been tested in serological assays for non-invasive diagnostic of EC. These include squamous cell carcinoma antigen (SCCA), Cyfra 21.1, Carcinoembryonic antigen (CEA) and sialylated Lewis (a) antigen (CA19-9) [17, 20]. SCCA consists of more than 10 protein fractions, which can be divided in two groups: acid SCCA and neutral SCCA where malignant squamous cells is found mainly in malignant cells [17]. CEA is a glycoprotein produced in normal cells that is overexpressed by adenocarcinomas [17]. CA 19-9 in serum is a carbohydrate present on mucins that are found in patients with malignancies like EC [17]. Cyfra 21.1 are molecules that structurally belong to the family of cytokeratins used for a wide range of neoplasms [17]. Nevertheless, of these biomarkers have not progressed to clinical practice due to the lack of sufficient sensitivity and specificity [11]. Therefore, at the moment, highly specificity and sensitivity biomarkers, for early diagnosis, aid therapeutic decision, or monitor disease, are still to be presented [20, 25].

Table 1. Serum biomarkers of esophageal cancer and their sensitivities and specificities.

Biomarkers	Sensitivity (%)	Specificity (%)	References
CEA	28-40	89	[20, 27]
CA 19-9	23-34	82	[20, 28]
SCCA	32-55	73	[20, 29]
Cyfra 21-1	72	53	[29]

1.1. General considerations about glycans as cancer biomarkers

Changes in the glycosylation of cell-surface proteins have been long recognized as hallmarks of malignant transformation [30], including esophageal cancer [4, 31, 32]. The disorganization of secretory organelles, the deregulated expression/activity glycosyltransferases, the availability of sugar donors for biosynthesis, are among some of the factors leading to these phenotypic changes [33]. Subtle alterations in the glycosylation of tumor cells can be often amplified by the over-expression of specific glycoproteins. Thus, identification of abnormally glycosylated proteins is regarded to increase both the sensitivity and specificity of glycoprotein-based cancer detection [34]. Tumor-associated glycans are frequently shared into the blood stream and may be explored as biomarkers. Some of these antigens, such as SLe^a (CA19-9), SLe^x (CD 15s), and sialyl-Tn (CA72-4) have already been introduced into clinical practice [30, 35].

Also, tumor-associated glycans are known to induce the production of autoantibodies that can be found in the serum of cancer patients [32, 36]. Proofs have been presented that autoantibodies may be raised in early stages of carcinogenesis and even in pre-malignant lesions [37]. Autoantibodies are considered more stable than the corresponding epitopes, and capable of amplifying molecular changes occurring in the tumor [38, 39]. This allows detection long before achieving the critical tumor mass need to obtain measurable amounts of tumor-associated molecules in serum [40]. Despite its potential, the use of autoantibodies for cancer detection has not yet been implemented in clinical settings. This is expected to occur in the near future, as awareness grows about its clinical value. Autoantibodies may also play a key role in the development of novel therapeutics, as they can be used to pinpoint tumor-specific immunogens [41]. As such, the integrated study of glycosylation and glycosylation-associated autoantibodies may provide insights to guide the development of non-invasive diagnostics for EC. It may also allow the identification of novel targets for immune-based therapies.

Based on these considerations, the Introduction section presents an overview on the role, biosynthesis and structural aspects of glycosylation. It then discusses the most studied tumor-associated glycans, which includes simple mucin-type *O*-GalNAc glycans, sLe^a and sLe^x, and systematically addresses the available information about these glycans in EC. It finishes by addressing the clinical value of autoantibodies to tumor-associated glycans.

1.2. Glycosylation in mammalian cells: structure and biosynthesis

Glycosylation is a common post-translational modification of proteins, and is required for the viability of mammalian cells [30, 42]. Glycosylation involves a covalent attachment of carbohydrate to a protein, catalyzed by glycosyltransferases, using specific sugar donor substrates Glycosyltranferases synthesized glycan chains, while glycosidases hydrolyze specify the glycan linkage [42]. The combine action of these enzymes in the endoplasmic reticulum and the Golgi apparatus, determine the glycosylation patterns that allows them to function as signaling, recognition, and adhesion molecules [42-44]. This glycans constitute a significant amount of the mass and structural variation in biological systems [45]. As such, cell surface glycans are involved in many physiologically important functions that include normal embryonic development, differentiation, growth, contact inhibition, cell–cell recognition, cell signaling, host–pathogen interaction during infection, host immune response, disease development, metastasis, intracellular trafficking and localization, rate of degradation and membrane rigidity [30, 42, 43].

There are two main types of glycosidic chain glycoproteins, *N*-glycans and *O*-glycans. Both types of glycosylation often coexist in the same protein and in the same cell [30, 43, 44] and comprehend more or less elongated chains, normally terminated by either ABO blood group determinants of Lewis related antigens [30]. During malignant transformations, both chains experience structural alterations [46]. In this thesis, particular attention is going to be devoted to transformations occurring in *O*-glycans.

O-Glycosylation refers to a class of carbohydrate chains *O*-linked to a serine or a threonine. [30, 44, 46]. The frequency of *O*-glycosylation of glycoproteins is high, particularly on secreted or membrane-bound mucins. These proteins are high-molecular assemblies predominantly expressed in the glandular epithelia [30], and present several serine (Ser) and Threonine (Thr)-rich tandem repeat regions known to be *O*-glycosylated [47, 48].

O-Glycans protect underlying proteins as well as epithelial cell surfaces, maintain protein conformations, control active epitopes and antigenicity; they participate in cell adhesion, binding to microbes and in the immune system [47]. They may determine the cell surface expression and function of cell surface receptors and may be involved in growth regulation [48].

The biosynthesis pathways of *O*-glycans occur in the Golgi complex, and comprehend sequential enzymatic steps that typically rely upon glycan structures produced by the previous enzyme to produce the substrate for the next [42]. *O*-Glycosylation is initiated by the transfer of *N*-acetyl-glactosamine (GalNAc) from a sugar donor UDP-GalNac on hydroxyl groups in serine (Ser) or threonine (Thr) residues [30]. A large of a family of proteins, constituted by more than 15 different isoforms in mammals, showing high overlapping substrate specificity, determine the sites and density of *O*- glycosylation [30]. These enzymes are termed poplypeptide N-acetyl-galactosamineyltransferases GalNac-transferases (ppGalNac-Ts) and catalyze the formation of the Tn antigen (GalNacα-O-SerThr) [36].

After the formation of Tn, chain extension may be terminated by sialylation, by sT6GalNac I-II [44, 48], responsible by the transfer of an N-acetyl-neuraminic acid (Neu5Ac) to the GalNAc residue of a Tn structure, generating the sTn antigen (Neu5Acα2-6GalNacα-o-Ser/Thr) [36, 49] However, sTn presents a restricted pattern of expression in healthy tissues [50].

The elongation of *O*- glycosylation continues when the Tn antigen is the substrate for core 1 β1-3 galactosyltransferases (C1GalT) or T synthase, that depends of molecular chaperone Cosmc, originating the core 1 or T antigen structure [36, 49]. The T antigens can also be sialylated by sT3Gal-I/II resulting in the S3T antigen (Neu5Acα2-3Galβ1-3GalNAcα-O-Ser/Thr) or by sT6GalNac-II/III and IV, originating the S6T antigen (Galβ1-3(Neu5Acα2-6) GalNAcα-O-Ser/Thr). If these enzymes act coordinately the resulting product is the disialyl-T antigen (dsT) (Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα-O-Ser/Thr). Sialylation stops O-chain elongation [46, 48]. Alternatively, in the presence of core 3 β1-3N-acetylglucosaminiltransferase (C3GnT), the core 3 (GlcNAcβ1-3GalNAcα-O-Ser/Thr) antigens is generated [36]. This antigen can be a precursor of core 4 (GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα-O-Ser/Thr) by action of M type β1-6-N-acetylglucosaminetransferase (C2GnT-2) [30]. Nevertheless, the Tn antigen can also be sialylated by sT3Gal-I/II resulting the S3T antigen (Neu5Acα2-3Galβ1-3GalNAcα-O-Ser/Thr) or by sT6GalNac-II/III and IV which originating the S6T antigen (Galβ1-3(Neu5Acα2-6) GalNAcα-O-Ser/Thr). If these enzymes act coordinately the resulting product is the disialylate-T antigen (dsT) (Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα-O-Ser/Thr). Such as the previous sialyltransferase, these also stopping the elongation [46, 48].

Though if not happen these sialylation the elongation of the T antigen continues with the action of the β1-6 N-acetilglucosaminiltransferase (C2GnT-1/3) results in the formation of core 2 (GlcNAcβ1-6(Galβ1-3)GalNAcα-O-Ser/Thr) [30]. The schematic representation is shown in the figure below, figure 2.

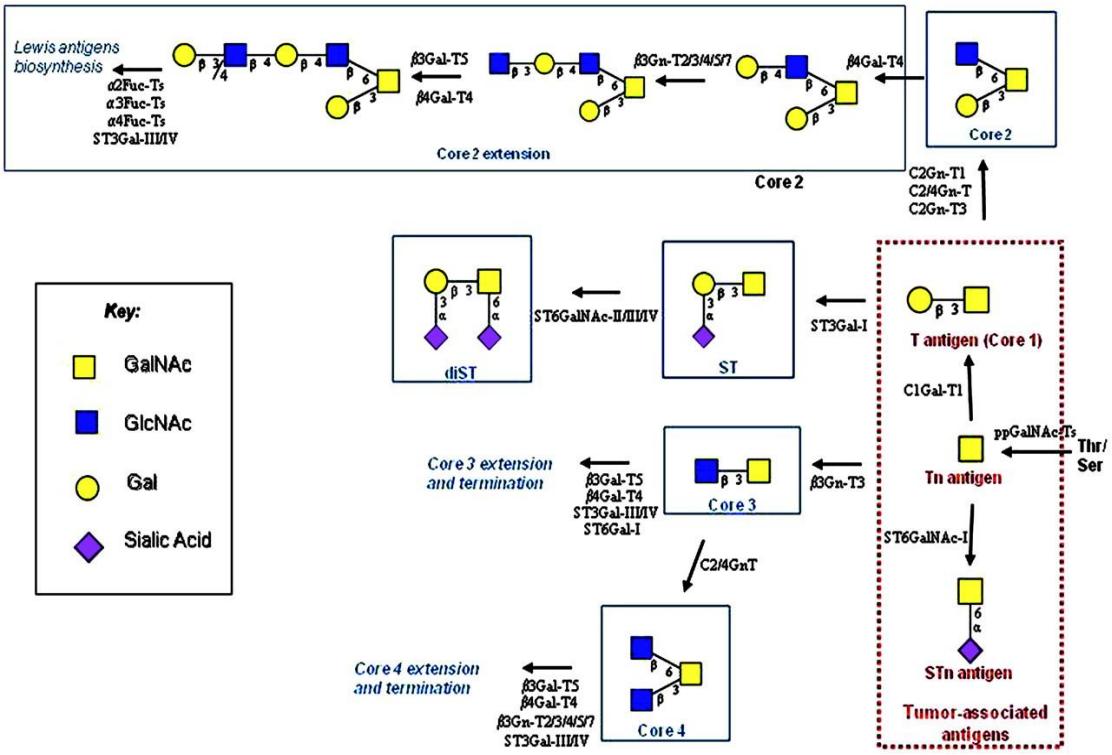

Figure 2. Biosynthesis of *O*-glycans of mucins common with the main tumor associated antigens from the precursor Tn and the action of N-acetylgalactosaminyltransferases (ppGalNAc-Ts); sialyltransferases ST6GalNAc,; core 1 β1-3 galactosyltransferase (C1GalT); core 3 β1-3 N-acetylglucosaminyltransferase (C3GnT); sT3/sT6Gal Core 2 β1-6-N acetylglucosaminyltransferase (C2GnT). (Adapted from [30]).

Core structures 1-4 in particular are elongated by Gal- an GlcNac-tranferases and/or terminated by Fuc-, sialyl-, GlcNac- and /or sulfo-transferases in many different ways [48] however the most common *O*- glycan core structures are cores 1 and 2 [46, 48].

The extension cores structures may be further extended, by the action of N-β3/4acetilglucosaminiltransferase (β3/4 GnT) and β3/4N-acetilgalactosiltransferase (β3/4 GalT), resulting in the formation of type 1 (Galβ1-3GlcNAc-R) and type 2 (Galβ1-4GlcNAc-R) chains [30, 49]. These chains are expressed on the surface of the esophagus, stomach and duodenum glands and not in human epithelia tissues [40, 54-56]. Another

modification, can occur in these chains, by action of fucosyltransferases and sialyltransferases [30], as shown in figure 3. In type 1 chains, FucT-III originates the Le^a antigen (Galβ1-3GalNAcα-*O*-Ser/Thr), that can be a precursor for FucT-I, that transfers a fucose to galactose residue resulting in the Le^b antigen (Fucα1-2Galβ1-3(Fucα1-4)GlcNAc-R) [30, 49]. Type 2 chains may originate the Le^x antigen (Galβ1-4(Fucα1-3)GlcNAc-R) and Le^y antigen (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc-R), that result from the transfer of fucose to GlcNac residue by act of FucT-IV/ VI/ IX and FucT- II, respectively [30, 49]. These structures also can be sialylated by sT3Gal-III generating the sLe^a (NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAc-R) antigen and by sT3Gal-IV/VI sLe^x antigen (NeuAcα2-3Galβ-4(Fucα1-3)GlcNAc-R) [30, 47]. The chain type 2 are most common in non-pathological condition [40, 54, 55].

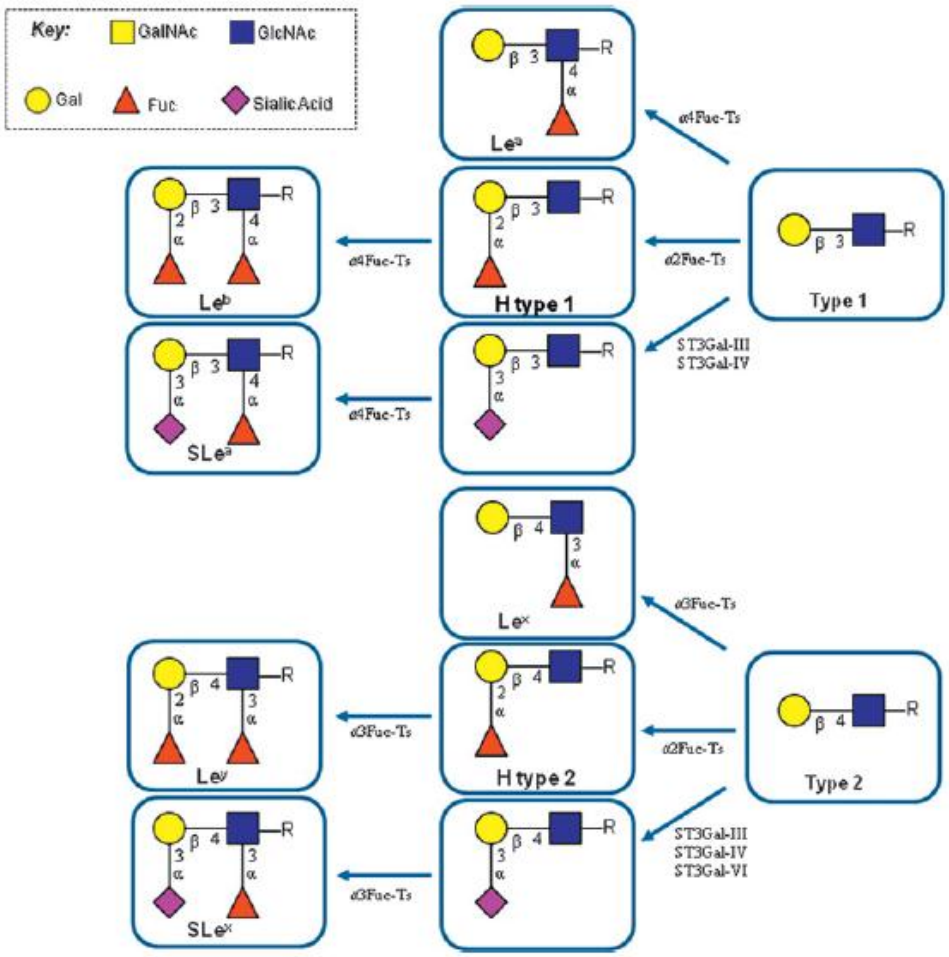

Figure 3. **Schematic representation of the biosynthesis of Lewis antigens. R represents precursor carbohydrate chain. Fuc-T, fucosyltransferase; Le^a, Le^b, Le^x, Le^y, sLe^a, sLe^x, sT3Gal, a2,3-Sialyltransferase** (Adapted from [30]).

1.3. Abnormal glycosylation in cancer

Abnormal protein *O*-glycosylation is considered a hallmark of malignant transformations [43, 51]. These alterations are present on the cell surface of cancer cells, thus easily accessible to antibodies or lectins as tissue biomarkers. Moreover, they are often expressed in the circulation, either on secreted products or by shedding from cell surfaces, and therefore may be explored in serological assays [34].

One of the main structural change results from a premature stop of *O*-chain elongation, normally by sialylation [48, 52]. These events, lead to the expression of a family of low molecular weight *O*-glycans that includes the Tn and T antigens and their sialylated counterparts [32]. Cell-surface and secreted mucins are major carriers of cancer associated *O*-glycans. Since these proteins present several repetitive sequences rich in serine and threonine, they amplify alterations in *O*-glycosylation [46]. As a result, short-chain tumor-associated *O*-glycans are often termed, in the literature, "simple mucin-type" *O*-GalNAc glycans [44]. These alterations stem from the disorganization of secretory pathway organelles (ER and Golgi) in cancer cells and altered glycosyltransferase expression [48]. Occasionally they may depend on mutations in a chaperone essential for glycosyltransferase function [53].

Another common structural feature of cancer cells is the expression of Lewis-type blood group antigens, sLe^a and sLe^x, mimicking their normal expression on blood cells (monocytes and neutrophils) and, consequently, mimicking their potential for migration through binding to endothelial cell selectins [48, 54, 55]. It has been also demonstrated that sLe^a and sLe^x are ligands of endothelial leucocyte adhesion molecule 1 (ELAM-1) in endothelial cells [32]. Moreover, cancer cells use these structures to adhere to activated endothelial cells events which have been recognized as promoters of haematogenous dissemination and metastisation [48]. As a result, sLe^x and sLe^a are generally overexpressed in carcinomas of several origins, *e.g.* cancerous gastric mucosa [48], breast [44, 49], head and neck [56] colon, lung, ovary [34].

Thus, simple-mucin type *O*-glycans, in particular sTn, as well as sLe^x and sLe^a are among the most studied and well characterized tumor-associated glycans. Moreover, these antigens have been transposed to clinical practice and explored as serological markers [57, 58]

1.3.1. T, Tn, sTn antigens

Tn, siayl-Tn, T are present in approximately of all 90 % of carcinomas specifically the gastrointestinal tract [48], colon [54], stomach [59] ovarian [48], breast [32], bladder [60], breast [44] lung [34] head and neck [56] among other tumors [61]. These antigens are generally absent from the normal tissue healthy individuals. However, sTn expression seems to be closely related to the upper digestive tract, still its expression is regulated and restricted to some specific cell types such as squamous cells in salivary glands and esophagus [22, 62].

In particular, T and Tn and sTn antigens often occur in advanced stage tumors [48, 54]. But sTn is also aberrantly detected in premalignant lesions neoplastic of the gastrointestinal tract [59, 63]. sTn has been also associated with a poor prognosis in several types of cancer including colon, gastric, breast and ovarian cancer [44, 48, 54]. It has also been demonstrated that the *de-novo* expression of sTn leads to major morphological and cell behavior alterations in gastric and breast cancer cells [50]. Sialyl-Tn is able to modulate a malignant phenotype inducing a more aggressive cell behavior, such as decreased cell–cell aggregation and increased ECM adhesion, migration and invasion [59].

Thus, simple-mucin type *O*-glycans are useful biomarkers for prognosis, diagnostic value and therapeutics [42, 44, 48]. In particular, the sTn antigen is currently explored as a serological biomarker throughout the CA72-4 assay. Raised CA72-4 concentration has been shown in patients with gastric, colorectal and pancreatic carcinomas [58, 64]. In gastric carcinoma, CA72-4 has been shown to be useful as an independent prognostic factor, and a predictor of tumor recurrence CA72-4 has also been proven to be an independent prognostic factor in pancreatic cancer [65].

The association of sTn with malignancy has also led to the development of an anti-cancer vaccine termed Theratope, comprehending a synthetic sTn coupled to the immunogenic-carrier keyhole limpet haemocyanin (KLH) [50, 66]. Tests in animal models and humans for breast and ovarian cancers have showed that the antigen is safe and produces a strong immune response against these tumors [67]. Recently, this vaccine has failed to provide an increased overall survival in phase III clinical trials for advance stage breast cancer. This was attributable to erroneous design of the study and research is ongoing to develop novel therapeutics based on these antigens [50].

These cancers associated antigens therefore are useful biomarkers for prognosis, diagnostic value and therapeutics [42, 44, 48, 54].

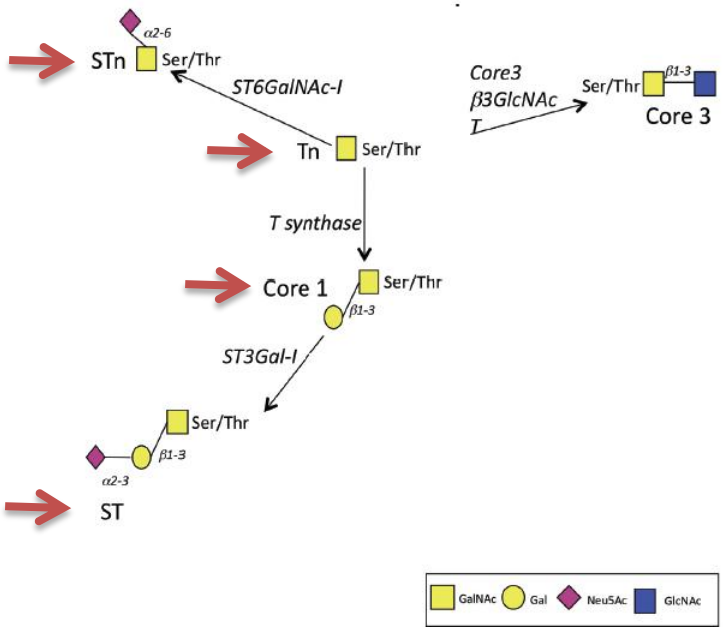

Figure 4. Structures *O*-glycosylated related in cancer (Adapted from [68]).

In cancer cells, many of the glycosyltransferases involved in *O*-glycan biosynthesis are -up or -down- regulated [48, 54, 69]. The increased expression of the T antigen in colon cancer tissues may be due to decreased activity of the C3GnT synthesizing core 3 structures, that favors core 1 synthesis by core 1 C3GnT, since both enzymes compete for GalNac-terminating substrates [33, 36, 48]. On the other hand, a down-regulation of C1GalT-1, resulting in the accumulation of Tn and sTn antigens, has also been observed in tumors [33]. Other glycosyltransferases may also be up-regulated, namely C2GnT1/3. This would result in a net decrease in core 2 synthesis, and a loss of core 4 synthesis [44, 48] as demonstrated in rat colonic mucosa [54]. A down-regulation of core 2 may lead also, an accumulation of T and sTn structures [33].

The up-regulation of sialyltransferases is also a relevant feature behind the premature *O*-glycosylation in cancer [33, 59]. So, the up-regulation of this enzyme in cancer cell might indicate that a specific signaling pathway as been activated [33, 47, 62]. Some studies, have shown that an overexpression of sT6GalNac-I by Ras oncogene overexpression, leads to the over-expression of sTn [33]. Several other studies have also highlighted a correlation between sTn expression and the expression of sT6GalNac-I [36,

62]. The sT6GalNac-I and C2GnT are also known to compete for the same substrate, and the competition between these enzymes may determine the switch from the core 2 to more simple and sialylated *O*-glycan structures [33]. Brockhausen *et al.* further suggests that C2GnT is down regulated during differentiation in colon carcinogens leading to sTn biosynthesis [54].

The overexpression of sT3Gal-I, responsible by the sialylation of the T antigen, has also been observed in primary breast carcinomas and relates to the grade of the tumor [36, 44]. This sialyltransferase, even in the presence of unaltered levels of C2GnT1/3, has been proven capable of inducing a shift from core 2 to sialylated core 1 structure [33].

The over-expression of simple mucin-type *O*-glycans has also been attributed to a rearrangement of a Golgi protein, as observed in colon cancer cells [48, 70]. The rearrangement of Golgi enzymes may prevent the synthesis of essential intermediates for subsequent enzymes or the premature synthesis of terminal structures [42, 48]. Also, the sequential addition of sugars in specific Golgi compartments is also controlled by the relative activities of glycosyltransferases or sulfotransferases acting on common acceptor substrates [44, 47, 48]. If these enzymes are present in the same Golgi compartment, competition may take place, which may unbalance biosynthesis [48, 54]. Change in metal ion concentrations and pH in the Golgi have also been reported in tumors, and are known to affect the activities of glycosyltransferases [47].

Alternative, premature stop in *O*-glycosylation may be induced by the loss of some chaperones [42]. A somatic mutation in the gene Cosmc that encodes a chaperon that is required for expression of active T-synthase, the enzyme responsible for the T antigen has been reported [53, 62] This loss leads to an accumulation of Tn epitope, which can be converted to sTn antigen, if this sT6GalNac-I is up-regulated [62].

1.3.2. sLe^a and sLe^x

Sialylated Lewis antigens, namely sLe^a and sLe^x, have been found over-expressed in gastric [48], breast [44, 49], head and neck [56] colon, lung, ovary cancers [34], whereas no or low positivity was detected in the corresponding histological healthy tissues [71]. The expression of these antigens is not restricted to *O*-glycans, as they can also be found in *N*-glycans as well as in glycolipids The over-expression was associated with increased

α2,3-sialyltransferase and/or α1,3/4-fucosyltransferase activities. Alterations in gene expression of these enzymes have also been reported [49, 52].

Sialyl-Le^a and sLe^x on cancer cells are able to bind to endothelial cells through E-selectin [48, 54, 55]. This interaction has been proposed as a factor in the attachment of cancer cells to the endothelium, and to contribute to promote invasion and metastasis [48]. The expression of these structures appears to be a marker of poorly differentiated adenocarcinomas and is associated with invasive and high proliferative property of the tumors, metastasis and poor clinical outcome [32, 48, 54].

The significance of sLe^a and sLe^x to cancer dissemination as led to attempts to use them, not only as cancer biomarkers, but also as therapeutic targets. One therapeutic strategy is based on the reduction of Le^x synthesis by using competitive disaccharide substrates as decoys [72]. Antisense strategies, directed to a1,3/4-fucosyltransferase, have also proven successful in reducing liver metastisation in a mouse model [73]. The identification of the mechanisms controlling gene expression (methylation, transcription factors, among others) and the identification of tumor-specific protein carriers will permit to improve their usefulness as cancer biomarkers and provide novel targets for immunotherapy [30]

1.4. Alteration in patterns of *O*-glycans in esophageal cancer

Very few studies have addressed the expression of tumor-associated glycans, namely the Tn, sTn, T, sLe^x, and sLe^a, in EC.

The results from a systematic review on the literature, presented from 20.07.12 to 6.12.12, about this subject has been presented in table 1. A total of 14 studies have evaluated at least one of the above described tumor-associated glycans in the esophagus. Three studies involved histologically normal epithelium from healthy individuals; three were on pre-malignant lesions and 12 on EC, 7 of which SCC, 2 EC, and 5 presented no information regarding its histological grade.

Simple mucin-type O-glycans are rarely expressed in normal tissue healthy individuals. However sTn expression seems to be closely related to the upper digestive tract, this expression is regulated and restricted to some specific cell types such as squamous cells in salivary glands and esophagus [22, 50]. Some studies have shown that

sTn is expressed in the submucosa and *lamina propria* of normal squamous epithelium in the esophageal glands. However, sTn was no detected in cells of either the basal layer or the parabasal layer using two different antibodies B72.3 and TKH2 [22]. Still, contradictory results were presented by De Faria *et al*., that did not observe sTn in a series of 8 cases [31].

De Faria *et al*. showed that sTn was mostly absent from dysplastic tissues, but always present in EB, which are precursors of AC. Approximately half of the EB cases also expressed the Tn antigen [31]. The T, sLe^x, sLe^a antigens were not evaluated. Sialyl-Tn was found in 50% of SCC and always in EC [70]. This data suggests that sTn may be characteristic EC and their precursor lesions [22]. The sLe^x was detected in approximately 30% of EC (31% in SCC and 28% in EC) while the sLe^a was found in 40-50% of EC, irrespectively of their histological nature [4, 74]. The T antigen has not been evaluated.

The collected data suggests that the Tn, sTn, sLe^x and sLe^a are expressed in EC but not in healthy esophagus epithelium [22, 31]. This studies further highlighted that these antigens are not correlated with TMN and other clinic-pathological variables, but are associated with poor survival and are present in early stages of carcinogenesis [4, 22, 74-76]. However, more studies should be conducted to validate these observations and establish a glycosylation pattern for EC.

Seven studies have also addressed the expression of tumor-associated glycans in the serum of EC (Table 2). Six evaluated the sLe^a antigen with variable results (16-48% positive cases) [20, 77, 78]. Türkyilmaz *et al.* analyzed 330 ESSC cases and only 16% of the cases were considered positive [77]. This contrasted with the higher sLe^a positivity observed by Mealy *et al*. for 33 AC [20]. This suggests that sLe^a is more expressed in AC than in SCC, however more studies are needed to confirm these observations. The sTn antigen was evaluated in two studies [23, 79], thus not allowing to with draw conclusions about their biomarker value. The Tn and sLe^x antigens have not been evaluated to the date.

Table 2. Summary of studies that correlated aberrant glycosylation and esophageal cancer

	n	Positive cases (%)					Method	Ref
		Tn	sTn	T	sLe^x	sLe^a		
Tissue								
Normal	20	-	0	-	-	-	Immunohistochemistry	[22]
	20	-	-	-	-	0	Imununohistochemistry	[74]
	8	100	0	-	-	-	Imununohistochemistry	[31]
Dysplasia	12	-	8	-	-	-	Immunohistochemistry	[22]
	11	-	-	-	-	4	Immunohistochemistry	[74]
EB	29	48	100	-	-	-	Immunohistochemistry	[31]
EC[1]	89	-	-	-	25	39	Immunohistochemistry	[80]
	74	-	-	-	-	40	Immunohistochemistry	[74]
ESSC	130	-	-	-	31	32	Immunohistochemistry	[4]
	54	-	-	-	-	52	Immunohistochemistry	[76]
	86	-	47	-	-	-	Immunohistochemistry	[22]
	16	75	-	-	-	-	Immunohistochemistry	[70]
	84	-	50	-	-	-	Immunohistochemistry	[75]
EA	7	100	100	-00	-	-	Immunohistochemistry	[31]
Serum								
EC	50	-	16	-	-	-	ELISA	[23]
	50	-	-	-	-	48	ELISA	[77]
	40	-	-	-	-	*	ELISA	[81]
SCC	25	-	-	-	-	24	ELISA	[20]
	330	-	-	-	-	16	ELISA	[78]
	43	-	*	-	*	*	Microarrays	[79]
AC	33	-	-	-		42	ELISA	[20]

* The percentage of positive cases was not reported and could not be inferred from the data provided in the manuscripts. Still, significant correlations with the pathological condition were reported in both cases ($p<0.05$).

1.5. Cancer associated antibodies

Antigenic changes in tumors can be recognized by the immune system leading to the production of immunoglobulins [82]. This promotes a very efficient biological amplification of tumor-associated events, with antigenic tumor proteins as templates, allowing an indirect, more sensitive detection [38].

The immune response is generated locally, or tumor-associated proteins can be processed by antigen-presenting cells and displayed by lymphocytes in the lymph node the tumor site [38]. This response may begin at an early stage as 5 years before a tumor is not clinically detectable and be detected in circulation [38, 40, 68, 83].

However, small populations of tumor cells that create a humoral immune response at an early may not continue to malignancy and are eliminated [68]. Moreover, advanced stage cancers may not only be able to grow and thrive within the constraints of the immune system, but also have an inhibitory effect on inflammatory function and recognition of antigens thus avoiding a immune surveillance [84]. Thus, stage-specific humoral response patterns can be generated [82] and explored in clinical practice.

Humoral responses can either be generated by a neo-epitopes or by enhanced self-epitope presentation to major histocompatibility complexes or the T-cell receptors. Another hypothesis involves tumor cell lyses and the presentation of intra-cellular molecules to the immune system in an inflammatory environment [41]. This event also releases proteases that may unveil cryptic epitopes that may trigger an autoimmune responses [40].

Although little is known about the origin of this immune response, it is now established that mutated proteins, misfolded, over-expressed, aberrantly degraded, aberrantly localized [38, 40] or aberrantly glycosylated proteins may induce autoantibody production [38, 85, 86]. The dominant immunoglobulins in autoimmune diseases and against tumor-associated antigens, is IgG with reactivity significantly greater within the IgG3 subclass [85, 87, 88]. In ovarian cancer patients, the immunoreactivities of IgG2 and IgG3 to nuclear and membrane antigens, in all stages of the disease, were greater than that observed in controls or patients with benign disease [85].

Some studies have reported the presence of autoantibodies against certain cellular proteins, including p53 and procathpsin D, using western blotting in advanced ovarian

cancer patient and correlated these observation with poor prognosis and poor survival [86, 89, 90].

Lacombe and colleagues using proteomic approach and serologic screening of breast cancer patients related autoantibodies against five antigens (GAL3, PAK2, PHB2, RACK1 and RUVBL1), these antigens are related with early-stage breast cancer [83]

The most common techniques used in the identification of autoantibodies and corresponding tumor-associated antigens are described in Table 2, and include the serological proteome analysis, immunoprecipitation of antigens followed by mass spectrometric analysis and antigen microarrays [39]. However, the most used remain ELISA (sandwish enzyme-linked immunosorbent assay) and Western blot [86, 91].

Table 3. **The methodologies that enable to detected autoantibodies**

Method	Description	Ref
ELISA	Tumor lysates are incubated with commercial antibody arrays so that each antigen is immobilized on a different spot.	[37, 38, 40, 87, 91]
Microarrays	The TAAs may be directly spotted on microarrays and probed with specific human sera.	[38, 40, 41, 82, 91, 9
Phase display	Autoantibodies in patient sera are captured by the phage display library through successive rounds of immunoprecipitations and the corresponding TAAs are sequenced for identification	[38-40]
Serologic Identification of Antigens Recombinant Expression (SEREX)	Identification of TAAs by screening patient sera against a cDNA expression library obtained from the autologous tumor tissue	[38-41]
Serological Proteome Analysis (SERPA)	Proteins from tumor tissue are separated by 2D electrophoresis, transfered onto membranes and probe with sera from healthy individual and cancer patients. Then the profiles are identified by mass spectrometry (MS)	[38, 40, 91]
Western Blot	The TAAs are transferred to a membrane and are incubated with autoantibodies against target protein	[40, 41, 90, 91, 93]

In resume, the identification of autoantibodies in the serum of cancer patients has created opportunities for a novel source of cancer biomarkers for cancer screening, diagnosis and immunotherapy [41, 83, 91]. There are various reasons for this, these autoantibodies can be detected in early and asymptomatic stage; autoantibodies are found in serum of patient and they are accessible to screening; and for last, autoantibodies are inherently stable and have a long half-life (7 days) than the corresponding epitopes, because they are generally not subjected to the types of proteolysis experienced by all other proteins [39, 40, 83, 92]

1.6. Autoantibodies present in esophageal cancer

Few studies exist addressing the humoral response against EC tumor-associated antigens, namely against intra-tumoral p53 protein accumulation [94, 95]. Others reports related autoantibodies against shock protein 70 (Hsp) and peroxiredoxin IV (Prx) in SCC [91, 93]. In particular, Fujita *et al*., described elevated levels of autoantibodies against Hsp70 in 93.7% of the sera from 16 patients with SCC, using western blot and a proteomics-based approach. Even though the levels of autoantibodies were significantly higher in the patients, they could also be found in healthy individuals, demonstrating autoantibodies can also be present in non-pathological conditions [93]. Kilic and colleagues also demonstraed, using microaarays in 18 patients, the presence of serum autoantibodies for 51 AC associated proteins. The most discriminating protein was FasL with 88.9% sensitivity and 100 % specificity [96]. A report concerning autoantibodies for the oncogenes CDC25B has also been presented. This showed higher sensitivity and specificity than tumor markers CEA, SCC-A and CYFRA21-1 (Table 1) for diagnosis of SCC [97]. Humoral responses for oncogene Bmi correlated with tumor stage and lymph node status [98]. All together, these reports point out that autoantibodies are promising, and more accurate biomarkers when compared to the ones presented in Table 1, for the non-invasive diagnostic of EC.

1.7. Humoral response against aberrant glycosylation

As described above the immune response can be the consequence of several mechanisms, including aberrant glycosylation [86, 88, 90]. Studies showed that Tn-glycoform induces potent IgG responses in MUC transgenic mice and cancer patients, that had no preexisting and all developed detectable IgG Tn-MUC1 antibodies after vaccination and a lower expression in healthy tissues [37, 99].

Blixt and colleagues, demonstrating using *O*-glycopeptide array-based assay, raised levels of autoantibodies against Tn-, core 3- and sTn-glycoforms in breast cancer patients [68]. These studies also concluded that these autoantibodies are found more frequently and at higher levels in early stage breast cancer patients than in women with benign breast diseases or healthy woman [68]. Also, individuals with higher autoantibodies titers against mucins have a better prognosis than patient with free circulating anti-mucin antibodies as (Core 3/Tn/sTn-MUC1) [37, 87, 99]. Similar results were found by Pedersen *et al*., in sera of colon cancer patients, with sTn-MUC1, and resulted in the detection of 79% of cancer patients, with a specificity of 92% [99]. However, autoantibodies for Tn- and sTn-MUC1 glycoforms showed more specificity in cancer patients, whereas the core 3-MUC1 were also detected in patients with chronic inflammatory lesions [92]. Others studies, demonstrated circulating autoantibodies that recognized these structures in breast, pancreas, ovarian, prostate and colon cancer patients at the time of diagnosis [37, 87, 99]. No difference in these autoantibody levels were detected among stage I, II, or III in colorectal cancer while levels were lower in patients with metastasis [99]. The same was found in pancreatic cancer where serum autoantibodies levels were more strongly associated with levels status than clinical prognosis factors [100]. The subclass of immunoglobulin predominant against core3/sTn-MUC1 is IgG2 [85, 99].

Additionally, Ravindranath *et al*., showed the presence of autoantibodies IgG against sLe^x in melanoma cells and are correlated with potential endpoint to assess the therapeutic effectiveness of vaccine therapy [101].

In esophageal cancer patients there are no studies relating circulating autoantibodies and altered glycosylation/glycoproteins.

II. Aims and scopes

2. Aims and scopes

EC has an extremely poor prognosis, an overall survival of 20 % at 3 years [20] and a 5-year recurrence rate of 71 % [21, 22]. It presents a poor prognosis due to late diagnosis, rapid growth and spread, and high rate of recurrence [23]. The diagnostic is mostly achieved by endoscopy which is invasive and fails to detect approximately 20% of early lesions, barely visible to naked eye [19]. Furthermore, this method has poor specificity and sensitivity and, at the moment, there are no biomarkers to improve the accuracy of diagnostic [20, 23].

The modification of cell glycosylation patterns is a recognized hallmark of cancer explored in non-invasive diagnostic, therapeutic decision, disease monitoring as well as therapeutics. Moreover, abnormally *O*-glycosylated proteins have been proven capable of eliciting humoral responses. Autoantibodies to tumor-associated antigens are regarded as the new generation of tumor biomarkers, as they are able to amplify events occurring in tumors and are more stable in circulation than the corresponding epitopes. Moreover, they can be used to pinpoint immunogenic proteins that may be explored in therapeutics. Despite this, little is known about EC associated glycosylation and no studies have been presented concerning its immunogenicity.

Based on these considerations, this thesis aims to identify humoral responses against abnormal EC glycosylation. The first part of the experimental section devotes to the characterization of the glycosylation of a series of EC and corresponding sera. The Tn, sTn, T, sLe^x, and sLe^a antigens, which are the most studied tumor-associated glycans, will be evaluated in an attempt to establish a glycosylation pattern for EC. The second part of the work will focus on the identification of humoral responses against EC proteins carrying abnormal glycosylations.

The generated information will allow guiding future studies for the development of an autoantibody-based non-invasive diagnostic method for EC. Important insights on the immunogenicity of EC glycosylated proteins will also be generated.

III. Material and methods

3. Material and methods

3.1. Overview

The first part of this thesis devotes to establishing a serological glycosylation profile for EC by slot blotting. Four of the most studied tumor-associated carbohydrate antigens, that include the Tn, T, sT, sLe^x, and sLe^a, were screened. In addition, these antigens were evaluated in some of the corresponding EC tissues by immunohistochemistry, to highlight common patterns. The s3T and s6T antigens were also evaluated, for the first time, in EC tissues using the combination of enzymatic treatments and antibodies shown in Figure 5. The generated data was comprehensively analyzed to determine tumor-associated antigens presenting the highest discriminative power.

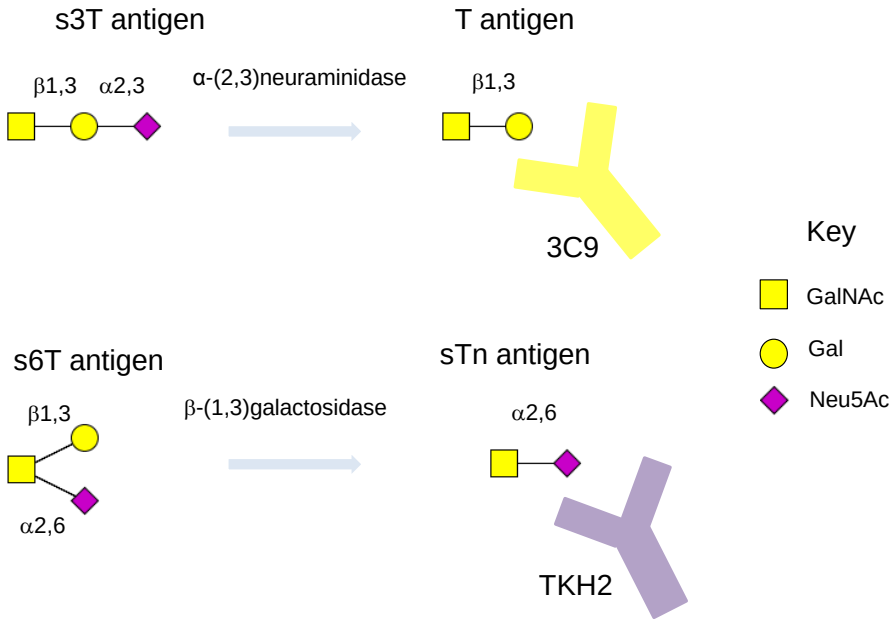

Figure 5. The s3T and s6T antigens structures using the combination of enzymatic treatments.

The second part of the thesis contemplated the characterization of the IgG profile of EC-associated sera and the mining of humoral responses against the EC proteins exhibiting altered glycosylation. Briefly, the proteins presenting altered glycosylation were recovered from EC tissues by immunoprecipitation and blotted with IgGs from the sera of EC

patients. The identification of immunoreactive bands allowed confirming humoral responses against tumor glycoproteins. An overview on the analytical strategy adopted is presented in Figure 6.

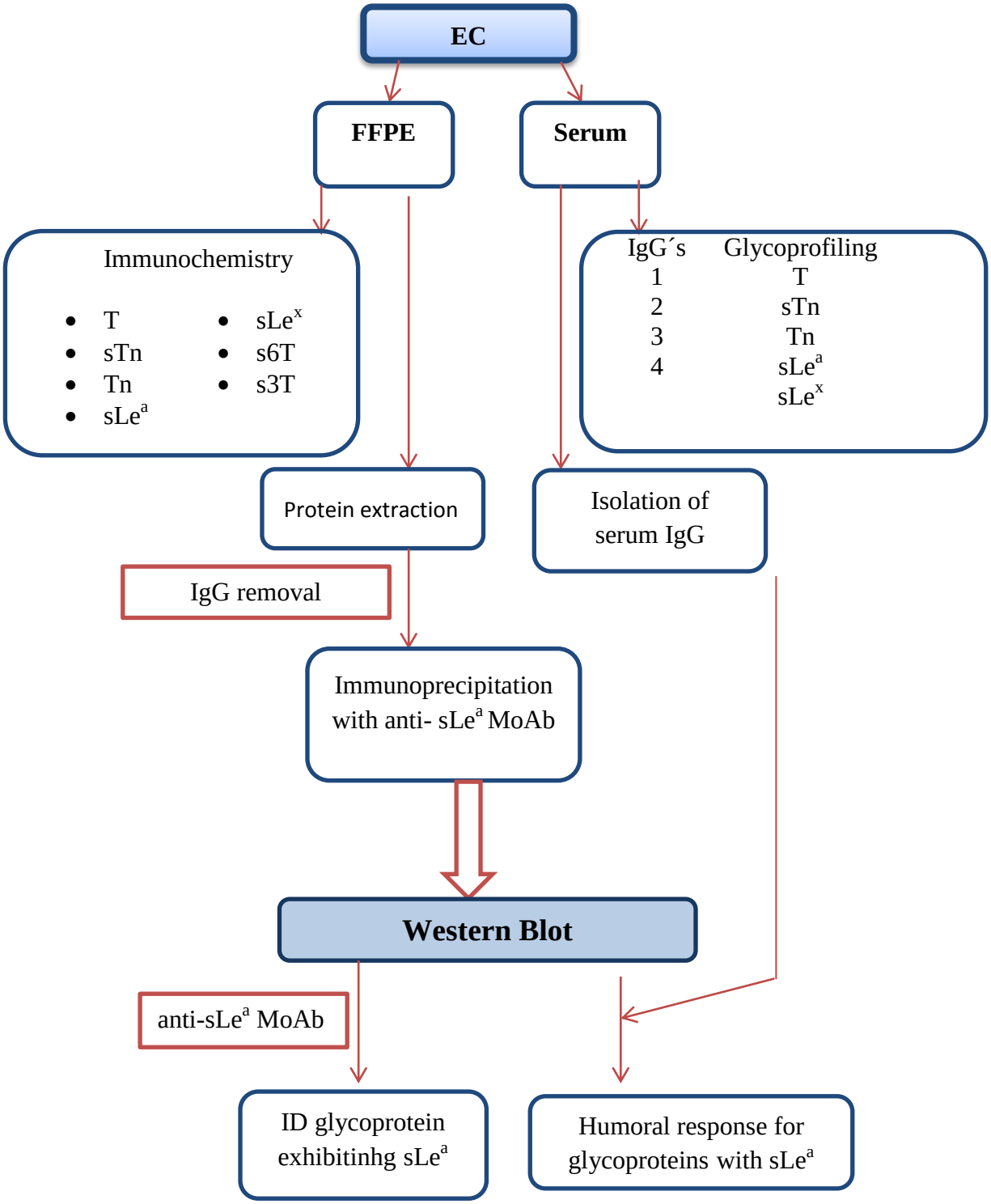

Figure 6. Overview on the analytical strategy.

3.2. Patient and sampling

Sixteen patients (14 men and 2 women), mean age of 65 (age range 51–89), facing primary diagnostic with esophageal cancer (EC) in Portuguese Institute for Oncology of Porto (IPO-Porto, Portugal) between March 2011 and May 2012, have been elected for this study. According to the patient's clinical records, 7 were classified as squamous cell carcinomas (SCC), 3 as esophageal adenocarcinomas (AC), whereas for 6 cases no information was available at the time of the study. None of these patients had received prior adjuvant therapy.

Whole blood was collected from all of the patients in tubes without anticoagulant. The samples were kept at room temperature for approximately 1 h and then centrifuged for 5 min at 3,000 rpm at room temperature. The supernatant (serum) was then collected and stored at -80ºC. Individuals within the same mean age and age range, without known neoplasia, were included as controls. The sampling also included 7 FFPE (Formalin-fixed paraffin embedded) tissues, (5 SCC and 2 AC) and the corresponding adjacent mucosa. All procedures were performed under the scope of Foundation for Science and Technology (FCT) financed project PIC/IC/82716/2007, under the approval of the Ethics Committee of IPO-Porto, after patient's informed consent.

3.3. Immunohistochemistry for simple mucin type *O*-GalNAc glycans, sLe^a, sLe^x and human IgG1

FFPE tissue sections were screened for Tn, sTn, T, s6T, s3T, sLe^a, sLe^x antigens and human IgG1 by immunohistochemistry (IHC) using the avidin/biotin peroxidase method. Briefly, 3 μm sections were dewaxed with xylene, rehydrated with graded ethanol series and microwaved for 15 min in boiling citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0). The slides were then exposed to 3% hydrogen peroxide in methanol for 20 min. The expression of the Tn, sTn, T, sLe^a, sLe^x, antigens and human IgG1 were then directly evaluated using mouse monoclonal antibodies IE3, TKH2, 3C9, anti-sLe^a clone 192, anti-sLe^x KM92, anti-human IgG1, respectively (Table 4). The expression of s3T was determined comparing the reactivity of the tissues with 3C9 antibody, before and after treatment with an α–(2→3)-neuraminidase from *Streptococcus pneumonia* (Sigma-

Aldrich) to yield the T antigen. The expression of s6T was evaluated comparing of the reactivity of the tissues with TKH2 antibody, before and after treatment with a recombinant β-(1-3)-Galactosidase from *Xanthomonas campestris* (R&D systems) to yield the sTn antigen. Prior to analysis, the sections were blocked with normal serum from Vectastain Elite ABC peroxidase kit (Vector Lab) for 30 minutes to avoid non-specific staining and then incubated with the enzymes, whenever the case, at 37ºC for 16 hours. The sections were then further incubated with specific antibodies at 37ºC for 16 hours. Afterwards the sections were washed with PBS-T (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4), incubated at room temperature for 30 minutes with the diluted biotinylated secondary antibody, and then with Vectastain Elite ABC reagent (Vector Lab). After washing in PBS-T, the sections were incubated with 3,3-diaminobenzidine tetrahydrochloride (ImmPACT™ DAB, Vector Labs) for 5 minutes at room temperature to visualize antibody binding sites. Finally, they were counterstained with Harri´s hematoxylin for 1 minute. Bladder carcinomas known to express the glycosylations of interest were used as positive controls. The negative control sections were performed by adding BSA (5% in PBS) devoid of primary antibody. A semi-quantitative approach was established to score the immunohistochemical labeling based on the intensity of staining and the percentage of cells that stained positively. The labeling was be assessed double-blindly by two independent observers and validated by an experienced pathologist. Whenever there was a disagreement, the slides were reviewed, and consensus was reached.

Table 4. Characterization of the primary antibodies used in this thesis.

Targeted Antigen	Antibody	Isotype	Host Specie	Conjugate	Company	Concentration (µg/mL) Western Blot	Slot Blot	IHC
Tn	1E3	IgG	Mouse	-	hybridome	1:15*	1.5:20*	1:10*
sTn	TKH2	IgG1	Mouse	-	hybridome	3:20*	3:20*	1:10*
T	3C9	IgM	Mouse	-	hybridome	1:15*	1.5:20*	1:10*
sLe^a	Anti-Human sLe^a, clone 192	IgG1	Mouse	-	Santa cruz Biotech	0.75	1	0.1
sLe^x	Anti-human sLe^x, clone KM92	IgM	Mouse	-	Millipore	1.25	1	0.2
s6T [1]	TKH2	IgG1	Mouse	-	hybridome	-	-	1:10*
s3T[2]	3C9	IgG1	Mouse		hybridome	-	-	1:10*
Human IgG1	Anti human IgG1, clone HP6069	IgG1	Mouse	HRP	Invitrogen	0.01	0.02	-
Human IgG2	Anti-human IgG2, clone HP6014	IgG1	Mouse	HRP	Invitrogen	0.01	0.02	-
Human IgG3	Anti-human IgG3, clone HP6047	IgG1	Mouse	HRP	Invitrogen	0.01	0.02	-
Human IgG4	Anti-human IgG4, clone HP6025	IgG1	Mouse	HRP	Invitrogen	0.01	0.02	-

* Dilution from culture supernatant
* After treatment with

3.4. Expression of glycosylated antigens and IgG profiling in serum

The expression of Tn, sTn, T, sLe^a and sLe^x antigens in serum was determined by Slot Blotting. Fifty micrograms of serum proteins diluted in TBS (100 mM Tris, 1.5 mM NaCl, pH 8.0) were slot-blotted on a nitrocellulose membrane (Whatman, Protan; pore size 0.45 μm) using the Hybri-slot apparatus (21052-014; Gibco BRL, Life Technologies). Equal amounts of BSA were used as negative controls. The membranes were first blocked with Carbo-Free Blocking Solution (SP-5040, Vector Laboratories) for 30 minutes at room temperature and incubated with the monoclonal antibodies presented in Table 4. The membranes were then washed with TBS-T (TBS with 0.5 % Tween 20) and incubated with either goat anti-mouse IgG1 heavy chain (Abcam; 1:35,000 in TBS), goat anti-mouse IgG (H+L; Jackson ImmunoResearch; dilution 1:30,000 in TBS) or rat anti-mouse IgM (mu chain; Abcam; dilution 1:30,000 in TBS) horseradish peroxidase conjugates, depending on the primary antibody being evaluated. Controls for the sTn, sLe^a, and sLe^x include samples digested with a α-neuraminidase from *Clostridium perfringens* (Sigma), and for the T antigen samples digested a β-(1-3)-Galactosidase from *Xanthomonas campestris* (R&D systems).

IgG subtypes 1, 2, 3, and 4 were also accessed by slot blotting using 15 μg of serum proteins. In this case, after blockage with carbo-free, the membrane was directly incubated with mouse anti-human IgG1, IgG2, IgG3 and IgG4 (Invitrogen).

Antibody-reactive bands were detected by enhanced chemiluminescence ECL prime (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Images were recorded using X-ray films (Kodak Biomax light Film, Sigma). The films were scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad) and analyzed with QuantityOne software (v 4.6.3 Bio-Rad). All analyses were performed in triplicates, and the presented results are the arithmetic average of these measurements.

3.5. Quantification of Total IgGs in serum

The quantitative determination of human IgG in serum was determined by an immunoturbidimetric tests with the Olympus 2700 auto-analyzer using commercial Olympus kits. Briefly, human serum was diluted in TBS and polyethylene glycol and

mixed with polyclonal goat anti human IgG. The generated immune complexes or cause an increase in light scattering that correlates with the concentration of IgG in the serum. Light scattering is measured by reading turbidity at 700 nm in an Olympus 2700. IgG standards 0.75-30.0 g/L were used to establish a calibration curve.

3.6. Protein extraction from tumors

Proteins were extracted from FFPE sections (3x10μm) after deparaffinization of the tissue using Q-proteome FFPE Tissue Kit (Quiagen) according to the manufacturer's instructions. The proteins were then recovered in the supernatant by centrifugation at 14,000 g at 4ºC for 30 min. The remaining pellets were then washed with cold RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM NaCl, pH 7.4) with 0.2 M NaOVa, Protease Inhibitor Cocktail (Roche) to increase protein yields. Total protein extracts were depleted of IgGs and other proteins with affinity for protein G by incubation with protein G-Sepharose beads (Sigma) for 4 h at 4 ºC. The amount of protein in each fraction was determined using the RC-DC kit (BioRad), based on a modification of the Lowry method.

3.7. Isolation/Removal of immunoglobulins

Serum immunoglobulins were recovered by incubation with protein G-Sepharose beads (Sigma). Prior to use, the beads were equilibrated with PBS and then incubated with human serum at 4º for 4 hours under gentle rotation. The unbound material was then removed by centrifugation at 1,000 *g* and the beads were extensively washed with PBS. The immunoglobulins were then recovered in 0.2 M glycine pH 3.0 and the buffer was immediately exchanged to PBS, using Amicon Ultra centrifugal 10 kDa MWCO filters (Sigma), to avoid protein denaturation. The same procedure was applied to remove immunoglobulins from protein extracts isolated from tumor tissues.

3.7.1. Isolation of sLe^a expressing proteins and western blotting

Briefly, the immunoglobulin-depleted protein extracts dissolved in RIPA buffer (approximately 100 μl) were incubated with mouse monoclonal anti-sLe^a IgG1 (Table 4)

for 16 h at 4ºC under gentle rotation, followed by incubation with protein G-Sepharose beads (Sigma) for 4 h at 4 ºC. The unbound material was removed by centrifugation at 1,000 *g* for 10 min at 4ºC and the beads were washed extensively with RIPA buffer. Bound proteins were eluted by boiling in sample buffer (125 mM Tris–HCl, pH 6.8, 4% (*v/v*) SDS, 10% (*v/v*) glycerol, 0.02% (*w/v*) bromophenol blue, 10% (*v/v*) β-mercaptoethanol) for 10 min. Supernatants were subjected SDS–PAGE electrophoresis in 4-12% Mini-PROTEAN TGX Precast Gels (BIORAD) and transferred onto an 0.45 μm Immobilon-P polyvinylidene di-fluoride (PVDF, Millipore) membrane in 25 mM Tris–HCl, pH 8.3, 192 mM glycine, 20% methanol at 50 mA for 2 h. Membranes were blocked with 1% carbo-free (Vector laboratories) for 1 h at room temperature, incubated for 45 min with monoclonal anti-sLe^a IgG1 (Table 4), washed with TBS-T for 30 min, and finally incubated for 1 h with goat anti-mouse IgG1 heavy chain horseradish peroxidase conjugate (Abcam; 1:35,000 in TBS). After washing, the bound antibodies were revealed by chemiluminescence using the ECL prime Kit (BIORAD).

The membranes were then submitted to mild stripping for further reproving. First the membranes were incubated with 1.5% glycine (*w/v*), 0.1% SDS (*w/v*), 1% Tween20 (*v/v*) pH 2.2 for 10 min two times at room temperature, followed by PBS and TBS-T. The efficiency of stripping was checked by incubating the membrane with chemiluminescent detection reagent. The protein content in the bands was monitored using MemCode™ Reversible Protein Stain Kit for PVDF membranes (Pierce).

3.7.2. Identification of humoral responses against tumor glycoproteins expressing sLe^a

The proteins expressing sLe^a, immobilized in PVDF membranes in section 3.7.1, were blotted with serum IgG from the corresponding patients, isolated as described in section3.7. These membranes were then incubated with mouse anti-human IgG1 horseradish peroxidase conjugates (Table 4) and revealed by chemiluminescence using the ECL prime Kit (BIORAD). Afterwards, the membranes were then mild stripped as described in section 3.7.1, blocked with 1% carbofree (Vector Laboratories) and incubated for 16 h with an α-neuraminidase from *Clostridium perfringens* (Sigma) at 37ºC. This was used as control experiment to determine the specificity of humoral responses against sLe^a.

3.8. Statistical Analysis

The statistical tests used in this thesis included Student t-test for unpaired samples. Differences were considered to be significant when $p < 0.05$. Also, a chi-square test was used to analyze correlations between clinicopathological features and antigen expression patterns. A Principal Components Analysis (PCA) was used to extract correlations between samples regarding the expression of tumor-associated carbohydrate antigens. Receiver operating curves (ROC) were used to estimate the sensitivity and sensibility of tumor-associated carbohydrate antigens in the serological detection of EC. The coefficient of correlation (R^2) was used to estimate the degree of adjustment of sLe^a *vs* IgG/IgG1 plots to a linear regression model**.**

IV. Glyco-serological profiling of EC

4. Glyco-serological profiling of EC

As described in detail in the Introduction chapter, alterations in the glycosylation patterns of tumor cells are considered a hallmark of malignant transformations [30]. These glycoproteins are often expressed in the peripheral blood circulation, either on secreted products or by shedding from cell surfaces [102]. Thus, several glycans are currently used as serum biomarkers in several cancers [59].

Despite the biomarker value of cancer-associated glycans, little information exists about serological glycosylation patterns associated with EC. Moreover, few studies have addressed the expression of the most studied tumor-associated carbohydrate antigens, such as the Tn, sTn, T, sLe^a, and sLe^x in EC. Thus, this chapter devotes to study the expression of these antigens both in the serum and EC tissues, with the intention of highlighting putative biomarkers.

4.1 Results

4.1.1. Screening of tumor-associated glycans in serum

A set of 16 sera from EC patients was characterized in relation to the expression of Tn, sTn, T, sLe^a, and sLe^x, which are the most studied tumor-associated glycans, by slot blotting. Ten individuals with matched age and gender, and without known oncological disease were included as a control group.

As shown in Figure 7B, the serological levels of sTn, T, sLe^a and sLe^x antigens were significant different between the two groups. The sTn and sLe^a were higher in the EC group ($p<0.05$) while T and sLe^x were significantly lower ($p<0.001$) when compared to the controls. The serum levels of the Tn antigens were also more elevated in the EC group, however this was not statistically significant. It was also attempted to correlate the expression of these antigens with clinicopathological nature of the tumor, sex, and age using a Chi-square test; however no correlations were found.

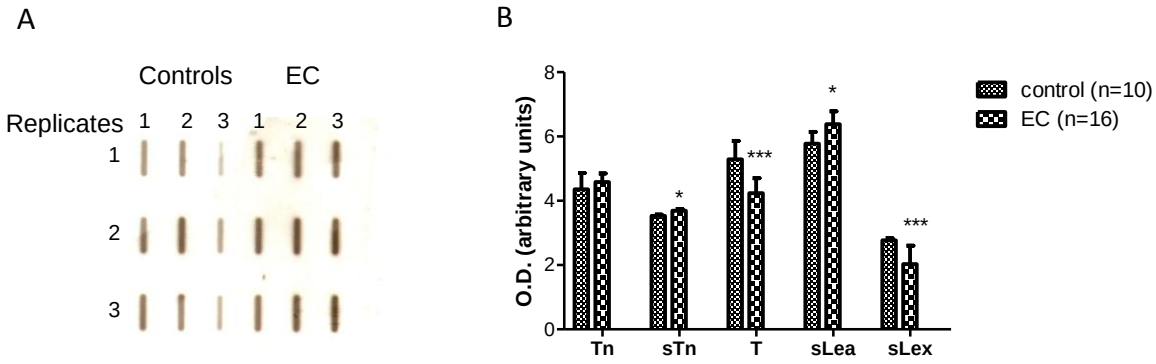

Figure 7. Serological levels of tumor-associated glycans sTn, T, sLe^a and sLe^x antigens in controls (n=10) and EC (n=16). A) Slot blotting for sLe^a showing a higher expression of this antigen in EC. B) Resume data from slot-blotting regarding the expression of Tn, sTn, sLe^a and sLe^x in serum. sTn and sLe^a were significantly increased in EC, whereas T and sLe^x were decreased. The Tn antigen was also increased, even though not statistically significantly, in the EC group. Data is presented as mean ± standard deviation (n=3 for each samples). * $p<0.05$; *** $p<0.001$ vs the preceding concentration.

A Principal Components Analysis (PCA) was then applied to the generated data in an attempt to isolate groups based on the most discriminative antigens. The Scores plot presented in Figure 8 show that the PC1 and PC2 accounts for 68% of the sample, with PC1 presenting 45.8% and PC2 the remaining 21.9%. The scores plot (samples plot; Figure 8-A) shows that approximately 88% of the EC are in the negative PC1 axis, whereas 80% of the control samples are in the positive PC1.

According to the Loadings (variables plot; Figure 8-B) the EC group is characterized by higher sTn and sLe^a and to some extent Tn, as previously observed in Figure 8- B. Conversely, the control group is discriminated based on higher levels of T and sLe^x. The PC2 axis does not allow discriminating more groups with the available information. Moreover, it was not possible to discriminate the tumors in relation to their clinic-pathological classification using PCA, thus in accordance with the results from the Chi-square test. Altogether, this serological analysis suggested that a combination of Tn, sTn and sLe^a could be used to better discriminate between EC and the control group. All together, this data demonstrates that, in addition to the sTn and sLe^a antigens, the Tn can also be used to characterize EC patients using serum

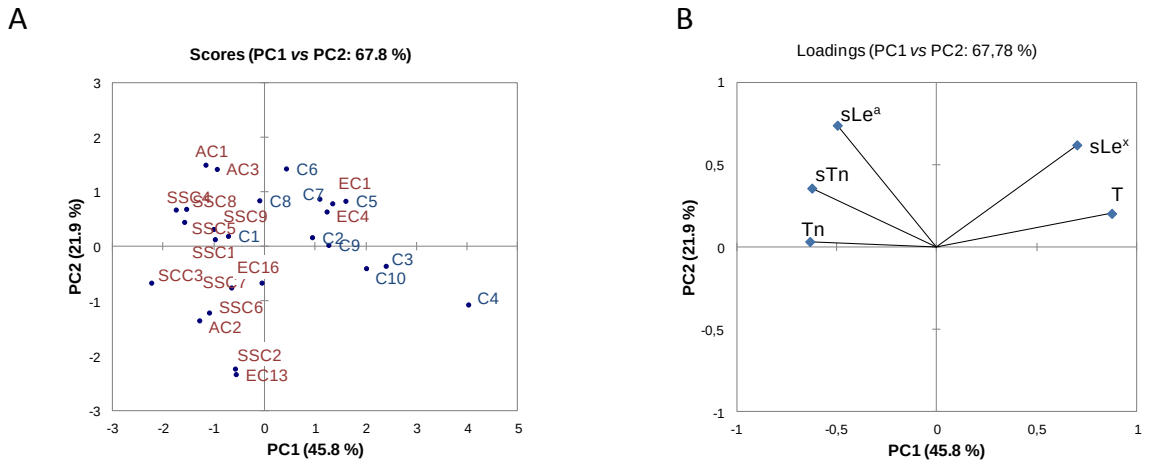

Figure 8. **Principal components analysis (PCA) for the expression of tumor-associated glycans Tn, sTn, T, sLe^a, and sLe^x in serum for controls (n=10) and EC (n=16).** A) Scores plot for PC1 *vs* PC2 accounting for 67.8% of sample variance. Samples marked red correspond to EC (SCC, AC, and those without available clinical data, termed generally as EC); controls were marked blue. EC mainly found in the negative PC1 (87.5%) whereas the controls are in the positive PC1 (80%). However, it was not possible to interpret the grouping presented by PC2 th the available information about the samples. B) Loading plot showing that PC1 negative sample was characterized by elevated Tn, sTn, and sLe^a and PC1 positive by elevated sLe^x and T

Given the tumor-associated nature of the Tn, sTn, and sLea antigens, ROC curves were applied to determine their sensitivity and specificity as serum biomarkers (Table 5) antigens. The sTn antigen presented the highest sensibility (81.3%) and the best combined specificity (80.0%). On the other hand, sLe^a showed 100% specificity, but only 50% sensibility. Based on the insights from PCA (Figure 8), showing that sTn and sLe^a and, to some extent, the Tn antigens are good discriminators of EC, the sensibility and specificity of these antigens was tested in combination (Table 5). As shown in Table 5, the combination of sTn and Tn retrieved the best results, with 93.8% sensibility and 100% sensitivity. The introduction of sLe^a in this array did not improve the quality of these indicators.

Table 5. Sensibility and specificity of Tn, sTn and sLe^a individually and in combination.

Antigen	Sensibility (%)	Specificity (%)
Tn	68.8	80.0
sTn	81.3	80.0
sLe^a	50.0	100
Array of antigens		
sLe^a + sTn	81.3	100
sLe^a + Tn	75.0	100
sTn + Tn	93.8	100
Sle^a + Tn + sTn	93.8	100

4.1.2. Screening of tumor-associated glycans in EC

Seven FFPE tissues were characterized in relation to Tn, sTn, T, sLe^a, and sLe^x expression by immunohistochemistry (IHC). The mono-sialylated forms of the T antigens, s6T and s3T were also included, even though they were not evaluated in serum. Both the T and sialylated T antigens were evaluated for the first time in EC. The FFPE tissues included the tumor and adjacent reactive mucosa.

The percentage of expression of the studied antigens in EC tumors and adjacent mucosa has been comprehensively resumed in Table 6. All antigens were detected in the cell membrane and also in the cytoplasm, even though in lower extent. They could also be observed in secreted mucins in adenocarcinomas. As shown in Table 6, all cases were negative for the Tn antigen, whereas the sTn, T, and sLe^x antigens presented lower expressions in comparison to s3T, s6T, and sLe^a. The sTn antigen was detected in 7/8 cases, diffusely expressed throughout the tumor (Figure 9B). It was also found in the basal luminal cells of the adjacent epithelia (Figure 9A). The T antigen was only found in mucins in adenocarcinomas (Figure 9D) and in the gastric metaplasia (Figure 9C). The sLe^x antigens presented an expression pattern similar to sTn, with the exception that it was not present in the adjacent mucosa (Figure 9- E). Still, it was extensively expressed in the stromal tissues (Figure 9- F).

Regarding the sialylated forms of the T antigens, s3T was detected after digestion of the FFPE tissues with a α-(2,3)neuraminidase to yield the T antigen, followed by detection with mouse monoclonal antibody 3C9. By comparison with the expression of the T antigen, 3 SCC and the gastric metaplasia were considered positive for s3T (Table 6 Figure 9I). Like the T antigen, s3T was not found in the adjacent mucosa. The s6T antigen was detected after digestion of the FFPE tissues with a β-(1,3)galactosidase to yield the sTn antigen and then detected using mouse monoclonal antibody TKH2. By comparison with the expression of the sTn antigen, all cases and adjacent mucosa were considered positive for s6T (Table 6, Figure 9J). Based on these observations, s6T was considered to be the most expressed of the two sialylated forms of the T antigen.

Table 6. Expression of tumor-associated carbohydrate antigens Tn, sTn, T, T+s3T, sTn+s6T, sLe^a, and sLe^x by immunohistochemistry.

	Tn		sTn		T		T+s3T[1]		sTn+s6T[2]		sLe^a		sLe^x	
	AM	T	AM	T	AM	T	AM	T	AM	T	AM	T	AM	T
ESSC														
Case 1	-	-	+	+	-	-	-	-	+	++++	+++	++++	-	+
Case 2	-	-	+	+	-	-	-	-	++	+++	+++	++++	-	+
Case 3[3]														
SCC	-	-	+	+	-	-	-	+	++++	+	+++	++++	-	+
Metaplasia	x	-	x	++	x	+	x	++++	x	++++	x	-	x	+
Case 4	-	-	+	+	-	-	-	+	++	+++	++	+++	-	+
Case 5	-	-	+	+	-	+	-	+						
AC														
Case 6	-	-	-	-	-	+	-	+	+	++++	++++	++	-	-
Case 7	-	-	+	+	-	+	-	+	+	++	++	+++	-	+

Antigen Expression: "x": not present; "-": negative; "+": >5-20%; "++": >20-35%; "+++": >35-50%; ++++: >50% (extensive); "AM" stands for tumor adjacent mucosa which was in all cases considered already as reactive mucosa, showing pronounced histological alterations. "T" stands for tumor; [1]Determined using mouse monoclonal antibody 3C9 anti-T antigen after digestion with a β-(1,3)galactosidase; [2]Determined using mouse monoclonal antibody TKH2 anti-sTn antigen after digested with a α-(2,3)neuraminidase; [3]This case presents a SCC and gastric metaplasia, as a result, the tumor and the EC precursor lesion were evaluated separately.

The sLe^a antigen was highly expressed in all tumors and adjacent mucosa, with the exception of the gastric metaplasia. Moreover, it was always more expressed in the tumors than in the adjacent mucosa (Figure 9H).

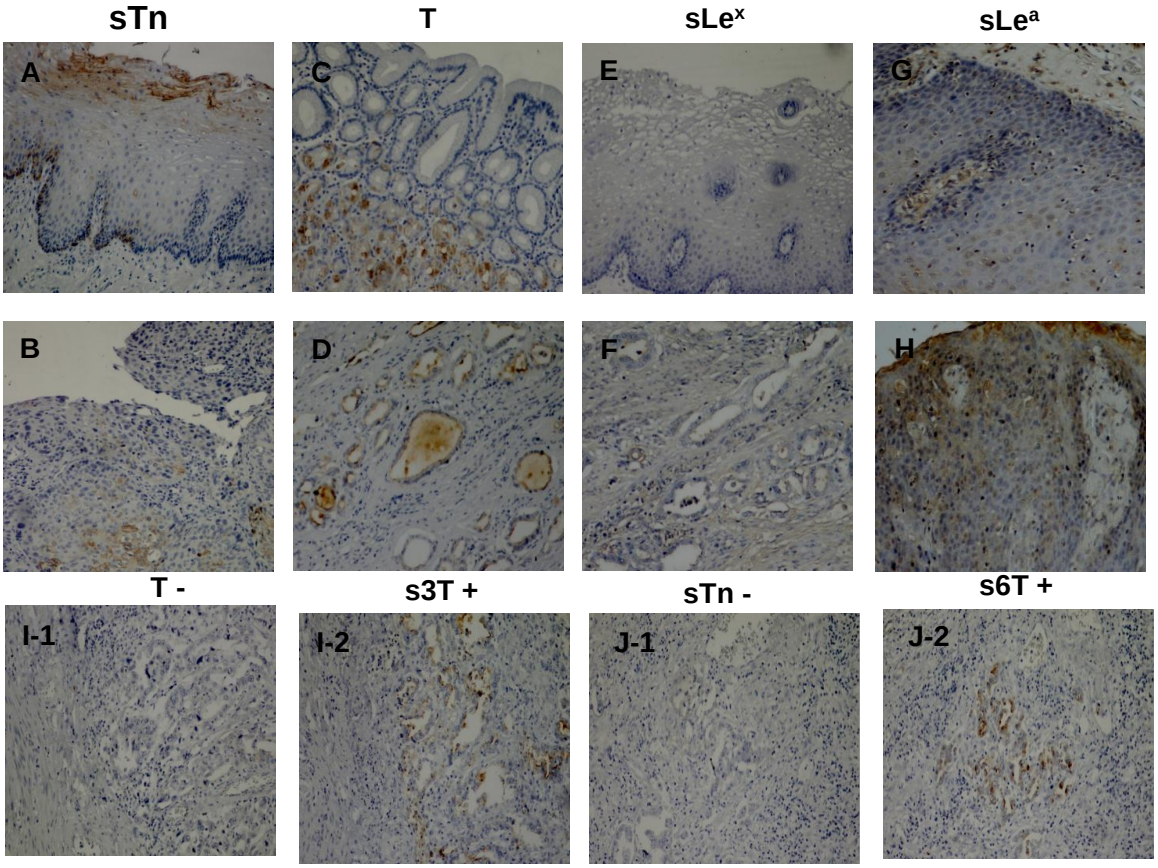

Figure 9. Expression of tumor-associated carbohydrate antigens Tn, sTn, T, T+s3T, sTn+s6T, sLe^a, and sLe^x by immunohistochemistry. Expression of the A) stn in a SCC adjacent mucosa; B) sTn in a SCC; C) T antigen in gastric metaplasia; D) T antigen in a AC; E) sLe^x antigen in a SCC adjacent mucosa; F) sLe^x antigen in SCC; G) sLe^a antigen in a SCC adjacent mucosa; H) sLe^a antigen in a SCC. I) SCC 1) negative for the T antigen and 2) positive for s3T; confirms the expression of the s3T antigens. J) SCC 1) negative for the sTn antigen and 2) positive for s6T antigen; confirms the expression of the s6T antigens

In resume, it has been highlighted that studied EC tissues extensively express sLe^a and do not express the Tn antigen. Based on the data provided in Table 6, the percentage of expression of the antigens in FFPE tissues can be ordered as follows: sLe^a>s6T>sTn>sLe^x>s3T>T.

4.1.3. Correlations between serum and tissue expression of tumor-associated antigens

The graphical matrix in Figure 10 highlights correlations between the serological levels of tumor-associated glycans (Figure 8) and the expression in the corresponding tissues (Table 6). Based on the Tn, sTn, and sLe^a serum levels and the cutoffs determined

using ROC curves (data not shown), the cases were classified has EC negative or positive. These cases were then matched against the results from immunohistochemistry. For the Tn antigen, 4 cases were considered positive by serology but the antigen was not detected in the tissue in any of the cases. Thus, no correlation was found between these events. Conversely, matched results were found in 5 out of the 7 cases expressing sTn, highlighting a significant correlation between the serum and EC tissues, regardless of the low levels of sTn expression presented by EC tissue (Table 6). The sLe^a antigen presented a matched positivity only in 2 out of 7 cases, despite the high levels of sLe^a expression presented by all the tissues (Figure 9H, Table 6).

Overall, this data suggests the lack of correlation between the expression for these glycosylated tumor-associated antigens in the serum and corresponding EC tumor tissue.

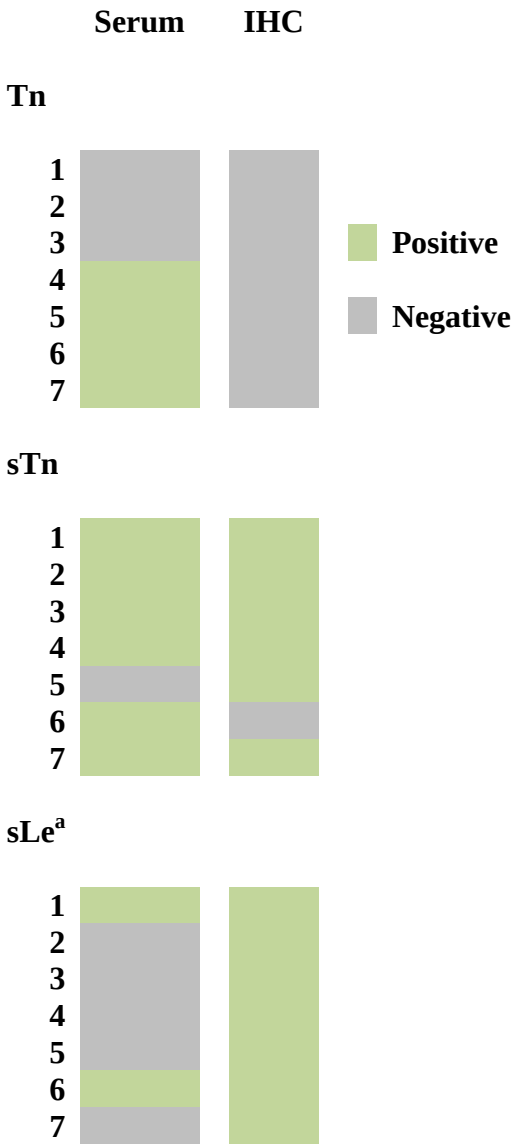

Figure 10. **Graphical matrix highlighting the expression of EC associated glycans Tn, sTn, and sLea in serum and in the corresponding EC tissues.** The sera were classified as positive or negative based on cutoffs given by ROC analysis (data not shown).

4.2. Discussion

This chapter described that the serum of a group of EC patients presented elevated levels of Tn, sTn and sLe^a antigens, when compared to a group of individuals, with matched in age and sex, and without any known oncological disease. It was also observed that the Tn and sTn could be used in combination to better discriminate the two groups. In accordance with these findings, three studies have previously reported high levels of sTn in the serum of EC patients using ELISA [23]. Elevated sLe^a were also detected in 50% of the

patients, which is in accordance with the values reported by Türkyilmaz *et al.* for a series of 50 EC using ELISA [77]. However, two other studies, comprehending a total of 335 SCC have reported significantly lower sLe^a expressions (approximately 20%) for EC [20, 78]; conversely one study involving 33 AC, reported 42% of sLe^a positive cases [20]. Altogether, this data suggests that elevated levels of sLe^a in the serum may be a characteristic feature of AC. Due to the low number of cases in our series, it was not possible to confirm these observations. Still, all serological studies conducted so far, consensually describe elevated sTn and sLe^a antigens in serum with EC. Our findings reinforce these observations and add that the Tn antigen, here evaluated for the first time, is also elevated in the sera of EC patients. More studied are now needed, using larger series, to determine the biomarker value of these antigens in the context of EC. Of note, both the T and sLe^x antigens that are normally elevated in the serum of cancer patients were significantly decreased in the sera of EC patients when compared to the controls. More studies should be conducted to determine whether this is a particular feature of the studied EC population. This suggests that Tn, sTn and T expression may stem from the same protein(s), and suggests that EC cells may be producing or inducing the production of low molecular weight glycans by other cells. On the other hand, the sLe^a is the type 1 chain (structure) positional isomer of sLe^x. A possible shift from type 2 to type 1 structures may be occurring in EC serum-associated glycoproteins. However, further studies are needed to address these observations

Additionally, tumor-associated antigens were evaluated in some of the EC tissues, in an attempt to highlight correlations with the serological observations. All the evaluated EC tumor tissue presented high levels sLe^a expression (>35% of tumor), irrespectively of the histopathological classification of the tumor. The sLe^a antigen was also observed in the tumor adjacent mucosa, however in lower extent than in the corresponding tumor. According to Ikeda *et al.,* sLe^a is not expressed in the "healthy" esophageal epithelia, thus demonstrating is tumor-associated nature [74], but it was observed in both EC precursor lesions and tumors [4], thus in accordance with the results presented in this work. However, significant inter-study variability was found regarding the percentage of positive cases (4-52%), which can either result from the use of different antibodies or be associated with sampling. The sTn and sLe^x antigens were also detected in the majority of the EC tumors, thus also in accordance with previous reports [22, 31, 75]. However, while sTn

could also be found in the adjacent mucosa, sLe^x expression was restricted to the tumor. Similar to what was observed for sLe^a, the sTn antigen has also absent from the "healthy" esophageal epithelia [22], but it can be found in EC precursor lesions [31]. The T antigen and their monosialylated forms, s3T and s6T. The T antigen were also studied. The T antigen mostly observed in mucins in AC while the s3T could also be found in SCC; however both presented low levels of expression (<10%, data not shown in Table 6). However, the s6T antigen was highly expressed in EC (>35% of the tumor) and, in less extent in the corresponding adjacent mucosa. Even though the s6T antigen was not evaluated in the serum, its elevated expression in the tumors reinforces the need for further studies. Contrasting with the above described antigens and also with previous findings [31, 74, 75], the T antigen could not detected in any of the studied EC tissues. The low abundance of T antigens in this tissues compared with the high expression of s6T denotes an overexpression of ST6GALNAc.II enzyme in this tissues

However, when the data from immunohistochemistry was matched with the levels of Tn, sTn, and sLe^a in serum, some lack of correlation discrepancies could be observed. The most salient was the lack of detection of the Tn antigen in tissues that contrasted with the elevated levels of expression in the sera of EC patients. Another contrasting feature was the lack of correlation between the elevated expression of sLe^a in the tumors and the corresponding sera. Such correlations were only evident for the sTn antigens. However, one is led to discuss, that the low levels of sTn in the tissues (generally <10% of the tumor, data not shown in Table 6) may not account for the high lvels of sTn found in serum. Tumor proteins are present in only minute quantities in complex physiological milieus such as serum, which is composed of thousands of proteins spanning a dynamic range of 10 orders of magnitude. The distribution of these proteins is dominated by a few highly abundant proteins, accounting for 99% of the total proteome [103]. Therefore, without prior enrichment steps, it unlikely that the glycosylation of tumor proteins could be responsible by the observed differences between the EC and control groups, even using highly sensitive analytical methods such as western blotting with chemoluminescent detection. These differences are most likely a result from alterations in the glycosylation pattern of more abundant serum glycoprotein, denoting tumor-driven systemic alterations. Nevertheless, glycoproteins carrying tumor-associated glycans can also be found in circulation [30]. In line with these observations, Shao *et al.* reported elevated levels of sLe^a

in IL6, IL10, and serum amyloid A (SAA) and sTn in IL10 and von Willebrand factor protein in the serum of EC patients [79]. Of note, sLe^a was also observed in MUC1 and sTn in cathepsin and gelsolin that are indeed proteins secreted by tumor cells [79].

Further studies should be conducted to determine the events leading to changes in the glycoprofile of the sera of EC patients. The identification of protein carriers may also bring more insights to this matter and provide novel, more specific and sensitive, biomarkers. Also, studies should be conducted in larger series of patients to confirm these findings.

V. Humoral responses against EC proteins expressing sLe^a

5. Humoral responses against EC proteins expressing sLe[a]

sLe[a] is expressed in cancer cells, mimicking their normal expression on blood cells (monocytes and neutrophils) and consequently their potential for migration through binding to endothelial cell selectins [30, 48]. Thus, sLea is explored in serological tests as a marker of poor prognosis (CA19-9 test) for several cancers [20].

Chapter IV described that all studied EC tissue presented high expression of sLe[a]. However, despite showing a specificity of 100%, sLe[a] was only elevated, by comparison with the control group, in 50% of the studied EC patients. Moreover, data suggested a lack of correlation between the sLe[a] levels in serum and the high expression in the tissues. Thus, given its contribution to malignancy, the development of highly sensitive and specific detection non-invasive tests to target sLe[a] is a critical matter.

Recent studies are demonstrating that tumor-associated proteins carrying altered glycosylated domains are responsible for exerting strong humoral responses [86, 88, 90]. IgGs are regarded as the next generation of tumor biomarkers, since they are highly sensible and specific, resistant to proteolysis, and capable of amplifying subtle molecular changes in the tumors [39, 83]. Based on these observations, chapter V describes an explorative study directed to identify humoral responses to EC glycoproteins carrying sLe[a]. The generated information is regarded of major importance to direct studies envisaging the non-invasive diagnoses of EC. Moreover it will allow pinpointing immunogenic glycoproteins with putative therapeutic value.

5.1. Results

5.1.1. IgG expression in serum

All sera were first characterized in relation to their IgG content. As shown by Figure 11A the IgG levels are elevated in the EC group when compared to the controls; however this difference is not statistically significant. However, when the IgG subtypes were analyzed individually by slot blotting (Figure 11B-1), it was observed that IgG1 was

significantly elevated in the EC group (Figure 11B-2). However, no differences were found between the two groups for the other subtypes of IgGs.

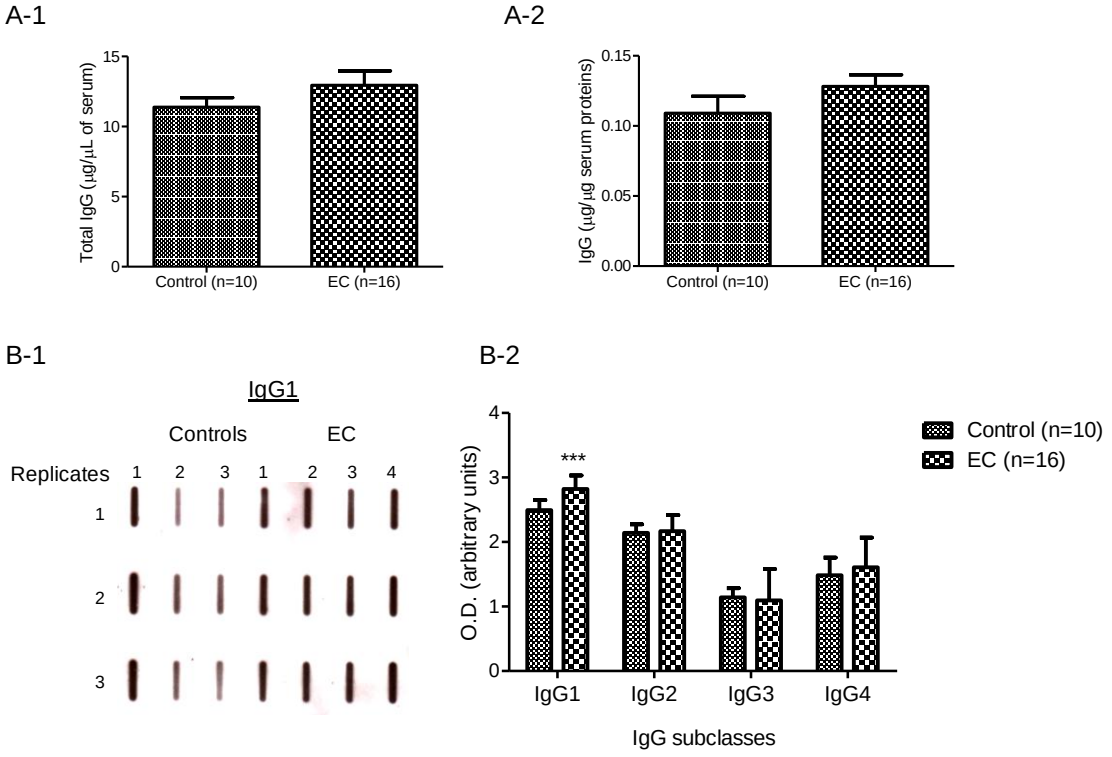

Figure 11. Correlations between IgG and sLe[a] levels in serum. A-1) shows the levels of IgG in the serum expressed in terms of μg/μL of serum; 2) shows the levels of IgG in serum expressed in terms of μg/μg of serum proteins. The IgG were elevated in the sera of the EC patients in comparison to the controls, however this difference is not statistically significant. B-1) Slotblot showing an elevated expression of IgG1 in the EC in comparison to the controls; 2) Expression of IgG subclasses (IgG1, IgG2, IgG3, IgG4) in the serum. IgG1 was found significantly elevated in the serum of EC patients ($p<0.001$)

Serum IgG concentrations were matched against the amounts of sLe[a] to mine possible associations between both events. As shown in Figure 12-A, for 50% of the controls and 69% of the EC a linear tendency was observed ($R^2 = 0.7011$); when plotted separately, a similar behavior was also observed (Figure 12- B and 12- C). Nevertheless, this data shows that IgG and sLe[a] were more correlated in EC than in the controls. Likewise, correlations of sLe[a] that showed higher differences between the control and EC groups, were studied. In this case, no linear behaviors were found among the controls

(Figure 12-D and 12- E), either in combination with the EC samples (Figure 12 D) or when plotted separately (Figure 12 E); however, approximately 63% of EC cases presented a significant linear correlation between the expression of sLe[a] and IgG1 (R^2 = 0.9804; Figure 12- F).

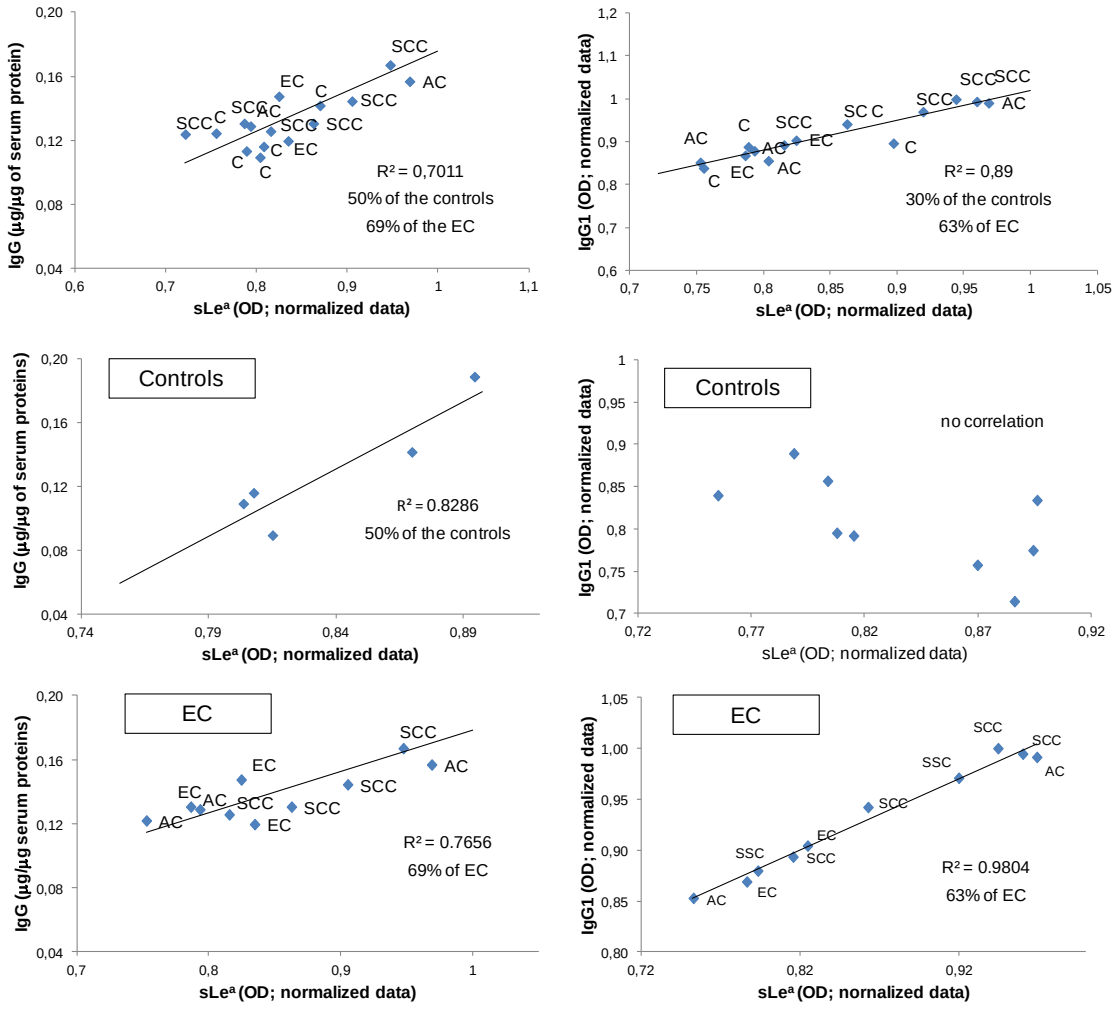

Figure 12. Figure 12. Scatterplot matrixes highlighting relationshiphs between sLea and IgG/IgG1 in serum A) IgG vs sLe[a] showing a correlation with an R^2 = 0.7011 for 50% of the controls and 69% of EC; B) IgG vs sLe[a] showing a correlation with an R^2 = 0.8286 for 50% of the controls; C) IgG vs sLe[a] showing a correlation with an R2 = 0.7656 for 69% of EC; D) IgG1 vs sLe[a] showing a correlation with an R2 = 0.89 for 30% of the controls and 63% of EC; E) IgG1 vs sLe[a] showing no correlation for the controls; F) IgG1 vs sLe[a] showing a correlation with an R2 = 0.9804 for 63% of EC. This data shows that the

expression of sLe[a] is correlated with the expression of IgG, and particularly with IgG1. These correlations are more significant in the EC than in the control group.

Altogether, data showed that the expression of sLe[a] is correlated with the IgG1, and that this is an EC-associated event. Moreover, it suggests that IgG, and in particular, IgG1 in the sera of EC patients, may express sLe[a].

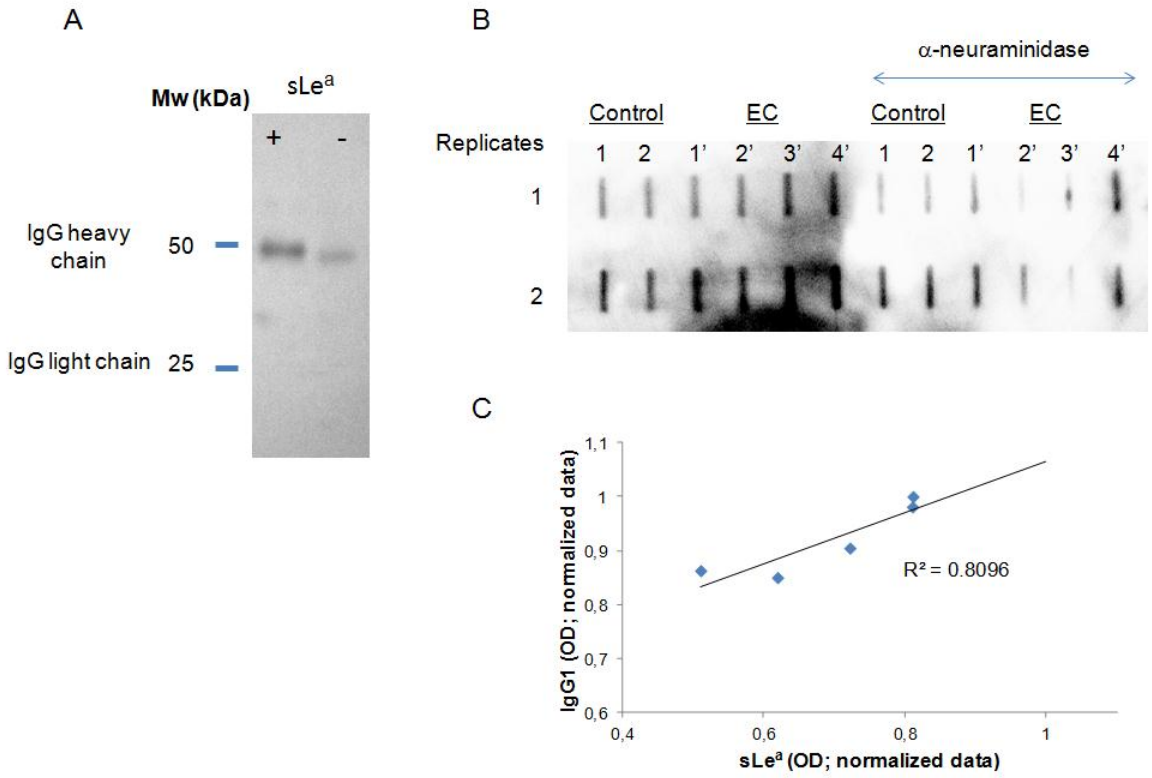

Figure 13. B) Expression of sLe[a] expression in the IgG recovered from whole serum by protein G-agarose affinity chromatography, for controls (n=2) and EC (n=4), before and after digestion with a α-neuraminidase. The decrease in chemiluminescence in the samples after enzymatic treatment confirms the expression of sLe[a]**. B) Scatterplot matrix highlighting relationships between sLe[a] expression in the IgG recovered from whole serum by protein G-agarose affinity chromatography, and IgG1 levels.** The observation of a linear correlation with an $R^2 = 0.8096$ confirms whole serum observations presented in Figure 12-F for the whole serum**.**

To determine if serum IgGs expressed sLe[a], these proteins were first isolated from the whole serum by affinity chromatography using protein G-Agarose and then slotblotted

for sLe^a (Figure 13). As shown in Figure 13A, both the controls (n=2) and EC IgGs (n=4) expressed sLe^a, as confirmed by the absence or decreased signals presented by the corresponding samples treated with α-neuraminidase. Like observed for the whole serum, the isolated IgGs also presented a linear correlation between IgG1 and sLe^a (Figure 13B), thus reinforcing previous associations between both events. The sLe^a levels in the serum of EC patients. Still, given its high abundance in serum, IgG are regarded to significantly contribute to this effect.

5.1.2. Humoral responses against EC proteins expressing sLe^a

The IgG1 levels were found significantly increased in the serum of EC patients as well as in the tissues (Figure 14), as shown by immunohistochemistry.

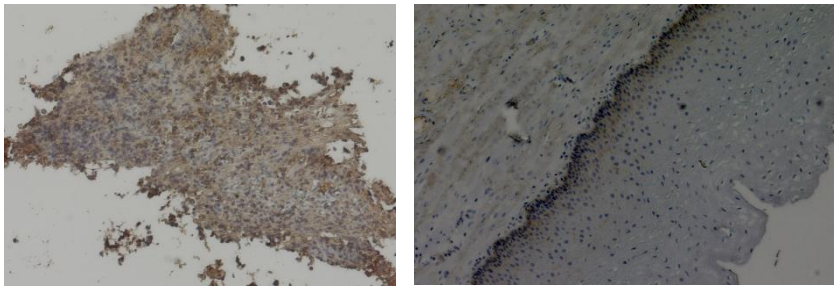

Figure 14. Expression of IgG1 in EC tissue

To identify possible IgG1-mediated humoral responses against glycoproteins carrying sLe^a, proteins were extracted from FFPE tissues. Three cases were elected for this explorative study: one non-invasive SCC, one non-invasive SCC presenting gastric metaplasia, and one invasive SCC. The protein extracts were then incubated with protein G-Agarose to remove tumor IgGs and other molecules with affinity for this protein. The glycoproteins carrying sLe^a were recovered by immunoprecipitation and blotted with a mouse anti-sLe^a monoclonal antibody. The western blots presented in Figure 14 show distinct patterns for each case. The invasive SCC showed more proteins expressing sLe^a , the majority of which of high molecular weight (>75 kDa). In the non-invasive SCC presenting intestinal metaplasia proteins between 50-75 kDa predominate, whereas in the SCC case 50-37 kDa predominate. The cross-reactivity of the secondary antibody to mouse IgG heavy and light chains that resulted from immunoprecipitation, did not allow

determining the presence of sLe[a] expressing proteins in the bands at 55 and 25 kDa. Despite these differences, the cases shared common features. Non-invasive SCC share a band at approximately 37 kDa and another above 50 kDa, which were significantly more intense in the case not presenting gastric metaplasia. These bands were not found in the invasive SCC. The case with gastric metaplasia and the invasive tumor also shared a common band below the 75 kDa maker. The invasive SCC presented three additional bands above 75 kDa. This demonstrates both the presence of sLe[a] expressing glycoprotein in EC, but also denotes similarities and differences between samples that should be further explored.

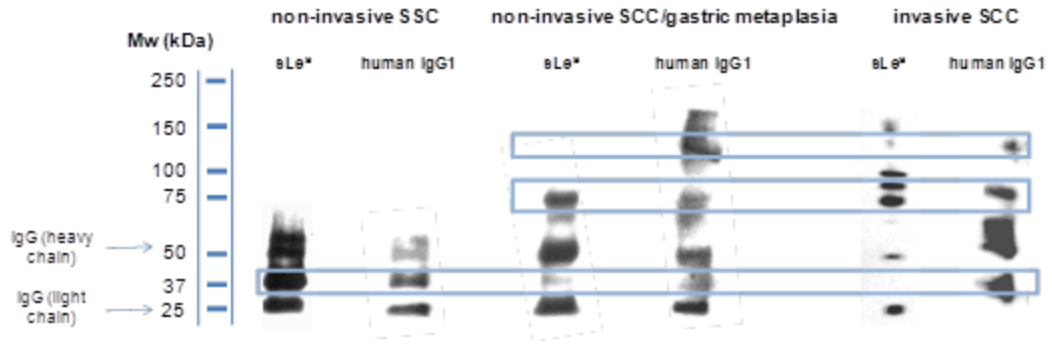

Figure 15. Expression of sLe[a] in EC proteins isolated by immunoprecipitated with mouse anti-sLe[a] monoclonal antibody, for a non-invasive SSC, a non-invasive SCC also with a gastric metaplasia, and an invasive SCC. The lanes more to the righ, for each group, show proteins recognized by serum IgG1 recovered from the same patients. The blue rectangles highlight common glycoproteins recognized by serum IgG1. This reactivity was lost after treatment with a α-neuraminidase (data not shown), confirming that sLe[a] is determinant for IgG1 recognition. Figure 15 further highlights, with blue arrows, the IgG (heavy and light chains) from the mouse monoclonal antibody using for immunoprecipitation.

IgGs were then recovered from the serum of the patients and used in the identification of IgG1 mediated humoral responses against tumor proteins carrying sLe[a] recovered from the corresponding tumors. As shown by Figure 14, the non-invasive SCC with intestinal metaplasia and the invasive SCC presented broader spectra of glycoprotein recognition, when compared to SCC alone. Also, reactivity was observed in all cases against the band at 37 kDa, including the invasive SCC where this glycoprotein was not observed with anti-sLe[a] monoclonal antibody (Figure 14). These observations denote a common pattern of IgG1 recognition among EC, irrespectively of their clinic-pathological

classification. Other common features between the non-invasive SCC with gastric metaplasia and invasive SCC include the recognition of the band at 37 kDa and bellow 150 kDa. To confirm this is the sLe^a-dependent recognition, all samples were also incubated with a α-neuraminidase prior to western blotting. The absence of bands in this blot confirmed that sLe^a is necessary for IgG1 recognition.

5.2. Discussion

This chapter described that IgG1 is significantly increased in the serum of EC patients in comparison to the control group. It also demonstrated that these proteins are glycosylated with sLe^a, an event that may account for the significantly high levels of this glycan found in the sera of EC patients.

The Fc fragment is known to bears two *N*-glycans linked to the conserved Asn-297 on both heavy chain-derived polypeptides [86]. The *N*-glycans found on IgG are of the complex biantennary type, differing in the levels of the terminal sialic acid, galactose, core fucose and bisecting *N*-acetylglucosamine (GlcNAc) [104, 105]. Additionally, 15–20% of serum-derived IgG molecules also have oligosaccharides attached to the Fab region. IgG functions rely on the interactions of the Fc region with other proteins [104]. Stimulation of the Fc receptor by IgG Fc region results in pronounced functional consequences, including antibody-dependent cell mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis and release of inflammation-associated mediator molecules [106]. The interaction of the Fc region with complement components results in complement-dependent cytotoxicity [107]. Also, different stimuli received by B cells during their activation and differentiation can modulate the Fc-linked glycosylation of secreted IgG1 without affecting the general cellular glycosylation machinery [104]. Also, the glycosylation profiles of IgG are tightly regulated by physiological and pathological determinants in an antigen-specific manner [105]. In healthy individuals, IgG glycosylation features are reflected in age, gender and pregnancy [86]. Thus, alterations in the glycosylation pattern of IgGs have been observed in several human malignancies, namely multiple myeloma patients [108], gastric [109], prostate [110], thyroid [111] cancer and found to be correlated with specific clinic-pathological features. However, to our knowledge, the cancer-associated expression of IgG1 carrying sLe^a is being observed for

the first time. Given the high correlation of this event with malignant transformation, this observation may allow to improve the sensitivity and specificity of the CA19-9 test. A deeper understanding of the IgG1 glycosylation patterns associated may further improve the diagnostic of EC.

Along with the over-expression of IgG1, it was also observed that this particular IgG subclass could recognize proteins carrying sLe^a in tumors, in a sLe^a-dependent manner. Even though this was a preliminary study involving only 3 SCC of different histopathological natures, common patterns of IgG1 recognition between the cases. However, tumor-specific immunogenic proteins were also observed. Reports of autoantibodies to glycosylated proteins expressing the Tn, sTn, and sLe^x have been presented [37, 68, 101]. Moreover, it was demonstrated that humoral responses are dependent on the particular peptide domain exhibiting the glycosylation [68]. To our knowledge this is the first study concerning sLea, despite its clinical importance. Additionally, this study highlighted both differences and similarities in the types of proteins expressing sLe^a from tumor to tumor. Further studies should now be conducted to determine the identity of these proteins, their biomarker value as well as the biomarker value of the IgGs generated against them. This may not only allow improving the diagnosis of EC, but also provide immunogenic molecules that can be explored in therapeutics.

These observations are of major relevance, since sLe^a is widely expressed not only in EC, but also on epithelial tumors of the gastrointestinal tract [48], breast [44] and on small-cell lung cancers [34] and is considered a key event in invasion and metastasis of many tumors [112].

VI. Conclusions

6. Conclusions

The first part of this work aimed to identify glycosylation patterns in serum associated with EC. It was concluded that the levels of sTn, and sLe^a antigens and, to some extent Tn, were significantly increased in the serum of the patients in comparison to a control group, matched in gender and age, without knowing malignant diseases. Moreover, it was demonstrated that the combination of the Tn and sTn antigens could be used to better discriminate the EC patients. Still, the expression of these antigens in the corresponding EC tissues, estimated by immunohistochemistry, showed a lack of correlation with the observations made in serum. Data suggests that glycosylation patterns of serum are mostly influenced by proteins that are not directly secreted or released from tumor cells, even though their contribution cannot be excluded.

The second part of the work aimed to identify humoral responses against proteins carrying the sLe^a, a glycosylated biomarker associated with increased potential of cellular migration and metastasis. The analysis of the IgG profile of the samples showed increased expression of IgG subclasse 1 (IgG1) in EC patients. *De novo* produced IgG1 were found to carry sLe^a, accounting for the increase in the levels of this glycan in the serum of EC patients. Even though the biological events underlying these observations remain to be clarified, this may allow improved the sensibility and specificity of the serological test for sLe^a (CA19-9 test). Using a combination of immunoprecipiations and western blotting techniques it was further demonstrated, for the first time, that tumor proteins carrying sLe^a could elicit IgG1 production. Furthermore, experiments using desialylated proteins confirmed that the expression of sLe^a expression was determined for IgG1 recognition.

These observations and the future identification of the immunogenic proteins carrying sLe^a will allow determining the clinical value of this explorative work and guiding the development of autoantibody-based serological tests.

Altogether, important insights have been provided to guide the development of non-invasive serological tests for the detection of EC.

VII. Future work and perspectives

7. Future works and perspectives

This work as provided important insights about the serum glycosylation patterns associated with EC. Future studies should be conducted to validate these observations in a series involving a higher number of patients, of variable ages and genders, encompassing the different clinic-pathological features. EC precursor lesions should be included in this dataset. A higher number of controls should also be studied. These should include also include controls with inflammatory disease, diabetes, among other pathologies, to isolate cancer-associated patterns. Proteomic studies will also allow identifying the proteins showing abnormal glycosylation and, consequently, improving the accuracy of the serological tests based on altered glycosylation. Furthermore, it will bring insights on the biologic events underlying the alteration of serum glycosylation patterns.

Regarding the humoral response against sLe^a in EC, it will be necessary to identify the protein carriers for this glycan and their correlation with clinic-pathological status. Then it will be necessary to determine the levels of autoantibodies in serum against the identified protein(s) to access their biomarker potential. The generated information may allow improving the accuracy of the CA19-9 in EC.

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6. References

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