# SULFATED AND SIALYLATED N-ACETYL-LACTOSAMINE AS BIOMARKER OF SUBPOPULATIONS OF PANCREATIC DUCTAL ADENOCARCINOMAS

By

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A DISERTATION

Submitted to
Michigan State University
in partial fulfilment of the requirements
for the degree of

Cell and Molecular Biology – Doctor of Philosophy

2019

## ABSTRACT

## SULFATED AND SIALYLATED N-ACETYL-LACTOSAMINE AS BIOMARKER OF SUBPOPULATIONS OF PANCREATIC DUCTAL ADENOCARCINOMAS

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The sialyl Lewis A (sLeA) glycan forms the basis of the CA19-9 blood test and is the current biomarker for pancreatic ductal adenocarcinoma (PDAC). However, it is not elevated in approximately 25% of PDAC patients and it also has difficulties in diagnosing early-stage PDAC. My overarching goal was focusing on improving precision of overall PDAC diagnostics. I hypothesized that other glycans within the Lewis blood group family besides sLeA are aberrantly increased in the subpopulation of PDAC patients who do not secret sLeA into their blood. To test the hypothesis, two specific approaches were implemented in this study: 1) Profile an isomer of sLeA, named sialyl-Lewis X (sLeX), and glycans with fucosylated motifs in the plasma of sLeA-low PDAC patients using antibody and lectin microarray method; and 2) Test the sulfated and sialylated glycans derived from type 2 N-acetyl-lactosamine precursor in subpopulations of PDACs using a novel on-chip analysis method.

In the first approach, I profiled the levels of multiple glycans and glycosylated mucins in plasma from two cohorts of 200 and 116 test subjects with PDACs and non-malignant disease patients. From these screens, I found significant increases in two categories of glycans: sialyl Lewis X variants, presented both in sulfated and non-sulfated forms, and the sialylated type 1 N-acetyl-lactosamine. These glycans are increased in distinct groups of PDAC patients and contribute to the improved accuracy of a biomarker panel.

Thus, I concluded that detecting other glycans within the Lewis blood-group besides sLeA has the potential to improve diagnoses of PDAC patients.

To further elucidate the structural nuances of sialyl Lewis X variants from initial screen, I developed a new assay called *On-chip Glycan Modification and Probing* and a complementary computational algorithm to accurately analyze novel sulfated and sialylated glycans from plasma of pancreatic cancer patients. In detailed structural information, I observed strong evidences of sulfated and sialylated type 2 N-acetyl-lactosamine glycans overexpressed in plasma of PDAC patients and pancreatic cancer cell lines, but not in the plasma of healthy people. In addition, the sulfated and sialylated type 2 N-acetyl-lactosamine glycans presented on a specific mucin, MUC5AC, was statistically associated ($p < 0.001$) with short time-to-progression of PDAC patients, but CA19-9 test was not. I concluded sulfated and sialylated type 2 N-acetyl-lactosamine glycans presented on MUC5AC were new serological biomarkers that could improve precision of current practices for diagnosis and prognosis of PDACs patients.

## ACKNOWLEDGEMENTS

This dissertation is dedicated to my family, friends, colleagues and everyone I encountered in the past five and half years. Without their generous support for my physical and mental growth, I would not have had the motivation, courage and resilience to accomplish this degree. I would like to specifically thank my immediate family for their unconditional love and support. I would like to show my gratitude to my thesis advisor, Dr. Brian Haab for his guidance, training, and allowing me the opportunity to pursue my passion in oncology research. Next, I like to show my gratitude to my thesis committee, Dr. John Wang, Dr. Karl Olson and Dr. Justin McCormick for their constructive inputs and supervision of my dissertation work. They all challenged me to be a better trained scientist. My sincere regards go to the Cell and Molecular Biology Program, for providing me this tremendous opportunity to pursue this PhD. I will like to send my deepest appreciation to all current and former lab members in the Haab Lab, as well as other colleagues at the Van Andel Research Institute and the Michigan State University. Lastly, I want to thank the Sponsorship from the Michigan State University College of Natural Sciences and the fellowship from the Aitch foundation.

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## KEY TO SYMBOLS AND ABBREVIATIONS

| Abbreviation/Symbol | Definition |
|---|---|
| AFP | Alpha-feto protein |
| ALSA | Antibody and lectin sandwich microarray assay |
| ApoB | Apolipoprotein B |
| Asn | Asparagine |
| AUC | Area under the ROC curve |
| BPL | Bauhinia purpurea lectin |
| BSA | Bovine serum albumin |
| CA | California |
| CA125 | Cancer antigen 125 |
| CA15-3 | Cancer antigen 15-3 |
| CA19-9 | Carbohydrate 19-9/Cancer antigen 19-9 |
| CCL2 | Coprinopsis cinera lectin 2 (Inky cap fungus) |
| CDKN2A | Cyclin-dependent kinase inhibitor 2A |
| CEA | Carcinoembryonic antigen |
| CEACAM1 | Carcinoembryonic antigen-related cell adhesion molecule1 |
| CFG | Consortium for Functional Glycomics |

| | |
|---|---|
| CHST4 | Carbohydrate sulfotransferase 4 |
| ConA | Concanavalin A lectin |
| COX2 | Cytochrome c oxidase polypeptide II |
| CTLA4 | Cytotoxic T-lymphocyte associated protein 4 |
| Cy3/5 | Cyanine 3/5 |
| DSL | Datura Stramonium Lectin |
| DTDST | Diastrophic dysplasia sulfate transporter |
| ECL | Erythrina Cristagalli Lectin |
| ECM | Extracellular matrix |
| EDRN | Early Detection Research Network |
| EDTA | Ethylenediaminetetraacetic acid |
| E-selectin | Endothelial selectin |
| FDA | Food and Drug Adminstration |
| FFPE | Formalin-Fixed Paraffin-Embedded |
| Fuc | Fucose |
| FUT2 | alpha 1-2 fuocsyltransferase 2 |
| FUT3 | alpha 1-4 fuocsyltransferase 3 |
| FUT7 | alpha 1-3 fuocsyltransferase 7 |
| GA | Georgia |

| | |
|---|---|
| Gal | Galactose |
| GI | Gastrointestinal |
| GlcNAc | N-acetylglucosamine |
| GlcNAc6ST-1 | GlcNAc 6-O-sulfotransferase 1 |
| GlcNAc6ST-2 | GlcNAc 6-O-sulfotransferase 2 |
| GSL II | Griffonia Simplicifolia Lectin I |
| HIV | Human immunodeficiency virus |
| HEV | High endothelial venules |
| H&E | Hematoxylin and eosin |
| IF | Immunofluorescent |
| IgG | Immunoglobulin G |
| IHC | Immunohistochemistry |
| IL | Illinois |
| IN | Indiana |
| IPMN | Intraductal papillary mucinous neoplasia |
| ITIM | Immunoreceptor tyrosine-based inhibitory motif |
| KRAS | V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| LacNAc | N-acetyl-lactosamine |
| LeX | Lewis X |

| | |
|---|---|
| LPS | Lipopolysaccharides |
| L-selectin | Leukocyte selectin |
| MA | Massachusetts |
| MAb | Monoclonal antibody |
| MAL-I/II | Maackia Amurensis Lectin I/II |
| MCN | Mucinous cystic neoplasm |
| MN | Minnesota |
| MS | Mass spectrometry |
| MUC5AC | Mucin 5AC |
| MUC16 | Mucin 16 |
| MI | Michigan |
| NET | Neuroendocrine tumors |
| NK cell | Natural killer cell |
| NY | New York |
| On-chip gmap | On-chip glycan modification and probing |
| PanIN | Pancreatic intraepithelial neoplasia |
| PBS | Phosphate buffered saline |
| PD-1 | Programmed cell death protein 1 |
| PDAC | Pancreatic ductal adenocarcinoma |

| | |
|---|---|
| PDMS | Polydimethylsiloxane |
| PHAL | Phaseolus Vulgaris Leucoagglutinin |
| PMN | Polymorphonuclear |
| PNGase F | Peptide:N-glycosidase F |
| PTM | Posttranslational modification |
| PSA | Prostate specific antigen |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| RCA-I | Ricinus Communis Agglutinin I |
| RFU | Relative fluorescent unit |
| ROC | Receiver Operating Characteristics |
| ROS | Reactive oxygen species |
| RSL | Ralstonia solanum lectin |
| RTKs | Receptor tyrosine kinases |
| TEM | Transendothelial migration |
| Thr | Threonine |
| SELE | E-selectin |
| Ser | Serine |
| SFT | Segment and Fit Thresholding |
| Sia | Sialic acid |

| | |
|---|---|
| Siglec | Sialic acid-binding immunoglobulins-like lectin |
| SLeA | Sialyl Lewis A |
| SLeX | Sialyl Lewis X |
| SMAD4 | SMAD family member protein 4 |
| SNA | Sambucus Nigra Lectin |
| SRL | Sclerotium rolfsii lectin |
| ST3Gal | Sialyltransferase |
| sTRA | Sialylated type 1 LacNAc |
| TAM | Tumor-associated macrophage |
| TMA | Tissue microarray |
| TP53 | Tumor protein p53 |
| U | Unit |
| U.S. | United States |
| VARI | Van Andel Research Institute |
| α | Alpha |
| β | Beta |
| µ | Micron |
| 2D/3D | 2-dimensional/3-dimensional |
| 6-sulfo-sLeX | 6-sulfated sialyl Lewis X |

| | |
|---|---|
| 6’-sulfo-sLeX | 6-sulfated sialyl Lewis X |
| 6,6’-sulfo-sLeX | 6,6’-sulfated sialyl Lewis X |

# CHAPTER ONE: INTRODUCTION

## Introduction

Pancreatic cancer continues to be a disease with a dismal prognosis and a five-year survival rate of about 5-8%. It typically presents at an advanced stage and is resistant to current treatment modalities. As a consequence, there is an urgent need to develop methods to achieve diagnoses at earlier, more treatable stages, and to identify effective means to treat the pancreatic cancers. Recent research has developed support for the concept that several biologically-distinct subtypes make up the broad category of pancreatic cancer. If so, each subtype likely would have its own molecular markers that would provide optimal detection. The long-term goal of my research is to develop molecular biomarkers to detect each subtype. In order to understand the basis for the approaches taken in the research described here, it is important to provide background on the pancreas, pancreatic cancer, the previous work done to define molecular subtypes of pancreatic cancer, the basis for pursuing glycans as biomarkers, and the specific glycans being studied here. The following introduction provides that background.

### Cell types of the pancreas

Pancreas performs two major functions: an endocrine function for regulating glucose metabolism throughout the body and an exocrine function for digestion. There are two major cell types that form the exocrine component of the pancreas, acinar cells and ductal cells, which make up 99% of the pancreatic mass. The acinar cells are responsible for producing approximately 22 different digestive enzymes, including trypsin, chymotrypsin, amylase, and lipase, that drain into branched pancreatic ducts formed by the ductal cells. The main pancreatic duct then joins the common bile duct to

form ampulla of Vater and connect with the duodenum. The enzymes are known as zymogens when they are secreted because they are in an inactive state to avoid self-destruction of the pancreas. The secretion of zymogens from the pancreas is tightly regulated by the hormones gastrin, cholecystokinin and secretin, which are secreted by the stomach in response to food intake [1].

The endocrine component of the pancreas consists of a cluster of cells known as islets of Langerhans. The islets are comprised of a variety of hormone-producing cells that are involved in the regulation of glucose metabolism. Two of the most important cell types within the islets are alpha cells and beta cells, which produce glucagon and insulin, respectively. These two hormones are the central controllers of blood glucose level. In addition to alpha and beta cells, delta cells and pancreatic polypeptide cells, which produce somatostatin and pancreatic polypeptide hormone, respectively, are also found within the islets of Langerhans and help in regulating food consumption and blood glucose levels [1, 2].

In cancers of the pancreas, the above physiological functions are partially retained and are thought to indicate the originating cell of the cancer. For example, cancers that express synatophysin, or other hormones of the pancreas endocrine system, and that have non-glandular morphologies are termed neuroendocrine tumors (NETs) and are thought to arise from the islets. But cancers that express mucins and others ductal-associated proteins, and that have glandular morphologies, are termed pancreatic ductal adenocarcinomas (PDACs) and are thought to arise from the acinar and ductal cells. In rare cases, the tumors express digestive enzymes; these tumors are termed acinar-cell carcinomas, as they are presumed to arise from the acini. Because the great

majority of pancreatic cancers are PDACs, and because PDACs have clearly different disease behaviors and molecular characteristic than NETs, this dissertation is focused on PDAC.

**Pancreatic Ductal Adenocarcinoma**

An overview of the statistics for PDAC shows that it represents one of the most devastating medical challenges in the U.S. and worldwide. In the U.S., pancreatic cancer is estimated as the fourth leading cause of cancer-related death with approximately 44,330 deaths out of 55,440 new cases in 2018, and it has the worst five-year survival rate (about 8%) among solid tumors [3]. The median survival ranges from three to six months, which means that 80% of newly diagnosed pancreatic cancer patients will die within the year. According to one study, pancreatic cancer was projected to elevate from the fourth place of leading cause of cancer-related death in 2018 to the second place by 2030 [4].

Improved cancer prevention potentially could reduce the death rate, but a major problem with pancreatic cancer is that many of the risk factors associated with this disease are unknown. Genetic diseases that predispose individuals to pancreatic cancer include Peutz-Jegher syndrome, familial atypical multiple mole melanoma, hereditary breast and ovarian cancer, Lynch syndrome, cystic fibrosis, hereditary pancreatitis, ataxia-telangiectasia, and non-O blood group [5]. However, 90% of pancreatic cancers are sporadic, which means modifiable risk factors have a greater contribution to the malignant transformation of pancreatic exocrine cells. These risk factors are lifestyle-related including smoking, obesity, diet, diabetes history, and long-

standing chronic pancreatitis [6-11]. The association between consumption of red meat and the risk of pancreatic cancer, although nonexclusive, was proposed [8].

Much PDAC research has focused on characterizing of morphology and genetic association. PDAC histology is often highly fibrotic with heterogeneous types of cancer-supporting cells, including fibroblasts, stellate cells, immune cells, and molecules, extracellular matrix (ECM), and growth factors. Genetic analyses have focused on alterations in driver genes, events that initiate malignant transformation in the pancreatic acinar cells. Activating mutations in driver gene, KRAS, typically arise first, followed by the inactivating mutations in tumor suppressor genes such as TP53, CDKN2A, and SMAD4 in acinar cells. With accumulation of mutations, histologically normal and well-defined acinar cells in normal pancreas can transform to low grade of Pancreatic intraepithelial neoplasia (PanIN), high grade of PanIN and then full-blown PDAC [12, 13]. More rarely, other pre-neoplastic PDAC precursors including intraductal papillary mucinous neoplasia (IPMN) and pancreatic mucinous cystic neoplasm, arise from pancreatic ductal cells with a different set of genetic drivers and passengers, can also transform to PDAC [14, 15].

**The need for molecular markers of PDAC**

PDACs are often asymptomatic during early stages of malignancy and when symptoms do arise, they can mimic non-malignant diseases, such as acute and chronic pancreatitis. Imaging techniques such as ultrasonography, computed tomography, magnetic resonance imaging, positron emission tomography and endoscopic ultrasonography, have limited ability to detect primary tumors early. For these reasons, at the time of initial diagnosis patients often present with metastatic lesions in the liver,

lung and peritoneum [16]. This leaves the majority of pancreatic cancer patients with the options of chemo- and radio-therapeutic regimes, which provide minimal benefit to morbidity and a lack of clear benefit to overall survival [17]. The prognosis is largely determined by the location of tumor; the five-year survival rate is 32% for local diseases, 12% for diseases with regional metastasis (cancer cells in local lymph nodes), and 3% for disease with distant metastasis, in which cancer cells are observed in other organs beside the primary tumor site and local lymph nodes. For the minority (20%) of patients who have non-metastatic disease and undergo surgical resection of the tumor, median survival is improved by approximately 20 months [18]. Therefore, an important goal is to develop methods of detecting cancers earlier, so that the existing treatment options are more successful. Molecular markers are the best option for testing for early progression of cancer.

One of the more active research areas is for tumor markers from serum or plasma samples, which are noninvasive, convenient, and have low associated costs. In recent studies, plasma thrombospondin-2 was combined with CA19-9, a biomarker for PDAC (described details below), to form a combination biomarker, and specific isoforms of apolipoprotein A-II were associated with pancreatic cancer, but also present in benign diseases [19, 20]. The detection of mutated, cell-free DNA in the circulation of cancer patients has also been a major area of research. Many pancreatic cancer patients harbor oncogenic mutations in the KRAS gene in their tumors, and a PCR-based assay to detect such mutated DNA in the circulation identified about 30% of pancreatic cancer patients with near-perfect specificity in comparison to healthy controls [21]. A multiplexed assay that includes additional mutations showed promise for screening for

eight common cancer types, including pancreatic cancer [22]. Other promising biomarkers include micro-RNAs, DNA and tumor cells in the circulation; proteins in the urine, and various types of biomarkers in the pancreatic juice or stool, all of which could help define biological subtypes of pancreatic cancer [23-28]. Moreover, certain tumor markers existed in the circulation before a sizable primary tumor was detected by imaging techniques, showing promise for early detection [29]. Other candidate biomarkers have been investigated, but no biomarker has been validated or approved for clinical use for pancreatic cancer except for CA19-9 (described below), which was discovered about 40 years ago [30-32].

**Pancreatic cancer biomarker: Carbohydrate Antigen 19-9 (CA19-9)**

The only Food and Drug Administration (FDA)-approved biomarker for pancreatic cancer is cancer antigen 19-9 (carbohydrate antigen 19-9), known as CA19-9. CA19-9 refers to a monoclonal antibody produced by Koprowski and colleagues that was raised against colorectal cancer cell line SW1116 in 1979 [33]. CA19-9 antibodies were later determined to primarily target a blood group antigen called sialyl Lewis A (sLeA). The sLeA was found on tumor cell membrane proteins, mucins and gangliosides, and it was determined to be an terminal tetrasaccharide structure [34, 35]. The serum CA19-9 levels are elevated in approximately 75% of pancreatic cancer patients and also elevated in other clinical diseases such as chronic pancreatitis, jaundice, biliary stricture, and other gastrointestinal cancers, which leads to a controversy about its usefulness to detect pancreatic cancers [36]. Regardless of its limitation as a diagnostic marker and screening method, it is routinely requested by clinicians for monitoring disease progression among patients for whom it is elevated.

Despite advances in the technology and knowledge relating to cancer biology, investigations are still searching for tumor markers that can outperform or complement CA19-9 in diagnosing pancreatic cancer. Previous research suggested that other glycans besides sLeA are overproduced in some pancreatic cancers; the most promising was the DUPAN2 antibody, which binds to a non-fucosylated variants of sLeA called sialyl-Lewis C [37]. A better understanding of these specific glycan structures will potentially enable researchers to correlate these markers with the progression of cancer to capture the early stage of disease. Since the sLeA antigen is a glycan in the Lewis blood group family, this introduction will provide a brief overview of important concepts in glycobiology, followed by background on some common members of the Lewis blood group family in the context of normal biology and cancer.

**Glycobiology**

Glycosylation is the most common posttranslational modification (PTM) on proteins, involving the attachment of glycan moieties to amino-acid sidechains. The amount and location of glycosylation on a given protein is regulated in a complex way and involves an interplay between a variety of transporters, glycosyltransferases, and glycosidases in the cell membrane, endoplasmic reticulum, and Golgi apparatus. Glycosylation plays an essential role in cellular functions such as protein folding, protein stability, physical protection and elasticity, lubrication, membrane organization, immune recognition, signal transduction, and reproduction [38, 39]. Consistent with these roles, deregulation in glycosylation has been implicated in an extensive number of conditional diseases, including inflammation, infectious diseases, diabetes, neurodegeneration and cancer [40-43].

Alterations in glycosylation also modulate key pathophysiological events in various aspects of tumor transformation. For example, increasing N-linked glycan complexity through increased branching on receptor tyrosine kinases (RTKs) promotes malignant cell proliferation [44]; truncation of O-linked glycans induces cancer cell growth and invasion [45]; and increasing the expression of sialylated glycans promotes cancer cell adhesion to endothelium and suppression of immune-surveillance and metastatic spread, leading to poor patient outcome [46-49].

It is notable that all of the tumor markers that are currently approved for clinical practice are either a specific glycan, such as CA19-9, or a glycosylated protein, CA125, prostate-specific antigen (PSA), and carcinoembryonic antigen (CEA) [50-54]. This fact supports further investigation into the role of glycosylation in tumorigenesis and the existence of other glycan-based biomarkers.

**Lewis blood group antigens**

The term ‘blood group antigen’ refers to an antigen on the surfaces of red blood cells with expression linked to a genotype that potentially influences compatibility in blood transfusions. In the case of the Lewis blood group family, these glycans were discovered through agglutination of blood samples from type O individuals and were named after the patient in the original study, a Mrs. Lewis [55]. Considering the potential importance of the sLeA and the related glycans as cancer biomarkers, researchers began intensive work to understand the expression and regulation of the Lewis blood group antigens in the state of normal biology and diseases.

The identification of the genes involved in Lewis blood antigen/glycan biosynthesis began with the genetic mapping of the Lewis phenotype. Researchers linked the presence of the Lewis A and B phenotypes to two genes called *Lewis* and *Secretor*. The *Secretor* gene was so named because it controlled the presence of ABO antigens on glandular secretions rather than red blood cells. Modern genetic and biochemical methods showed that the genes encoded α1-2 fucosyltransferases, FUT1 and FUT2. The *Lewis* gene, later named α1-3/ α1-4 fucosyltransferases, catalyzes the linkage of a fucose monosaccharide to the 3' and 4' carbon of N-acetylglucosamine (GlcNAc) in N-acetyl-lactosamine (LacNAc), and the *Secretor* gene catalyze the linkage of fucose to the 2' carbon of galactose (Gal) in LacNAc.

The unifying feature among the Lewis glycan biosynthetic steps is that they add various components—fucose, sialic acid, and sulfate ester —to the core backbone of the LacNAc precursor, which is a Gal linked to a GlcNAc. The major division among the Lewis glycans is based on the linkage of the galactose, whether α1-3 or α1-4, the former called "type 1" and the later "type 2" (Fig. 1). The type 1 and type 2 LacNAc precursors, with addition of fucose to the 3' or 4' carbon of GlcNAc, yield the Lewis A and Lewis X (LeX) antigens, respectively (Fig. 1). In addition to their presence on red-blood cells, Lewis antigens are produced mainly by certain types of endothelial cells, epithelial cells of the gastrointestinal (GI) tract, other glandular areas, immune cells, and developing cells.

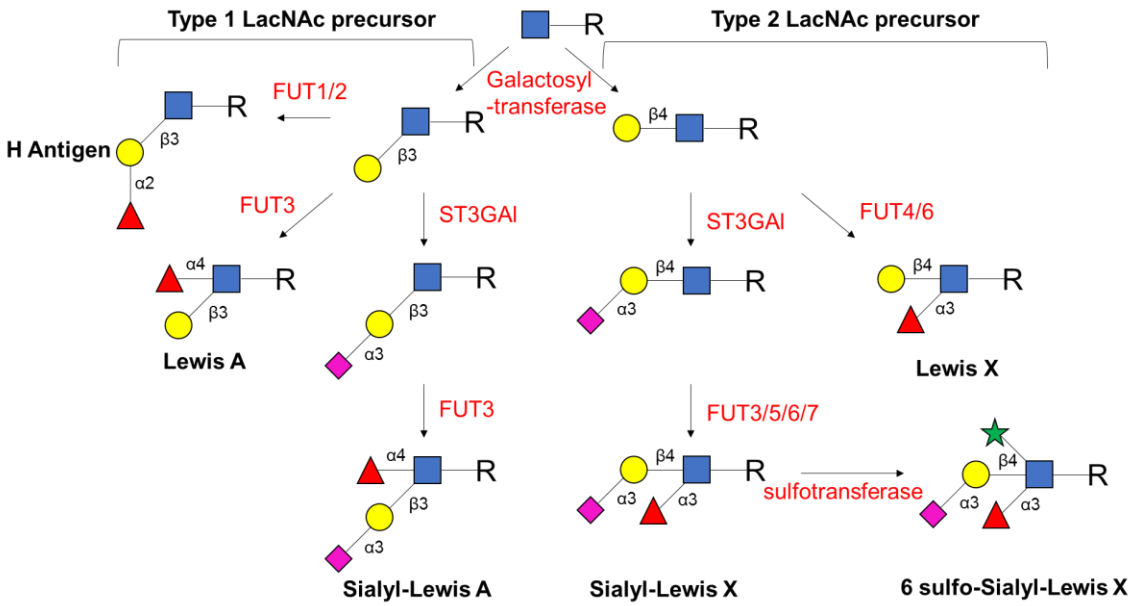



**Figure 1. Structure and biosynthesis of Lewis blood group family.**
Monosaccharides are represented – blue square: N-acetylglucosamine (GlcNAc); yellow circle: galactose (Gal); red triangle: fucose (Fuc); pink diamond: sialic acid; and green star: sulfate. Anomers, linkages, and enzymes involved in the reactions are indicated. The fucosyltransferase and sialyltransferase are abbreviated as FUT and ST3Gal in this figure. The two major groups of Lewis blood group antigens are divided by the type of N-acetyl-lactosamine (LacNAc) precursor structures (indicated on the top panel). * H antigen is not part of Lewis blood group, but we include it as indication of biosynthetic and structural relationship between Lewis antigens and ABO blood group antigens.

No affirmative connection between genotype and phenotype exists for other features of Lewis glycans, because multiple enzymes can catalyze each of the other steps. For example, at least six different sialyltransferases, ST3Gal-I to VI, encoded in the human genome can catalyze the addition of sialic acid to the 3’ and 4’ carbon of Gal, and loss of a single sialyltransferase does not lead to noticeable deficits even in processes that require sialic acid such as leukocyte rolling, tethering and homing [56]. Likewise, β1-4 galactosyltransferases can attach Gal in β1-4 linkage to GlcNAc residue to form the disaccharide precursors of ABO blood and Lewis blood antigens. Some genes,

however, are predominant in specific cell types. For example, in the creation of sLeX and Lewis X glycans, FUT7 appears to be the most important in leukocyte trafficking [57].

The biosynthetic steps outlined above suggest that scientists cannot make predictions on relative expression of the Lewis family members solely based on gene expression in the majority of circumstances. This is because these glycans also depend on the activities of glycosyltransferases and glycosidases, availability of donor substrates, and the correct sequence of precursor substrates. Therefore, the regulation of glycosylation is more complicated than the mRNA expression of certain glycosyltransferases. For example, the reduction in sulfated sLeX and increase in sLeX on the surface of colon cancer cells could be mediated by epigenetic silencing of a sulfate transporter, DTDST, without directly influencing N-acetylglucosamine-6-O-sulfotransferases expression, the main enzymes responsible for assembling the sulfated sLeX [58].

**Sialyl Lewis A and Sialyl Lewis X in normal biology**

The sLeA has limited expression in the adult, appearing mainly in the pancreas on centroacinar cells and intralobular ducts, seminal fluid, bile, saliva and as a free glycan in milk [59-62]. It is also concentrated in embryonic tissue and increased in certain inflammatory conditions [63, 64]. Proteins carrying sLeA include mucins, carcinoembryonic antigen, apolipoproteins and kininogen [35, 65, 66]. The presence of sLeA on ApoB occurs in about 25% of people, independent of the presence of pancreatic disease [67]. It also appears on CD44 in the inflamed colon [68].

In contrast, sLeX has less frequent expression on normal ductal and glandular cells of the GI tract but is more common on cells of the myeloid lineage [69, 70]. It is also a marker for neural stem and progenitor cells in mice and appears on glycolipids in human kidneys and as a free glycan in human milk [71-73]. The proteins that carry sLeX include CD11b/CD18 and CEACAM1, PSGL-1, leukosialin, and CD44 [74-77].

The most well-known function of the sialylated Lewis antigens, sLeA/X, involves their function as main ligands for a family of adhesion molecules called selectin, consisting of E-, P-, and L-selectin. E-selectin, expressed on the endothelial cells, interacts with both sLeX and sLeA but not their non-sialylated counterparts [78, 79]. Glycosylation of intestinal epithelia with sLeA mediates polymorphonuclear leukocyte (PMN) transendothelial migration (TEM) [80]. The sLeX on both linear and branched O-glycans is involved in lymphocyte rolling on activated endothelial cells [81]. It is highly expressed in inflamed colonic epithelial cells and mediates PMN detachment and migration into the lumen [80]. A carrier of sLeA on inflamed intestinal epithelia is CD44v6, which also mediates PMN detachment from epithelium during TEM [68]. As part of the multistep leukocyte recruitment and migration during inflammation, E-selectin and P-selectin expressed on cytokine-activated vascular endothelium are responsible for initial tethering and the subsequent rolling movement of leukocytes [82, 83]. Selectin-mediated leukocyte trafficking is crucial in both normal and disease states during inflammation.

**Sialyl Lewis A and Sialyl Lewis X in cancer**

Pathogens can modulate a host's immune system by modifying the glycan patterns on the host's cell membrane; similarity, cancer cells are capable of modifying cell-cell

interactions and gaining advantage in survival by modifying glycan patterns, such as expressing sialylated Lewis glycans on cancer cell membrane. For example, the role of sLeX in tumor metastasis is potentially mediated through interactions with E-selectin receptor [84]. sLeA is another ligand for E-selectin, so the relative levels of sLeX and sLeA could affect cancer cell behavior, disease progression, and metastasis [85]. More evidences for the involvement of sLeA/X glycans in metastasis comes from in *vitro* studies, mouse models, and correlations with clinical information.

The adhesion of cancer cell lines to activated endothelial cells *in vitro* is enhanced through the interaction of sLeX or sLeA ligand with E-selectin [86-88]. The sLeX-mediated interactions between cancer cells and circulating platelets through P-selectin also could influence metastasis, although in order to bind to P-selectin, cancer cells require certain sulfated glycan epitopes besides sLeX, which include the PSGL-1 core protein and the sulfated tyrosine on PSGL-1 [89, 90]. Breast cancer cells with decreased sLeA/X have increased clumping and formation of emboli, because expression of sLeA/X leads to greater cell-cell repulsion [91]. Biophysical experiments with glycan-coated beads confirmed the ability of a non-sialylated version of sLeX, called LeX, to confer adhesive forces [92]. Vocadlo and colleagues, alternatively, demonstrated that decreases in sLeX expression with a fucose analogue, 5-thio-L-fucose, leads to impairment of selectin-mediated adhesion in liver cancer cells [93]. Pancreatic cancer cells exhibit increased sLeX level on E-cadherin and α2β1 integrin, resulting in the reduction of cell-cell aggregation capacity and increased migration and invasion characteristics [94].

Complementing the *in vitro* studies, *in vivo* studies further demonstrate that higher sLeX expression on cancer cells in mice leads to higher metastasis [95]. Colon cancer cells selected for high sLeX had higher liver metastases than their counterparts with low sLeX expression [87]. A study involving staining for sLeA/X showed the association with the invasive front of primary colorectal cancer specimens [96]. The staining of the sLeX pattern at the invasive front, particularly in areas with dedifferentiated histology, was associated with the presence of liver metastases.

In clinical applications besides pancreatic cancer, high serum levels of sLeA but not high sLeX is a prognostic indicator for shorter survival among patients with colorectal cancer [97, 98], whereas in tissue the reverse is true [99]. Some antigens related to the one detected by the CA19-9 antibody that differ in the linkage of the sialic acid residues are not as specific for cancer showing, in contrast, higher rates of elevation in non-malignant disorders [100].

Conversely, sLeX has not been as useful as a serological biomarker as sLeA, mainly because it appears to be elevated more frequently in benign disease conditions, probably owing to its broader expression in infiltrating leukocytes. The sLeX is elevated in the serum of various cancers, mostly of the GI cancers, at a frequency of about 7-42% of patients, while LeX is elevated at a frequency of 2-22% [101]. Serum glycoproteins bearing sLeX are significantly upregulated in several epithelial cancers, including prostate, colorectal, liver and lung cancers [86, 102-104]. Interestingly, studies demonstrated general agreement between blood levels and tissue levels of sLeA, but no reports examined the relationships with various histomorphologies or the relationships with sLeX [105, 106].

As a tissue marker, despite the multiple studies showing an association between sLeX staining and disease progression, the clinical use of immunohistochemistry for sLeX is not established. The difficulty might have to do with establishing objective standards by which conclusions may be drawn. Another difficulty may be variability in staining levels due to prominent expression among certain immune cells, the levels of which are highly irregular.

**Sulfated Lewis glycans in normal biology**

In comparison with sLeA/X, the sulfated Lewis glycans are rare and less studied. The expression and function of these glycans are primarily investigated through the lens of their receptor counterparts. The three members of the selectin family mentioned above require distinct ligands involve with either sLeX or sulfated variants of sLeX present on a variety of glycoproteins [75, 82, 107]. Sulfated sLeX on O-glycans appears as an essential ligand in the high endothelial venules found in secondary lymphoid organs. Two key sulfotransferases, N-acetylglucosamine-6-O-sulfotransferase 1 (GlcNAc6ST-1) and N-acetylglucosamine-6-O-sulfotransferase 2 (GlcNAc6ST-2), are responsible for assembling the 6-sulfated sialyl Lewis X (6-sulfo-sLeX) in normal immunology [108, 109]. In term of ligand specificity, the bindings between carbohydrate ligands and the L-selectin expressed on all leukocytes mediates important lymphocyte homing processes during acute inflammation [107]. 6-suflo-sLeX and tyrosine sulfations (sulfate modification on amino acid) on PSGL-1 are essential ligands required for P-selectin that are found in the storage granules of platelets and Weibel-Palade bodies of activated endothelial cells [75, 82, 110]. L-selectin prefers ligands with 6-sulfo-sLeX more than tyrosine sulfation on the endoglycan and PSGL-1 [110-112].

The MECA-79 antibody directly against the 6-sulfo-LacNAc motif on extended core-1 O-glycans [113]. The MECA-79 antigen is found induced on pancreatic ducts in type-1 autoimmune pancreatitis and may contribute to the dense lymphocyte infiltration seen in that condition [114].

Beside the selectin family, sulfated variants of sLeX glycans also associate with three CD33-related I-type lectins, Siglecs-7, -8 and -9. Siglec-7 is predominately expressed by natural killer (NK) cells and serves as an inhibitory receptor. Siglec-8 is predominately expressed on eosinophils, with weaker expression on basophils. Siglec-9 is expressed on neutrophils, monocytes, a subset of NK cells and CD8+ T cells. These lectins contain the immunoglobulin variable region (V)-set domain that specifically interacts with sialylated and sulfated glycans.

Glycan array analyses revealed tremendous details of ligand specificities for these Siglec receptors. Although both Siglec-7 and -8 demonstrate preferential binding to 6,6'-disulfated sLeX, Siglec-8 exhibits a stronger binding toward the 6'-sulfo-sLeX and 6'-sulfo-sialylated LacNAc [115, 116]. On the other hand, Siglec-9 has demonstrated preferential bindings to 6-sulfo-sLeX and 6-sulfo-sialylated LacNAc [115, 116].

Cross-linking of Siglec-7 with monoclonal antibody on total peripheral blood mononuclear cells triggers a remarkably high production of several pro-inflammatory cytokines and chemokines exclusively within the monocyte compartment [117]. Thus, it suggests that Siglec-7 and sulfated sLeX play a role in generating a monocyte-mediated inflammatory outcome following pathogen recognition. In a virology study, Siglec-7 on NK cell surface expression is significantly decreased in HIV-1 infected patients with high

levels of ongoing viral replication, thus also suggested that a direct binding between Siglec-7 and HIV-1 causes modulation of immune response [118].

Similarly, the cross-linking of Siglec-9 on neutrophils in an inflammatory environment has been shown to induce both caspase-dependent and caspase-independent cell death of innate immune cells and subsequently generate reactive oxygen species (ROS) [119]. Resident macrophages express Siglec-7 and Siglec-9 receptors, and their engagement with sulfated glycans is immunosuppressive to Lipopolysaccharides (LPS) induction of cytochrome c oxidase polypeptide II (COX2) in colonic epithelial mucosa. Moreover, the engagement of Siglec7 and Siglec-9 receptors with their sulfated ligands is also maintaining immunological homeostasis in colonic mucosal membranes [120].

Human Siglec-8 and its murine paralog Siglec-F recognize 6’ sulfo-sLeX [121]. These receptors appear on eosinophils, and their activity can be blocked by the expression of the glycan ligand in cis [122]. The engagement of these receptors is inhibitory, acting through an immunoreceptor tyrosine-based inhibitory motif (ITIM) on their cytoplasmic domains. Engagement of the receptor by the ligand is potentially pro-apoptotic in eosinophils [123]. Overall, these Siglec receptors on innate immune cells and engagement with their respective sulfated and sialylated glycans plays a significant role in the normal inhibitory mechanism of leukocytes to downregulate inflammation.

**Sulfated Lewis glycans in cancer**

The highly sialylated glycocalyx of some cancer cell surfaces functions as a self-associated molecular pattern, which can inhibit the innate immune response similar to pathogens mimicking host self-antigen mechanism [124]. Recently, there is evidence

suggesting that Siglec-7 and Siglec-9 expressed by NK cells are associated with impairing NK cell immunosurveillance in several cancers [47, 125, 126]. By manipulating the sialylated glycocalyx of cancer cell, researchers found decreased susceptibility of NK cells to cancer cells via Siglec-7 engagement and facilitated inhibition of NK cell-mediated cytotoxicity toward cancer cells [47]. In an *in vivo* model, immunodeficient mice with reconstituted human NK cells and human cancer cells showed a subset of Siglec-9+ NK cells and their 6-sulfo-sLeX ligands to be responsible for the inhibition of NK cell mediated tumor cell killing [125].

In addition, 6-sulfo-sLeX detected using the MECA-79 antibody is correlated with depth of invasion, venous invasion, TNM stage and distant metastasis in gastric cancer [108]; and a similar connection was observed in bladder urothelial carcinoma [127]. Siglec-8, recognizes 6'-sulfo-sLeX, which also suggests as that this is an independent prognostic indicator of overall survival for gastric cancer patients. In immunohistochemistry study of ovarian cancer patients, the expression of GlcNAc6ST-2, the sulfotransferase responsible for 6-sulfo-sLeX production, is correlated with poor prognosis due to the association with chemo-resistant subtypes of adenocarcinomas [128].

The data from these *in vitro* and *in vivo* experiments present an insufficient understanding of specific interactions between sulfated sLeX glycans and Siglec. One obvious question is how to properly identify and quantify specific sulfated sLeX variants in clinical samples. Currently, technological limitations prohibit researchers to fully investigate the links between these sulfated glycans and cancer. These limitations primarily associate with technological developments, however, a small number of studies were able to perform serum glycomic analyses with antibody/lectin profiling and

mass spectrometry-based methods and provided some evidence to begin addressing this gap [84, 108, 129, 130]. To investigate the expression levels of these sulfated and sialylated Lewis glycans in a large-scale cancer versus control study, I desperately need to develop a feasible and robust method to facilitate basic and translational research.

**The possibility of subtypes of PDAC**

Pancreatic cancers display significant diversity in their rates of growth and dissemination and in their responses to drugs. Recent research has found support for the concept that PDACs comprise several biological subtypes. One of genomic classification studies reported three subtypes, “classical”, “quasi-mesenchymal”, and “exocrine-like”, which have different clinical outcomes and therapeutic response [131]. Subsequently, more whole-genome sequencing studies have identified numerous and different subtypes of PDAC with patient prognostic application and diverse tumorigenesis signaling pathways [132-134]. Although these studies collectively reported potential prognostic and predictive genomic signatures, few have been validated, and practical molecular markers from whole-genome expression profiling are not available. Nevertheless, these studies support the idea that distinct subtypes make up the overall category of PDACs. The implications of that finding are that the molecular markers could be greatly different for each subtype, and that in research to find new markers, I should be searching for markers to detect complementary subgroups. That in principle guided the approach taken in this dissertation.

## Hypothesis

My previous analyses, which focused on examining multiple CA19-9 monoclonal antibodies and their cross-reactivities, indicated that other Lewis blood group antigens besides sLeA, such as sLeX and 6-sulfo-sLeX, could be alternative tumor markers for PDACs [135]. Therefore, I planned to test the hypothesis that other glycans within the Lewis blood group family besides sLeA are aberrantly increased in the subpopulation of PDAC patients who do not secret sLeA into their blood.

Other studies used enzymatic, chromatographic and mass spectrometry methods to provide detailed information about these cancer-associated glycans [84, 108, 129, 130]. However, the requirement for large amounts of experimental material limits the application of these methods to clinical specimens, thus prohibiting extensive analyses of relationships with disease. Affinity-based methods, using reagents such as lectins and glycan-binding antibodies, are an ideal alternative to allow measurements of glycan motifs in large case control studies. Depending on the experimental design and specificity of the affinity reagents, the antibody-lectin sandwich microarray method described previously can enable the profiling of specific glycans on multiple proteins across multiple samples [66].

To test the overarching hypothesis, I initially focused on the type 2 LacNAc glycans bearing α1-3 fucosylated motifs, using monoclonal antibodies and lectins.

To more extensively examine the overarching hypothesis on other members within the Lewis blood group antigens, especially sulfated type 2 LacNAc glycans, it was necessary to advance technological aspects in both the benchtop methods and

computational methods to decipher less-studied glycan structures, for example, sulfated variants of sLeX. Therefore, I used a new method developed in my laboratory, named on-chip Glycan Modification and Probing (on-chip gmap), to provide high-confidence analysis of multiple glycosylated antigens in clinical specimens. I used antibody-lectin sandwich microarray methods and on-chip gmap to pursue the following goals.

1. Improve diagnostic biomarker performance by detecting sLeX and α1-3 fucosylated glycans in the sLeA-low pancreatic cancer subgroup.
2. Identify novel pancreatic tumor biomarkers that complement CA19-9 through probing the sulfated variants of α2-3 sialylated type 2 LacNAc glycans.

# CHAPTER TWO: IMPROVE DIAGNOSTIC BIOMARKER PERFORMANCE BY DETECTING SLEX AND α1-3 FUCOSYLATED GLYCANS IN THE SLEA-LOW PANCREATIC CANCER SUBGROUP.

The results of this chapter have been published as

1. Tang H, Singh S, Partyka K, Kletter D, **Hsueh P**, Yadav J, Ensink E, Bern M, Hostetter G, Hartman D, Huang Y, Brand RE, Haab BB. (2015) Glycan motif profiling revels plasma sialyl-Lewis X elevations in pancreatic cancers that are negative for sialyl-Lewis A. Mol Cell Proteomics 14(5):1323-33

**Author contributions**

I was involved in data collection and analysis for Fig. 3 and Table 3.

2. Singh S, Pal K, Yadav J, Tang H, Partyka K, Kletter D, **Hsueh P**, Ensink E, Kc B, Hostetter G, Xu HE, Bern M, Smith DF, Mehta AS, Brand R, Melcher K, Haab BB. (2015) Upregulation of glycans containing 3' fucose in a subset of pancreatic cancers uncovered using fusion-tagged lectins. J Proteome Res. 14(6):2594-605

**Author contributions**

I was involved in data collection for Fig. 4B and 4C.

3. Tang H, Partyka K, **Hsueh P**, Sinha JY, Kletter D, Zeh H, Huang Y, Brand RE, Haab BB. (2016) Glycans related to the CA19-9 antigen are elevated in distinct subsets of pancreatic cancers and improve diagnostic accuracy over CA19-9. Cell Mol Gastroenterol Hepatol. 2(2):201-221

**Author contributions**

I conducted the study shown in Fig. 7, and contributed to the design, data collection and analyses for Fig. 8, Fig. 9 and Table 4.

## Introduction

A patient with an uncertain lesion of the pancreas typically is referred to a specialist for dedicated scans of the pancreas and additional endoscopic imaging with fine-needle aspiration to obtain material for cytopathology. The greatest diagnostic challenge associated with the most common type of pancreatic cancer, pancreatic ductal adenocarcinoma (PDAC), is differentiating non-malignant from malignant conditions [136-139]. Solely based on imaging and biopsy, non-malignant diseases such as chronic pancreatitis and biliary obstriction can resemble PDAC and result in false positive diagnoses. In addition, the biopsy procedure is not always possible for PDAC patients [140]. The discovery of an ideal molecular marker for PDAC is of great interest as it will provide an objective and detailed assessment of each patient's condition, with the potential for less invasive screening.

Many PDAC tumors secrete glycoproteins that bear a glycan called sLeA into the circulatory system [34, 35]. This glycan forms the basis for the FDA-approved CA19-9 blood test for pancreatic cancer. The CA19-9 test was named after the monoclonal antibody first developed against the sLeA [141], and is currently used as the best indicator of disease recurrence. However, approximately 25-35% of PDAC patients do not show elevated sLeA using a typical cutoff value of 37 U/mL, rendering the test inconclusive for the diagnosis or monitoring of cancer in those patients. Therefore, identification of another marker to detect cancer among patients with low sLeA levels could lead to an improved diagnostic test.

Previous research suggested that abnormal glycan biosynthesis is a common feature of pancreatic cancer. The discovery that CA19-9 antibodies recognize a glycan further

revealed the prevalent nature of glycosylation alteration in pancreatic cancer. Through understanding the cause of sLeA elevations in pancreatic cancers, we may gain information why sLeA not elevated in 25-35% of cases. The increase of sLeA in the blood likely results from accumulation in the stroma followed by leakage into the capillaries or lymph [142]. The lack of sLeA expression is partly linked with genetics; FUT3, a glycosyltransferase enzyme, is critical for the biosynthesis of sLeA and is inactive in approximately 5% of the North American population as a result of homozygous mutations in the active part of the gene [143]. However, the cause of low sLeA levels is not clear for patients with wild-type FUT3.

Previous research indicated that alternative Lewis blood group glycans could be associated with pancreatic cancer. An isomer of sLeA called sLeX was shown to be up-regulated in the tissue of some pancreatic cancers [144]. Our previous study examined the binding of three different monoclonal antibodies against sLeA, and we found variations between these antibodies[135]. Glycan array analyses revealed that one of the antibodies (clone M081221, Table 1) bound to other types of glycans, including sLeX, the sulfated variant of sLeX, and the canonical target, sLeA. Other studies have demonstrated that fucose levels (related to Lewis blood group) are increased in GI cancers on specific proteins [145-148]. The specific linkage of fucose can be a critical difference in distinguishing malignant diseases from a healthy condition. For example, if fucose is on the 2’ carbon of a galactose, it can contribute to the precursor for ABO blood group structures, whereas if it presents on the 3’ or 4’ carbon of the GlcNAc in type 1 and type 2 LacNAc, it is a precursor for Lewis blood group glycans and can contribute to the formation of sLeA and sLeX (Fig. 1). These observations led to the

hypothesis that a subgroup of PDAC patients with low CA19-9 could have alternative Lewis blood group biosynthesis and thereby produce other similar glycans to sLeA.

These observations suggest that PDACs produce a variety of Lewis blood group glycans, and these glycans are secreted into the circulation, with individual cancers presenting different glycan “signatures”. Thus, to capture and characterize the full diversity of PDAC patients, our strategy was to define the various glycans that pancreatic tumors express at altered levels as compared to healthy tissue. Through this approach, we tested the hypothesis that other glycans within the Lewis blood group family besides sLeA are aberrantly increased in the subpopulation of PDAC patients who do not secret sLeA into their blood.

## Methods and materials

### *Human plasma and tissue samples*

All plasma samples were collected at the University of Pittsburgh Medical Center following informed consent of the participants and prior to any surgical or medical procedures. The donors consisted of patients with pancreatic cancer, pancreatitis, or biliary stricture, and healthy subjects (Table 2). Early-stage cancer included stages I and II, and late-stage cancer included stages III and IV. The healthy subjects had no evidence of pancreatic, biliary or liver disease. All blood samples were collected according to the standard operating procedure from the Early Detection Research Network (EDRN) and were frozen at -70°C or colder within 4 h of time of collection. Aliquots were shipped on dry ice and thawed no more than three times prior to analysis.

In addition, the Van Andel Research Institute Biospecimen facility provided formalin-fixed, paraffin-embedded tissue from patients who underwent pancreatic resections at a regional hospital affiliate in Grand Rapids, MI. The Institutional Review Boards at the University of Pittsburgh Medical Center and the VARI approved this research project (protocol #12008).

***Biological reagents***

The plasma samples were diluted two-fold into 1X PBS containing 0.1% Tween-20, 0.1% Brij-35, and IgG blocking cocktail (200 µg/mL mouse and rabbit IgG and 100 µg/mL goat and sheep IgG (Jackson ImmunoResearch)) and 1X protease inhibitor (Complete Mini EDTAfree Tablet, Roche Applied Science). The CA19-9 standard human CA19-9 Gastrointestinal Cancer Antigen Grade from RayBiotech, Norcross, GA) was prepared the same way as the plasma samples. This protein served as a positive control and allowed us to calibrate sLeA glycan levels from relative fluorescent unit to Unit/mL.

The antibodies and lectins were acquired from various sources (Table 1). Affinity reagents used as capture antibodies were dialyzed against 1X PBS and ultracentrifuged (47,000 x g for 1 h at 4°C) as a standard antibody processing protocol. Affinity reagents used as detection probes, antibodies and lectins, were labeled with a biotin tag by using the EZ-Link-sulfo-NHS-LC-Biotin kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocols.

### *Antibody microarray fabrication*

The antibodies were prepared at 250 µg/mL in 1X PBS with 0.05% Tween-20 and 15% glycerol. We used a robotic microarray printer (2470, Aushon Biosystems, Billerica, MA) to print the capture antibodies (Table 1) onto microscope slides coated with a thin layer of nitrocellulose (PATH slides, Grace Bio-Labs, Bend, OR). Each slide contained 48 identical arrays arranged in a 4 x 12 grid with 4.5 mm spacing between arrays, and each array had three to six replicate spots of each antibody. A wax-based hydrophobic border was imprinted to define boundaries between the arrays (SlideImprinter, The Gel Company, San Francisco, CA). The printed slides were stored at 4°C in a desiccated, vacuum-sealed slide box until use.

### *Antibody and lectin sandwich microarray assay (ALSA)*

The ALSA experiments allowed multiplexed comparisons of all combinations of capture antibodies and detection probes, antibodies and lectins (Fig. 2). After the antibody microarrays were fabricated, the slides were rinsed in 1X PBS plus 0.5% Tween-20 (PBST0.5), washed in the same buffer for 15 min, and dried by centrifugation (Eppendorf 5810R, rotor A-4-62, 1500 x g for 3-6 min), with the printed arrays facing outside. Arrays were then blocked to avoid nonspecific interaction by using 1% bovine serum albumin (BSA) in PBST0.5 buffer for 1 h at room temperature (RT). The arrays were washed in three changes of 1X PBS plus 0.1% Tween-20 (PBST0.1) for 3 min each and dried by centrifugation (1500 x g for 3-6 min). 6 µL of each plasma sample was applied to each array and incubated overnight at 4°C. Each unique sample was applied to three separate arrays. After overnight incubation, the arrays were washed in three changes of PBST0.1 for 3 min each and dried by centrifugation (1500 x g for 3-6

min), and various biotinylated lectins or antibodies were incubated on the arrays for 1 h at room temperature (Table 1). In this study, the lectins and antibodies were prepared at 3 µg/mL in 1X PBS with 0.1% BSA and 0.1% Tween-20, except for the anti-LeA (clone 7LE) antibody, which was at 15 µg/mL. For *Coprinopsis cinerea lectin 2* (CCL2) detection, we pre-complexed the CCL2 with Cy5-conjugated streptavidin (Roche Applied Science) at a 4:1 molar ratio [149].

After washing and drying the arrays as described above, Cy5-conjugated streptavidin was prepared at 2 µg/mL in 1X PBS with 0.1% BSA and 0.1% Tween-20 and incubated on arrays for 1 h at RT, followed by a final wash (three changes of PBST0.1 for 3 min) each and dried by centrifugation (1500 x g for 3-6 min)). The arrays detected with pre-complexed CCL2/streptavidin were washed once and dried by centrifugation (1500 x g for 3-6 min). Fluorescence detection was performed using 633 nm excitation (LS Reloaded, TECAN) to obtain raw fluorescent images.

To analyze the fluorescent images, first the software GenePix Pro 5.1 (Molecular Devices, Sunnyvale, CA) was used to quantify and analyze the array images using both automatic and manual spot finding features. The intensity of each spot was calculated by subtracting the local background from the median intensity of each spot. The quantified results for each image were further processed using the Grubb's test to remove outlier spots among the three to six replicates for each spotted antibody or protein based the individual experiment.

Quantification of the raw fluorescent array images was also performed using in-house software written in Matlab (version R2014a, Mathworks). A custom script was used to remove any outliers from the six replicate spots according to the Grubbs' test. The script

calculated the Grubbs' statistic for the spot farthest from the mean of the replicates and rejected the spot if the Grubbs' statistic exceeded a preset threshold, in this case $p < 0.1$. This process was repeated until no outliers remain or to a minimum of four spots. It then calculates the geometric mean of the remaining replicate spots as the final output for each array. The program also averages signal values between replicate arrays and reports the associated coefficient of variation. Assays with measurements that had a coefficient of variation (CV) > 0.4 for signals in the quantifiable response range of the assay (determined by dilution series of pooled samples) were repeated [150].

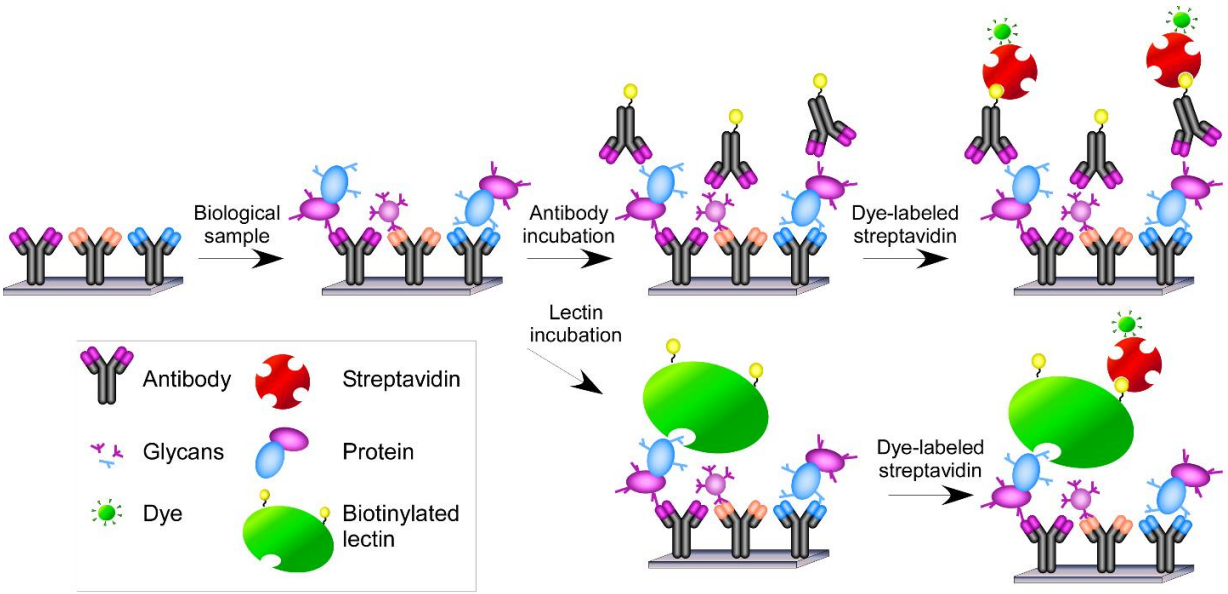



**Figure 2. Antibody-lectin sandwich microarray (ALSA)**. The high throughput and multiplexed sandwich array-based assay measures specific glycoforms of glycoproteins and the co-expression of different glycans on individual proteins. A plasma sample or supernatant was incubated on customized antibody array, and specific affinity reagents, antibodies and lectins were used to probe glycans of interest.

***Statistics and analysis methods***

To characterize classification performance of individual biomarkers, nonparametric estimates of the Receiver Operating Characteristics (ROC) curves were generated. Performance of each biomarker was compared to CA19-9 based on the area under the ROC curve (AUC). Specifically, a nonparametric bootstrap procedure stratified on case and control status was performed with 500 bootstrap samples. Two-sided p-values for testing the equivalence in AUC between a pair of biomarkers were calculated based on the Wald test and the bootstrap estimated standard error. Also reported were 95% confidence intervals of the difference in AUC based on bootstrap samples. All statistical calculations were carried out using R program R-3.2.2 (https://cran.r-project.org/).

The three markers panels were selected by using the Marker State Space (MSS) method with 10-fold cross-validation to select individual markers [151]. This approach limits the initial size of panels to three markers, with the option of adding markers iteratively. The MSS software is available upon request. GraphPad Prism and Microsoft Excel for graph preparation and Canvas XIV (ACD Systems) utilized for figure preparation.

***Immunohistochemistry***

Immunohistochemistry (IHC) was performed using automated staining (Ventana Discovery Ultra) on sections cut from Formalin-Fixed Paraffin-Embedded (FFPE) blocks. The standard protocol for the anti-sLeA (9L426), anti-sLeX (CSLEX1) primary antibodies and CCL2 lectin was as follows: antigen retrieval using the Ventana CC1 buffer for 36 min at 95°C; primary antibody incubation at a 1:200 dilution and CCL2 incubation at 15 µg/mL for 32 min at 37°C; and secondary antibody (Ventana Umap HRP-conjugated anti-mouse) for 12 min at 37°C. The development step used the diaminobenzadine chromagen according to preset parameters in the Ventana platform.

***Immunofluorescence (IF) staining method***

Immunofluorescence staining was performed on 5 µm FFPE sections by first removing paraffin by three citrosol washes followed by ethanol/H2O rehydration (twice each at 100%, 95%, 70%) and two washes in 1X PBS. Antigen retrieval was achieved by incubating the slides in citrate buffer at 100°C for 20 min followed by blocking the slides in 1X PBS containing 0.05% Tween-20 (PBST0.05) and 3% BSA for 1 h at RT.

One round of immunofluorescence staining was incubated in PBST0.05 with 3% BSA containing anti-MUC5AC antibody or SELE (10 μg/mL each), SELE was labeled with sulfo-Cyanine5 (13320, Lumiprobe) and anti-MUC5AC antibody was labeled with sulfo-Cyanine3 (11320, Lumiprobe) according to the supplier protocol. The antibody/lectin solution was incubated with a tissue section overnight at 4°C in a humidified chamber. The antibody solution was subsequently decanted, and the slide was washed three times for 3 min each, twice in PBST0.05 and once in 1X PBS. The slide was blotted dry and incubated with Hoechst 33258 (1:1000 dilution in 1X PBS) for 10 min at RT. Slides were then given two final washes in 1X PBS twice for 5 min before imaging using a scanning-fluorescence microscope (Vectra, PerkinElmer, Waltham, MA).

## Results

### *Testing the plasma sLeX levels in sLeA-low PDAC patients*

We hypothesized that other glycans within the Lewis blood group family besides sLeA are aberrantly increased in the subpopulation of PDAC patients who do not secrete sLeA into the blood. We first planned to measure the sLeX level in the plasma samples of PDAC patients with an antibody that has the primary specificity for sLeX.

A cohort of 200 plasma samples (Discovery cohort), consisting of samples from PDAC patients and patients with chronic or acute pancreatitis or biliary stricture (Table 2), was screened by the ALSA method using antibody microarrays containing both anti-sLeA/CA19-9 (9L426) and anti-sLeX antibodies (Table 1). The arrays were incubated with plasma samples and probed with anti-sLeA and anti-sLeX antibodies. To objectively evaluate our data relative to results from the clinical CA19-9 assay, we calibrated the sLeA levels using a control protein standard, called the CA19-9 standard, to convert the data to Units/mL as used with the clinical assay. From our data, we observed sLeA levels were elevated in 40 of 109 PDAC patients (37% sensitivity) based on the cutoff of 100 U/mL, as used clinically to give higher specificity for cancer (37 U/mL is the standard cutoff). Among the PDAC patients who had below 100 U/mL sLeA level, 13 of 69 (19%) displayed elevated sLeX, based on a threshold that produced no additional control subjects showed elevations (Fig. 3A). In addition, sLeX levels were significantly different ($p < .001$, Mann-Whitney test) between sLeA-low PDAC patients and all control subjects (Table 3). Therefore, distinct groups of PDAC patients showed sLeA and sLeX elevations found in plasma, while most of control subjects showed low to no elevations in either.

In order to optimize this approach, other combinations of capture:detection assays were assessed for improvements in discriminating pancreatic cancer patients from controls. The combination assay showing the greatest difference between sLeA-low PDAC patients and controls was anti-sLeA antibody as capture with anti-sLeX antibody as detection (Table 3). This result, representing the presence of sLeA and sLeX on the same glycoproteins, was significantly elevated in PDAC patients. Among the PDAC patients that were below the cutoff of 100 U/mL of sLeA, 24 of 69 (35%) displayed elevated sLeA:sLeX signals using a cutoff in that marker added only one new false positive control (Fig. 3B). Interestingly, we found that a two-marker panel consisting of the sLeX sandwich assay and the hybrid sLeA:sLeX assay, that had a sensitivity of 76% (83/109 cases, 95% Confidence Interval, represents the frequency of possible confidence intervals that contain the true value of the unknown population parameter, (CI): 59.6 –89.0%), a specificity of 78% (71/91 controls, 95% CI: 65.9 –97.8%), and an accuracy of 77% (154/200, 95% CI: 74.0 –84.0%) (Fig. 3C). These values were significantly better ($p < .009$) than the best accuracy of 68% (136/200) for the CA19-9 test. In addition, it should be noted that the controls in this study did not include healthy subjects, so the performance of CA19-9 is slightly worse than in studies comparing PDAC patients to healthy people.

**Table 1. Capture antibodies and detection reagents.**

| Name | Clone Name | Primary Target | Source | Cat. No. |
|---|---|---|---|---|
| **Capture antibodies** | | | | |
| **anti-MUC16** | X325 | MUC16 | AbCam | AB10033 |
| **anti-MUC16 (Ab2)** | X306 | MUC16 | Novus Biologicals | NB120-10032 |
| **anti-MUC5AC** | 45M1 | MUC5AC | ThermoScientific | MS-145-P1ABX |
| **anti-MUC5AC (Ab2)** | 2-11M1 | MUC5AC | Affinity BioReagents | MA1-35704 |
| **Anti-Sialyl Lewis A (CA19-9, Ab1)** | 9L426 | Sialyl Lewis A | USBio | C0075-03A |
| **Anti-Sialyl Lewis A (CA19-9, Ab2)** | 121SLE | Sialyl Lewis A | AbCam | AB3982 |
| **Anti-Sialyl Lewis X** | CSLEX1 | Sialyl Lewis X | BD Pharmingen | 551344 |
| **Mouse IgG, biotin labeled** | N/A | N/A | Jackson ImmunoResearch | 015-000-003 |
| **Detection antibodies and lectins** | | | | |
| **Anti-Sialyl Lewis A (CA19-9, Ab1)** | 9L426 | Sialyl Lewis A | USBio | C0075-03A |
| **TRA-1-60** | TRA-1-60 | Terminal N-acetyl-lactosamine, type 1 | Novus Biologicals | NB100-730 |
| **Anti-Sialyl Lewis X** | CSLEX1 | Sialyl Lewis X | BD Pharmingen | 551344 |
| **Recombinant Mouse E-Selectin/CD62E Fc Chimera, CF** | SELE | Sulfated Lewis Structure | R&D Systems | 575-ES-100 |
| **Anti-Blood Group Lewis A** | 7LE | Lewis A and Terminal N-acetyl-lactosamine, type 1 | Abcam | ab3967 |
| **Coprinopsis cinerea (Inky cap fungus) lectin 2** | CCL2 | Lewis X variants: sialylated, sulfated, internal | Recombinant production | N/A |
| **Enzyme** | | | | |
| **α2-3,6,8 Neuraminidase (sialidase)** | N/A | α2-3,6,8 salic acids | NEB | P0720 |

**Table 2. Sample characteristics.** The Discovery cohort was the same set used in Figures. 3, 4, 8, and 9, but one cancer sample was excluded out of this study due to lack of sufficient material.

| Discovery | *n* | Age (SD) | % Male |
|---|---|---|---|
| All Cancer | 108 | 68.1 (9.8) | 48.1 |
| Stage I | 2 | | |
| Stage II | 36 | | |
| Stage III | 32 | | |
| Stage IV | 32 | | |
| Unknown Stage | 6 | | |
| Neuroendocrine Tumor | 0 | | |
| All Control | 91 | 57.5 (15.3) | 49.5 |
| Pancreatitis | 61 | | |
| Benign Stricture | 30 | | |
| Abnormal Imaging | 0 | | |
| p-value* | | <0.05 | NS |

| Validation | *n* | Age (SD) | % Male |
|---|---|---|---|
| All Cancer | 48 | 65.9 (9.3) | 58.3 |
| Stage I | 0 | | |
| Stage II | 21 | | |
| Stage III | 6 | | |
| Stage IV | 20 | | |
| Unknown Stage | 0 | | |
| Neuroendocrine Tumor | 1 | | |
| All Control | 69 | 54.0 (15.4) | 40.6 |
| Pancreatitis | 12 | | |
| Benign Stricture | 9 | | |
| Abnormal Imaging | 48 | | |
| p-value* | | <0.05 | NS |

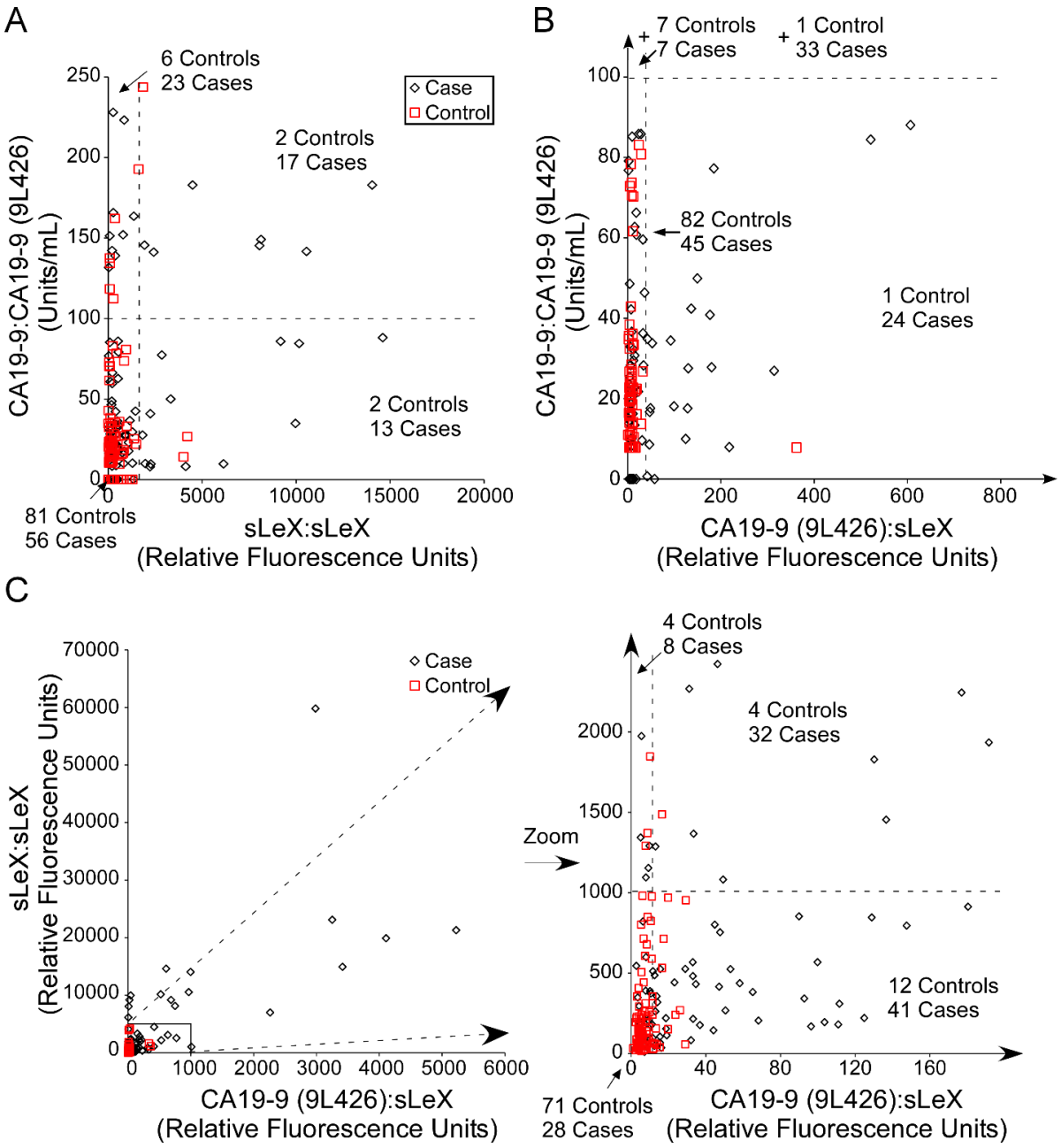



**Figure 3. sLeX and dual expression of sLeA and sLeX are elevated in subpopulation of sLeA-low PDAC patients.** These figures are adapted from Tang H et al (2015) [177]. A) A scatter plot displaying sLeA levels (using anti-sLeA/CA19-9 antibody, 9L426, as capture and detection, y axis) and sLeX levels (using anti-sLeX antibody, CSLEX1, as capture and detection, x axis) in the plasma of both PDACs (cases, black diamonds) and control subjects (controls, red squares). Each point represents a patient sample, and each value is the average of experimental replicates. The dashed lines represent thresholds defining elevations for each assay. B) Identical to the graph A), the y axis indicates the sLeA level is measured by anti-sLeA sandwich assay. The x axis indicates measurement of the dual expression of sLeA and sLeX on

Figure 3 (cont'd)

the same glycoprotein is found in the plasma samples (using anti-sLeA antibody as capture and anti-sLeX as detection). At the thresholds defined by the dashed lines, many PDAC patients are elevated only in the sLeA levels or in the sLeA:sLeX dual expression marker. C) A scatter plot comparing the sLeX level (y axis) to the sLeA:sLeX dual expression (x axis) in the plasma sample of PDACs and control subjects. The right panel is a magnification of the indicated region. At the thresholds defined by the dashed lines, 8 and 41 cases are elevated only in the sLeX levels or in the sLeA:sLeX dual expression, respectively.

**Table 3. Statistical comparisons between the patient groups within the Discovery cohort.** This table is taken from Tang H et al (2015) [177]. Each marker is indicated by the capture:detection antibodies (left). The first p value* (middle) is based on the Mann-Whitney test for comparing sLeA-low (< 37 U/mL, the standard cutoff) PDAC patients (n = 51) to all non-malignant disease patients (controls) (n = 91). The second p value^ (right) is for comparison all PDAC patients (n = 109) to all controls (n = 91). NS = not significant.

| Marker | *p* value* | *p* value^ |
|---|---|---|
| Sialyl Lewis A(9L426):sialyl Lewis X(CSLEX1) | 9.20E-06 | 3.09E-13 |
| Sialyl Lewis X(CSLEX1):sialyl Lewis X(CSLEX1) | 2.24E-04 | 4.06E-08 |
| Lewis A(7LE):sialyl Lewis X(CSLEX1) | NS | 1.08E-03 |
| Sialyl Lewis A(121SLE):sialyl Lewis X(CSLEX1) | NS | 1.01E-02 |
| Sialyl Lewis A(M081221):sialyl Lewis X(CSLEX1) | NS | NS |
| Sialyl Lewis X(9L648):sialyl Lewis X(CSLEX1) | NS | NS |
| DUPAN2:sialyl Lewis X(CSLEX1) | NS | NS |
| Lewis X(P12):sialyl Lewis X(CSLEX1) | NS | NS |

### *Assessing α1-3 fucosylated glycan as a candidate biomarker*

To potentially further improve detection of sLeA-low cancers, other Lewis X variants or type 2 LacNAc structures containing the α1-3 fucose motif were screened for significant expression in PDAC patients. Preliminary results revealed that recombinant *Coprinopsis cinerea lectin 2* (CCL2), a α1-3 fucose motif binder, was capable of detecting four additional PDAC patients and only one control that were not detected by CA19-9 (Fig. 4A). To build upon these findings, we analyzed the statistical differences between cancers and controls in CCL2 binding in the Discovery cohort (Table 2). The three assays using CCL2 lectin as detection, sLeA:CCL2, sLeX:CCL2 and MUC5AC:CCL2 (Fig. 4B), gave significant discrimination of PDAC patients from controls, which indicated α1-3 fucose was a prevalent feature exhibited on certain glycoforms of MUC5AC and other glycoproteins likely produced by the tumors. Using the stringent cutoff of only allowing one false-positive control, the sLeA:CCL2 assay improved the sensitivity by detecting five additional PDAC patients (Fig. 4C).

The sLeX glycan, which contains the α1-3 fucose motif, could potentially be the main glycan bound by CCL2 lectin. To determine whether CCL2 was detecting glycans besides sLeX, we directly compared the CCL2, sLeA and sLeX results. Different groups of cancer patients had higher levels of different glycans; some PDAC patients were exclusively elevated with CCL2 assay (sLeA:CCL2) (Fig. 4C). The results indirectly indicated that CCL2 was not binding only sLeX and that CCL2 provides a complementary candidate marker to both sLeA and sLeX.

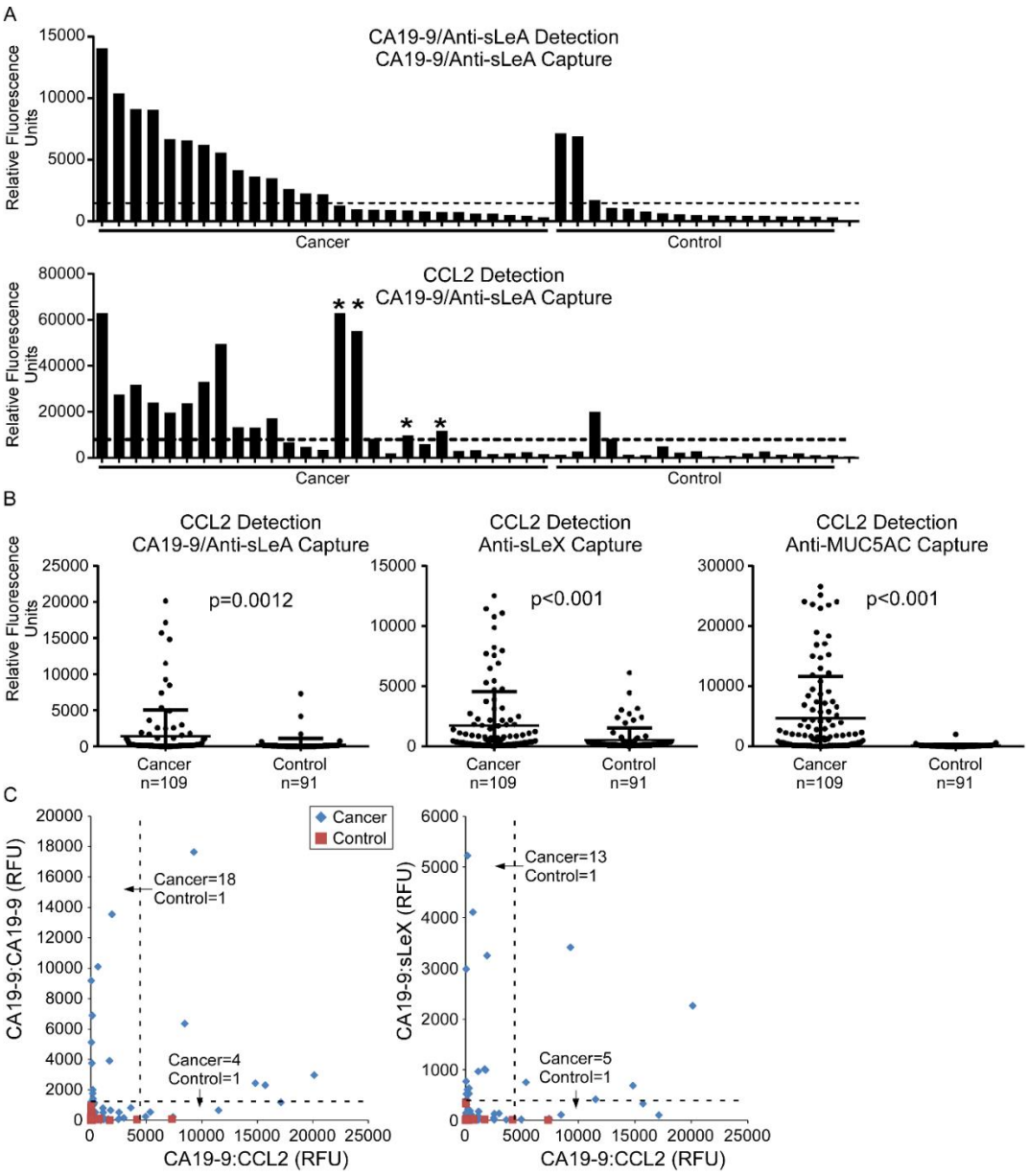



**Figure 4. CCL2 detection as a marker can complement sLeA.** These figures are adapted from Singh S et al (2015) [166]. A) The sLeA levels (upper panel) of plasma samples were measured by the sLeA/CA19-9 sandwich assay (anti-sLeA antibody, clone 9L426). The sLeA:CCL2 (CA19-9:CCL2) dual glycan expression marker (lower panel) was measured by using the CA19-9/anti-sLeA antibody as capture and lectin,CCL2, as the detection reagent. The glycan levels of individual samples (PDACs and controls) were plotted and organized from high to low sLeA level separated by PDAC patients (left) and control subjects (right). The dashed line in the graph represents a cutoff chosen to give no false-positive elevations in addition to those identified using the CA19-9 sandwich assay in upper panel. The asterisks indicate individual samples that showed elevations using sLeA:CCL2 dual expression marker

Figure 4 (cont'd)

but not with the sLeA/CA19-9 sandwich assay. B) the three dot plots represent three individual sandwich assays. The y axis indicates the relative fluorescence signals that were measured from individual assay. Each point represents a patient sample (cancer or control), detected by each unique assay described by combination of capture and detection reagents (labeled on the top of each graph). C) Scatter plots comparing glycan marker levels of PDAC and control patients for the indicated combination assays. In these two graphs, the CA19-9 antibody was used as the capture antibody, with differing detection reagents. The measurements of glycan level obtained from detection with CCL2 are presented on the x axes, and the measurements obtained using sLeA (left) and sLeX (right) are on the y axes. The dashed line represents cutoffs chosen to give no false positives. Importantly, some of the PDAC patients were detected only using CCL2 as detection.

***Comparisons of sLeA, sLeX and CCL2-bound glycans expressions in the PDAC tumors***

We next asked whether glycans detected by CCL2 lectin were produced in the tumor tissue of PDAC patients with elevated plasma levels and whether CCL2-bound glycans have similar tissue distributions as the glycans detected by sLeA and sLeX antibodies. We obtained matched tumor tissue and plasma from 11 patients who had a surgical resection for pancreatic cancer. The plasma levels of sLeA and CCL2-bound glycans were compared among 11 PDAC patients and four healthy controls. Four PDAC cases displayed higher blood levels of sLeA and seven PDAC cases displayed higher levels of CCL2-bound glycans (Fig. 5A).

The CCL2 was heavily stained in the malignant epithelia of 10 out of 11 PDAC tumors (Fig. 5B). Some tumors had high CCL2 staining in the secretory epithelial cells, consistent with the concept that the glycoproteins elevations in the plasma samples were due to secretions from the cancer cells. However, the glycan levels did not necessarily match between plasma and tissue. Interestingly, the CCL2-bound glycans were heavier stained than sLeA in some cases (Fig. 5B, #5769, #5775, and #5770), further showing the potential of CCL2 lectin as a complementary marker to CA19-9. Furthermore, the staining pattern of CCL2 was also different than sLeX staining, which indicated that the cancer-associated glycan bound by CCL2 was not simply sLeX (Fig. 5B, # 5769, #5776, #5774, # 5775, and # 5770). Unfortunately, we couldn't establish the baseline level of these glycan expression in the healthy pancreas due to lack of matched healthy pancreatic tissue specimens.

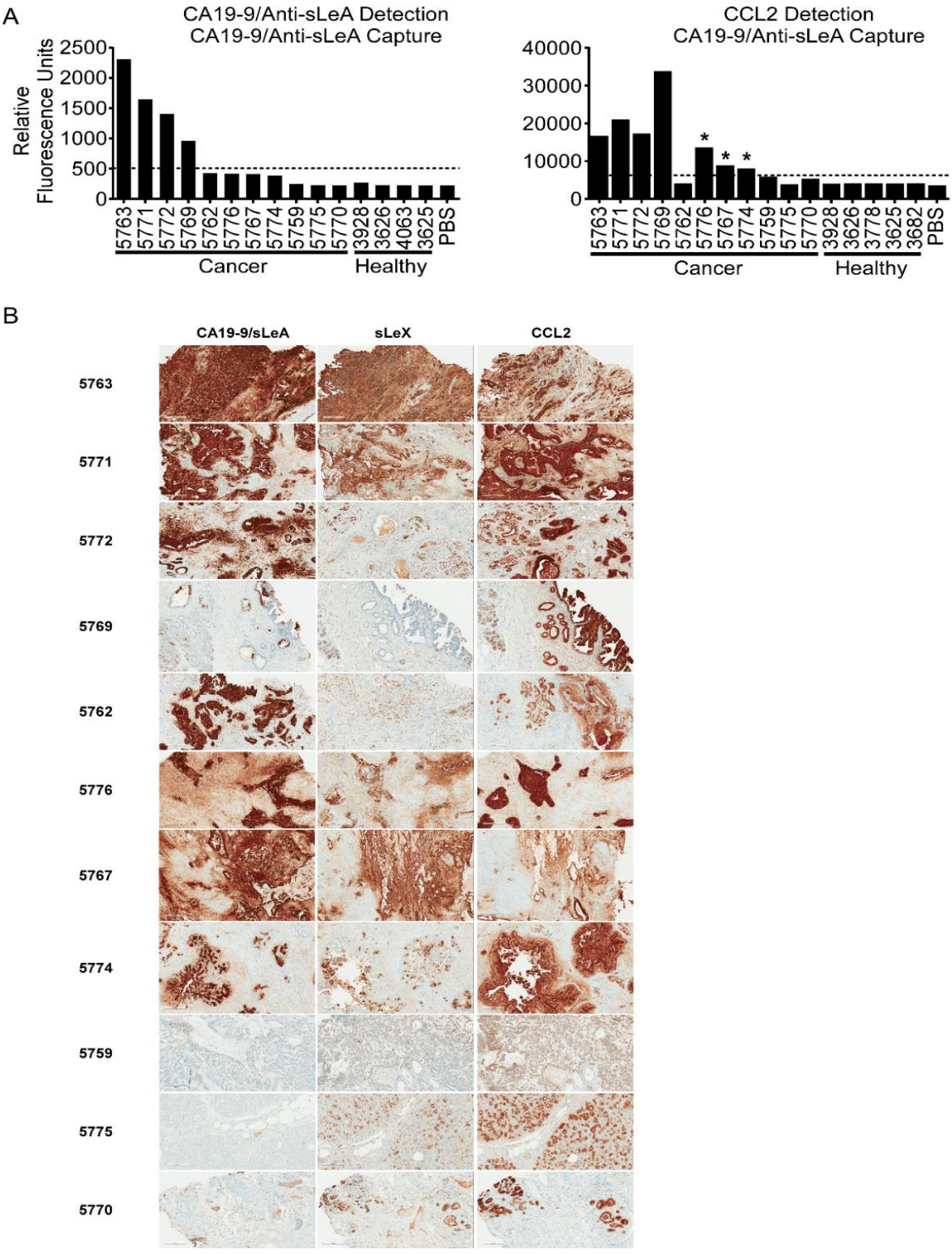



**Figure 5. Expression of glycans between plasma and tissue levels.** These figures are adapted from Singh S et al (2015) [166]. A) The glycan levels of selected plasma samples from PDAC patients and healthy people were measured. The measurements

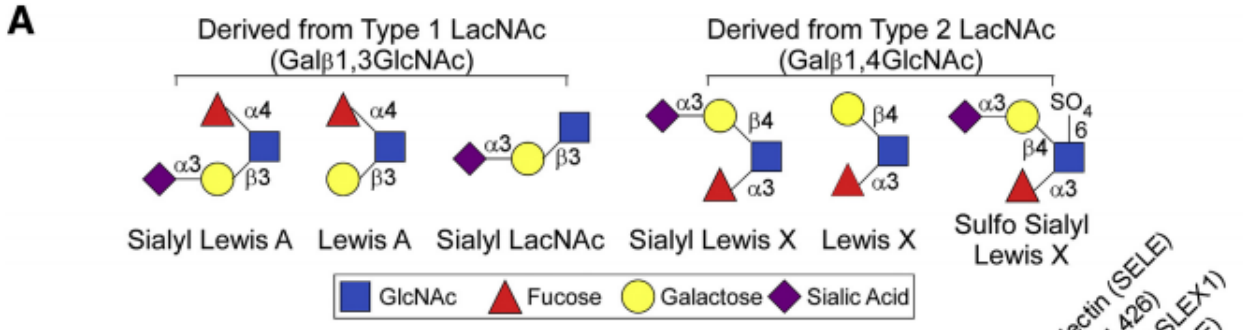
Figure 5 (cont'd)

obtained from sLeA/CA19-9 sandwich assay (left) and hybrid sLeA:CCL2 assay (right) are organized by decreasing level of sLeA. B) Three glycan markers, sLeA (left), sLeX (middle) and CCL2 (right) were visualized by the immunohistochemistry staining of primary tumor tissue.

### *Evaluating candidate glycans as biomarkers for PDACs*

To objectively and comprehensively evaluate the potential of Lewis blood group glycans as biomarkers for PDAC diagnosis, a comparison of glycans bearing structural similarity to sLeA was made in two cohorts of plasma samples, previously mentioned Discovery cohort and the new Validation cohort (Table 2). The glycans in plasma samples were profiled with lectins and glycan antibodies, targeting the Lewis blood group glycans (Fig. 6B). The candidate glycan markers could be categorized into two main groups based on type 1 or type 2 LacNAc cores (Fig. 6A). Glycan array data available from the Consortium for Functional Glycomics (CFG) database allowed for the determination of specificities of these lectins and antibodies. Some of the reagents bound only one glycan motif with high specificity, but others bound multiple motifs. Of note, the 7LE antibody, bound both Lewis A and nonfucosylated type 1 LacNAc (Fig. 6B), while the mouse E-selectin (SELE) bound sLeA, sLeX and sulfated sLeX. We validated SELE as a detection reagent with a broad specificity using cell lines and tissue specimens (Fig .7). To probe sialylated and nonfucosylated type 1 LacNAc structures, we used an enzyme, called sialidase, to remove the sialic acids before probing with two affinity reagents, 7LE and TRA1-60 (type 1 LacNAc binder) antibodies (Fig. 6B).

A




B

| Glycan | Structure | Abbreviation | Mouse E-selectin (SELE) | Anti-sLeA (9L426) | Anti-sLeX (CSLEX1) | Anti-LeA (7LE) | TRA-1-60 | CCL2 |
|---|---|---|---|---|---|---|---|---|
| Sialyl-Lewis A | Siaα2,3Galβ1,3(Fucα1,4)GlcNAcβ | sLeA | ■ | ■ | | | | |
| Lewis A | Galβ1,3(Fucα1,4)GlcNAcβ | LeA | | | | ■ | | |
| Sialyl LacNAc type1 | Siaα2,3Galβ1,3GlcNAcβ | sLacNAc t1 | | | | | | |
| Terminal LacNAc type1 | Galβ1,3GlcNAcβ | LacNAc t1 | | | | ■ | | |
| Sialyl LacNAc type1-type2 repeat | Siaα2,3Galβ1,3GlcNAcβ1,3Galβ1,4GlcNAc | sLacNAc t1t2 | | | | | | |
| Terminal LacNAc type1-type2 repeat | Galβ1,3GlcNAcβ1,3Galβ1,4GlcNAc | LacNAc t1t2 | | | | | ■ | |
| Sialyl-Lewis X | Siaα2,3Galβ1,4(Fucα1,3)GlcNAcβ | sLeX | ■ | | ■ | | | ■ |
| Sulfo/sialyl Lewis X | Siaα2,3Galβ1,4(Fucα1,3)(6SO4)GlcNAcβ | sulfo-sLeX | ■ | | | | | ■ |
| Terminal Lewis X | Galβ1,4(Fucα1,3)GlcNAcβ | LeX | | | | | | ■ |
| Internal Lewis X | (Any)Galβ1,4(Fucα1,3)GlcNAcβ | Int LeX | | | | | | ■ |

**Figure 6. Identifying binders to candidate glycans related to sLeA.** These figures are taken from Tang H et al (2016) [165]. A) Glycans with structures related to sLeA. Monosaccharides are represented – blue square: N-acetylglucosamine (GlcNAc); yellow circle: galactose (Gal); red triangle: fucose (Fuc); purple diamond: sialic acid. B) A table representing individual glycan of interest, sequence of glycans (structure), abbreviations and affinity reagents were used to detect these glycans. A red square indicates each specific reagent's specificity toward that particular structure, and the boxes in bold indicate structures that are not detected in the reagent screen.

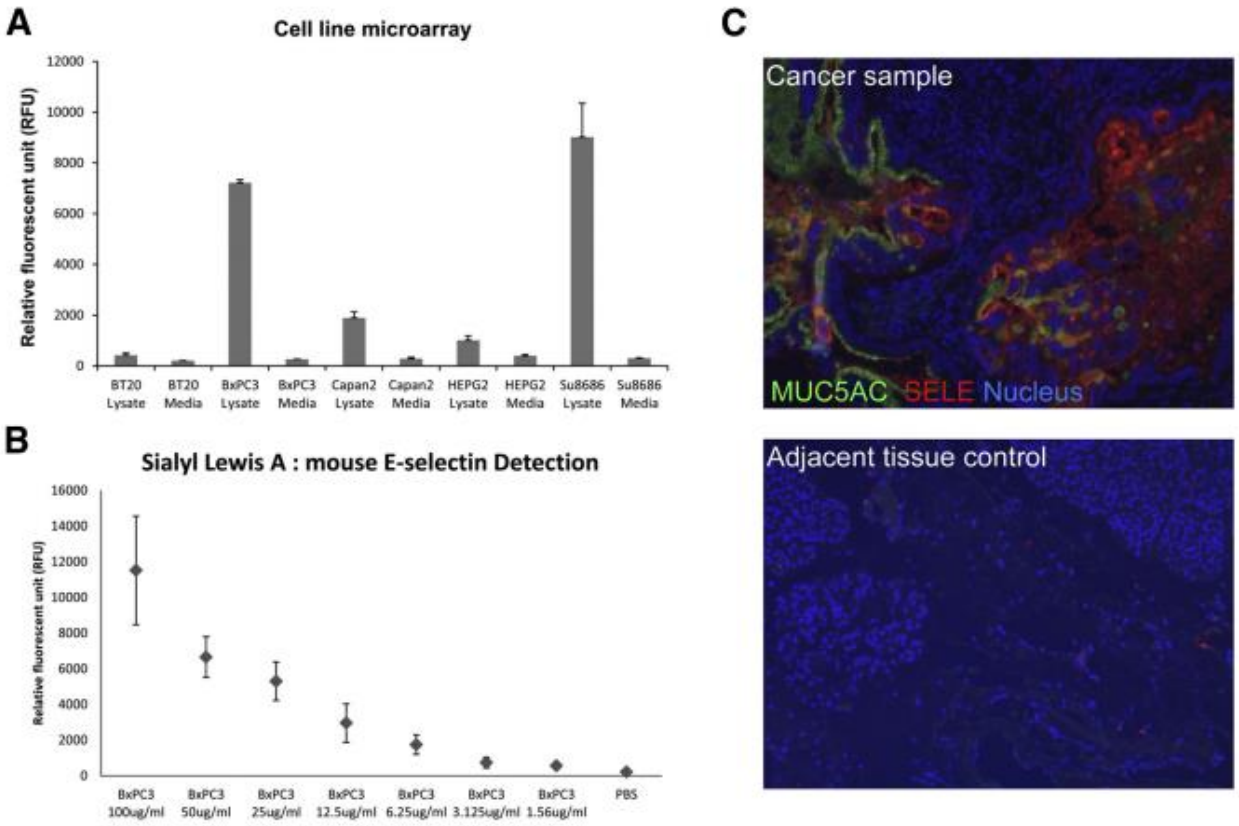



**Figure 7. Validation of SELE as a detection reagent.** These figures are taken from Tang H et al (2016) [165]. A) Cell line microarray. Lysates and conditioned media of cell lines known to express sLeA (BxPC3, Capan2, and Su8686) or to not express sLeA (BT20 and HEPG2) were printed onto the nitrocellulose microscope slides and probed with biotinylated SELE followed by Cy5-labeled streptavidin. The fluorescence values show binding mainly on the cell lines expressing sLeA. B) Antibody-lectin sandwich arrays. Anti-sLeA antibody (CA19-9), was spotted onto microscope slides and incubated with dilutions of a lysate from BxPC3 and probed with SELE. The fluorescence shows a good dose response curve with low nonspecific binding at the spot incubated with PBS. C) Validation in immunofluorescence staining. The Cy3-labeled anti-MUC5AC, Cy5-labeled SELE, and hoechst (blue) were incubated on a section of pancreatic tumor tissue and adjacent pancreatic control tissue (bottom). SELE binding appears on various glycoproteins that associates with the cancer cells.

In the Discovery cohort, 33 individual markers had significant increases in PDACs compared to controls (Table 4). Highly represented markers included CA19-9 (sLeA sandwich assay), two distinct glycoforms of MUC5AC, the sialylated type 1 LacNAc, and the sulfated and/or sialylated Lewis A/Lewis X (Fig. 8A and Table 4.). A reduced number of markers (five capture antibodies and five detection reagents) were tested on the Validation cohort which resulted in significant increases to 19 markers in PDAC samples as compared to controls (Table 4). The sLeA, sialylated type 1 LacNAc carried by MUC5AC, and sulfated and/or sialylated Lewis A/Lewis X markers also showed significant increases in PDACs (Fig. 8B). These markers showed significant improvements in AUC (area under the curve) over sLeA in the Discovery cohort, although not in the Validation cohort (Fig. 8C & 8D), perhaps owing to the higher performance of sLeA in the Validation cohort. Since a recent definitive characterization of CA19-9 assay showed an AUC = 0.77 for discriminating PDAC from pancreatitis patients, with lower performance when including the patients with biliary stricture, we viewed this CA19-9 performance in the Validation cohort as an aberration [135].

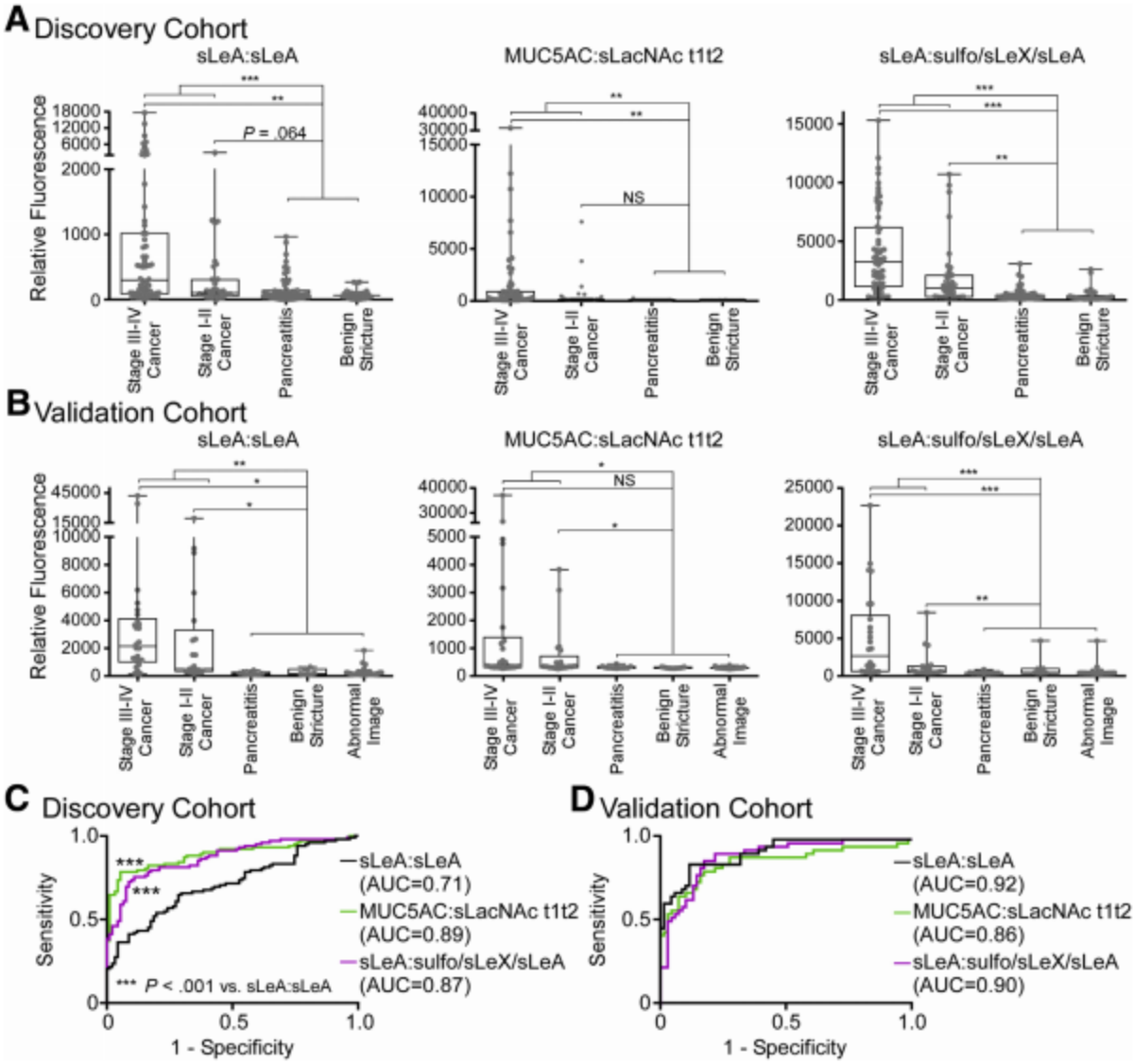



**Figure 8. Novel glycan biomarkers of PDAC.** These figures are taken from Tang H et al (2016) [165]. A) Discovery cohort. The heading of each graph indicates the capture and detection reagents, separated by a colon. A glycoform of MUC5AC showing sialylated type 1 LacNAc (detected by TRA-1-60 antibody after sialidase treatment) and a sandwich assay by using CA19-9 antibody as capture and sulfated and/or sialylated sLeA/sLeX detection (detected by SELE) showed significant expressions in PDAC. B) Validation cohort. We observed similar glycans overexpressed in this cohort of PDAC patient samples as the Discovery cohort. The ROC curves showed C) improvement over sLeA in the Discovery cohort but D) performances of MUC5AC:LacNAc type 1 & 2 (t1t2) and sLeA:sulfo/sLeX/sLeA in the Validation cohort were not significant over sLeA.

**Table 4. Individual assay performance in the Discovery and Validation cohorts**. This table is taken from Tang H et al (2016) [165]. The p value was based on the Mann-Whitney test for comparing PDACs and control subjects. The assays previously shown in the biomarker panels are shown in italic font, and the CA19-9 assay (capture and detection of sLeA) is shown in bold. ESEL = SELE in this table.

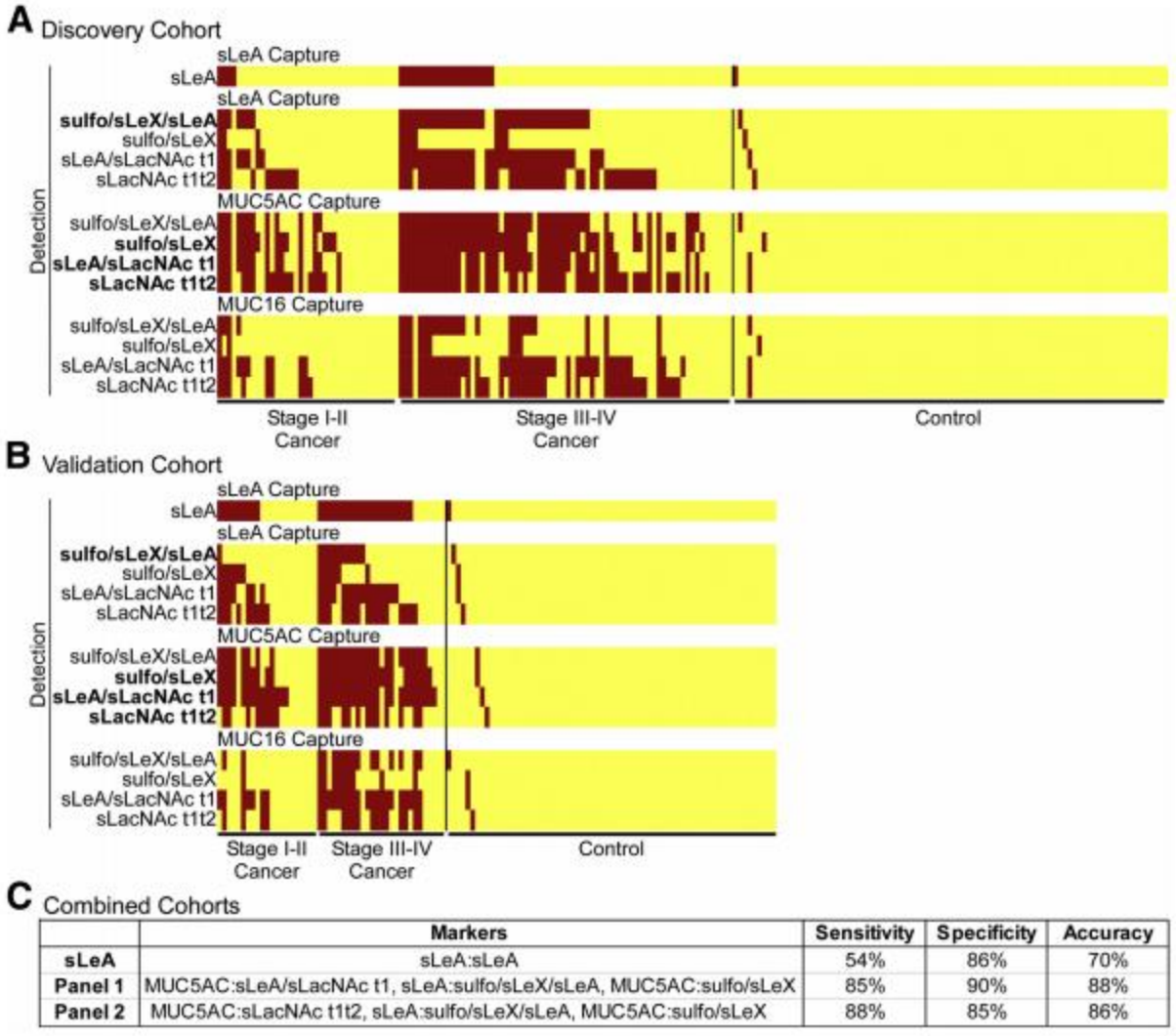
| Assay | Discovery | Validation |
|---|---|---|
| *sLeA: sulfo/sLeX/sLeA (ESEL)* | *6.06E-14* | *1.81E-04* |
| MUC5AC: sulfo/sLeX/sLeA (ESEL) | 1.30E-11 | 1.44E-05 |
| sLeA: sLeA/sLacNAc t1 (7LE) | 8.62E-11 | 1.04E-06 |
| *MUC5AC: sulfo/sLeX (CCL2)* | *3.66E-10* | *2.80E-05* |
| *MUC5AC: sLeA/sLacNAc t1 (7LE)* | *5.02E-10* | *6.46E-05* |
| sLeX: sulfo/sLeX/sLeA (ESEL) | 9.01E-09 | NS |
| **sLeA: sLeA** | **1.02E-07** | **2.00E-03** |
| MUC16: sulfo/sLeX/sLeA (ESEL) | 3.54E-07 | 7.58E-04 |
| MUC16: sLeA/sLacNAc t1 (7LE) | 1.17E-06 | 5.09E-04 |
| sLeA(Ab2): sLeA/sLacNAc t1 (7LE) | 5.45E-06 | 6.88E-05 |
| MUC16(Ab2): sulfo/sLeX/sLeA (ESEL) | 9.56E-06 | - |
| LeA: sulfo/sLeX/sLeA (ESEL) | 1.73E-05 | - |
| MUC16: sLeA | 3.29E-05 | 3.83E-02 |
| sLeA(Ab2): sLeA | 3.88E-05 | 6.45E-03 |
| sLeA(Ab2): sLacNAc t1t2 (TRA-1-60) | 5.76E-05 | NS |
| sLeX: sulfo/sLeX (CCL2) | 5.90E-05 | 7.11E-05 |
| LeA: sLacNAc t1t2 (TRA-1-60) | 7.20E-05 | - |
| sLeX: sLeA/sLacNAc t1 (7LE) | 9.54E-05 | 1.22E-03 |
| sLeA: sLacNAc t1t2 (TRA-1-60) | 1.05E-04 | 8.32E-03 |
| MUC5AC(Ab2): sulfo/sLeX/sLeA (ESEL) | 1.46E-04 | - |
| MUC1: sLacNAc t1t2 (TRA-1-60) | 2.44E-04 | - |
| sLeA: sLeX | 3.43E-04 | 5.09E-03 |
| sLeX: sLeX | 3.78E-04 | NS |
| MUC16: sLacNAc t1t2 (TRA-1-60) | 7.66E-04 | 1.46E-02 |
| MUC16: sulfo/sLeX (CCL2) | 8.75E-04 | 5.78E-03 |
| sLeA: sulfo/sLeX (CCL2) | 1.19E-03 | 1.86E-04 |
| *MUC5AC: sLacNAc t1t2 (TRA-1-60)* | *1.21E-03* | *4.93E-02* |
| LeA: sLeX | 4.59E-03 | - |
| MUC5AC(Ab2): sLacNAc t1t2 (TRA-1-60) | 1.23E-02 | - |
| sLeX: sLacNAc t1t2 (TRA-1-60) | 1.81E-02 | NS |
| sLeA(Ab2): sLeX | 3.42E-02 | NS |
| LeX: sLacNAc t1t2 (TRA-1-60) | 3.79E-02 | - |
| LeX: sulfo/sLeX/sLeA (ESEL) | 4.90E-02 | - |

***Biomarker panels outperformed the CA19-9 marker alone***

A goal of our work was to develop a biomarker panel that could exceed the sensitivity and specificity of the clinical CA19-9 assay for detecting pancreatic cancer. We hypothesized that individual markers can complement sLeA detecting distinct subpopulations of PDAC patients that do not express sLeA. For each individual marker, we set a threshold to allow one false positive control, thus providing an overview of each marker's elevation that were specific to cancer. At such a high-specificity threshold, sLeA was elevated in the plasma of only 22% of the cancers in the Discovery cohort. In contrast, several other markers were highly enriched in the early stage (stage I-II) and late stage (stages III-IV) cancers (Fig. 9A). The trends were similar in the Validation cohort (Fig. 9B). These results indicated that the markers had increases in distinct groups of PDAC patients, independent of stage.

By combining all 316 plasma samples from the Discovery and Validation cohorts, we found that two three-markers panels provided better accuracy than sLeA alone (Fig. 9C). Panel 1 consisted of a glycoform of MUC5AC with sulfated and sialylated Lewis X (detected by CCL2); another glycoform of MUC5AC with sialylated type 1 LacNAc and sLeA (detected by the 7LE antibody after sialidase treatment); and a sandwich assay comprising the capture of sLeA and the detection of sulfated and/or sialylated Lewis A/Lewis X (detected by SELE). A second panel (panel 2) differed from panel 1 by one marker (Fig. 9C). The marker selection program (MSS) did not choose sLeA (CA19-9 sandwich assay) for inclusion in the panels, indicating that sLeA at best provided only marginal additional diagnostic information beyond what already was detected by the markers in the panel 1 & 2. These data demonstrated that the profiling analyses of the

new glycan markers was increased independently of sLeA subsets of PDAC patients, and that together they formed two biomarker panels with improved accuracy compared with sLeA alone.




C Combined Cohorts

| | Markers | Sensitivity | Specificity | Accuracy |
|---|---|---|---|---|
| **sLeA** | sLeA:sLeA | 54% | 86% | 70% |
| **Panel 1** | MUC5AC:sLeA/sLacNAc t1, sLeA:sulfo/sLeX/sLeA, MUC5AC:sulfo/sLeX | 85% | 90% | 88% |
| **Panel 2** | MUC5AC:sLacNAc t1t2, sLeA:sulfo/sLeX/sLeA, MUC5AC:sulfo/sLeX | 88% | 85% | 86% |

**Figure 9. Glycans related to sLeA could improve accuracy of diagnosis for early-stage and late-stage PDAC patients**. These figures are taken from Tang H et al (2016) [165]. A and B) The rows present glycan markers elevated in the PDAC patients compare to patients with non-malignant disease (controls). The glycan markers are indicated by the capture (listed above) and detection targets (on left). In contrast, the columns are represented individual plasma samples from PDAC patients and non-malignant disease patients. For each glycan marker, we set a cutoff to allow one false-positive control sample. A red box indicates a measurement greater than the threshold, and a yellow box is a measurement below the threshold. Panel A displays the data from the Discovery cohort, while panel B shows the Validation cohort. C) Two candidate biomarker panels (Panel 1 & 2) provide improved performance in sensitivity and accuracy compared with sLeA (measured by CA19-9 sandwich assay) in the combined sample sets (Discovery and Validation cohorts).

## Discussion

We reported here detailed investigations examining the hypothesis that glycans within the Lewis blood group family other than sLeA are aberrantly increased in the subpopulation of PDAC patients who do not secrete sLeA into their blood. We identified glycan markers in addition to sLeA that characterized such subgroups. The useful glycan markers described here could be categorized into three structural categories: sLeA, sLeX variants, and sialylated type 1 LacNAc. Each category has its own biosynthetic pathways, cell types on which the glycans are shown, and protein receptors, suggesting that the glycans reflect biological subtypes of pancreatic cancers. We demonstrated these markers, in combination, could improve diagnostic accuracy: the biomarker panels improved accuracy by 16-18% relative to CA19-9 alone. However, further research is necessary to validate the application of this panel to the diagnosis of pancreatic cancer. Further research also could explore the application to other needs in clinical practice, including surveillance among people with an increased risk for pancreatic cancer, improving the determination of likelihood of rapid progression after surgery, and monitoring the course of the pancreatic cancer after treatment.

Previous studies have investigated possible origins and functions of the glycans described in this work. The sialylated type 1 LacNAc probed by the TRA 1-60 and 7LE antibodies after sialidase treatment serves as a marker for pluripotent stem cells [152-154]. Other reports have found sialylated type 1 LacNAc presents on glycolipids in malignant glioma and embryonal carcinoma [155, 156]. Accordingly, the expression of sialylated type 1 LacNAc may indicate a stem-cell origin of the pancreatic cancer cells. In light of these findings, we propose that pancreatic cancer cells alter biosynthetic

pathways, down-regulating fucosylation while increasing expression of sialylated type 1 LacNAc.

In this study, we initially focused on sLeX as a malignant marker and investigated its potential role as a complementary biomarker to the CA19-9 test. sLeX was thought to play an impactful role in hematogenous metastasis of certain solid tumors. Several studies indicated sLeA and sLeX served as critical ligands to the activated E-selectin receptors on the endothelial cells and facilitated extravasation, arrest and metastasis for circulating tumor cells [85, 157, 158]. Therefore, the relative levels of sLeX and sLeA could affect cancer cell behavior, disease progression and metastasis. In contrast, a previous study revealed that sLeX is also found on the acute-phase proteins, including haptoglobin, fetuin, α-1-acid glycoprotein, transferrin and α-1-antitrypsin produced from the liver in response to chronic inflammation [159]. Together, these findings suggested that the sLeX glycan is not solely associated with the pancreatic cancer, but also with chronic inflammatory diseases within the gastrointestinal tract. Thus, a more specific marker than sLeX was required to differentiate between chronic inflammation and PDAC.

The glycans detected by the CCL2 were significantly enriched in PDAC patient plasma (Fig .4). Based on the fact that CCL2 detection did not correlate with sLeX detection in plasma and tissue samples (Fig. 5), we reasoned that the glycan elevations in CCL2 detection were not simply due to sLeX; they must be due to other glycans that contained α1-3 fucose motifs. A strong possibility is Lewis blood group glycans with sulfation motifs, because sulfated variants of sLeX contain the α1-3 fucose motif and have increased affinity for mouse E-selectin receptors. In addition, one of the sulfated

Lewis blood group glycans, called 6-sulfo-sLeX, was found to be a primary ligand to the L-selectin (leukocyte-selectin) receptor. The L-selectin is expressed by general leukocytes and plays a significant role in inducing hematogenous metastasis by suppressing immunological response [160-162].

Additional research will facilitate understanding of the relationship between the glycan biomarkers found here and other promising candidate markers for the detection of early stage PDACs. Other promising biomarkers for PDAC include micro-RNAs, DNA, and tumor cells in circulation; proteins in the urine; and various types of biomarkers in the pancreatic juice or stool. All could help define the biological subtype of pancreatic cancer. Future directions of this work would involve a comparison of all published biomarkers to the glycan markers to explore a panel of complementary markers for a more precise diagnosis in a broader range of PDAC patients.

In summary, we demonstrated that glycans other than sLeA – the glycan detected by the CA19-9 – are increased in distinct subpopulations of PDAC patients. Collectively, these glycan markers could contribute to the development of a biomarker panel with improved accuracy over the CA19-9. Among the biomarkers, three categories of glycans with structural differences– sLeA, sLeX variants, and sialylated type 1 LacNAc – showed level differences in distinct groups of PDACs. Importantly, this new glycan-based biomarker panel has the potential to achieve more accurate diagnoses at earlier and more treatable stages of PDACs comparing to CA19-9.

# CHAPTER THREE: IDENTIFY NOVEL PANCREATIC TUMOR BIOMARKERS THAT COMPLEMENT CA19-9 THROUGH PROBING THE SULFATED VARIANTS OF α2-3 SIALYLATED TYPE 2 LACNAC GLYCANS.

## Introduction

Pancreatic cancers, like other cancer types, exhibit tremendous diversity between tumors in many features, such as cellular morphology, gene and protein expression, and most importantly, resistance to therapeutics and propensity to metastasize. This diversity produces challenges in patient care. First, the diagnosis of pancreatic cancer can be a challenge, since no defined set of molecular markers or other features captures all cancers without confounding with non-malignant conditions. Second, making decisions about surgery or other treatment options can be difficult because biomarkers of prognosis are not very accurate. Therefore, new biomarkers are needed both for specifically detecting a greater percentage of pancreatic cancers and for rendering an accurate prognosis.

In previous work, I investigated the possibility that specific glycans are useful as markers of subpopulations of pancreatic cancers. That direction was based on the usefulness of certain glycans as indicators of cell type and differentiation, and on the fact that CA19-9, the well-studied biomarker of pancreatic cancer, is a glycan that detects a subset of pancreatic cancers. The CA19-9 antigen is a member of the Lewis family of blood group antigens. The Lewis structures are built off the LacNAc core, a di-saccharide of Gal and GlcNAc. The linkage between the Gal and GlcNAc is either on the 3’ carbon of the GlcNAc, referred to as type 1, or on the 4’ carbon of the GlcNAc, referred to as type 2 (Fig. 1). The LacNAc core is variously modified with sialic acid, fucose, or sulfate, and the specific type and positions of the modifications have major implications for the functional activities of the structures. The CA19-9 antigen has the type 1 core and is both sialylated and fucosylated, but not sulfated. The sialylated type 1

LacNAc (sTRA) glycan, previously shown to be a valuable biomarker of pancreatic cancer, also has the type 1 core [163, 164]. This glycan is sialylated but not fucosylated or sulfated [152].

Thus, the type 1 LacNAc core seems more strongly associated with pancreatic cancer than the type 2 LacNAc core, but I previously found evidence that glycans built off the type 2 LacNAc core also are biomarkers of pancreatic cancer. Type 2 LacNAc structures are more common than type 1 LacNAc and form the repeating units in the extended antennae of most epithelial surfaces. A structural isomer of CA19-9 called sLeX, differing from CA19-9 only by the type 2 LacNAc core instead of type 1 LacNAc core, is elevated in approximately 30% of pancreatic cancers but also in some benign conditions of the pancreas, limiting its usefulness as a biomarker. In addition to sLeX, we found evidence that sulfated glycans built off the type 2 LacNAc core are markers of pancreatic cancer, but the methods used in the previous work did not provide details on the structures [165, 166]. Therefore, I hypothesized the novel pancreatic tumor biomarkers that complement CA19-9 are sulfated variants of sLeX and α2-3 sialylated type 2 LacNAc glycans.

Sulfated type 2 LacNAc glycans are particularly difficult to analyze by mass spectrometry or other conventional methods, because they are relatively low-abundance and are labile when ionized. Antibodies or lectins against specific sulfated groups are lacking or not well characterized. I recently developed a method that potentially gives better access to the analysis of sulfated glycans. The method, called on-chip gmap, elucidates the glycan structure by taking advantage of the high specificities of lectins and glycosidases for particular glycan isomers [167, 168]. On-chip

gmap is made practical and effective by the use of detailed information about the specificities from glycan array analyses [169, 170]. The information from glycan arrays is computationally integrated with the lectin-profiling data to give results on the glycan structures that are most likely in the sample. As a result, the method provides details about the isomeric variants of glycans, such as sequence, branching, linkage position, and linkage direction (α or β)—information that is otherwise difficult to obtain. Another advantage of the method for this research is that I desired to analyze proteins derived from clinical samples and culture media, which are limited in quantities. Because on-chip gmap analyzes proteins captured on antibody microarrays, it is compatible with the analysis of low-abundance proteins. These features enabled me to test whether specific sulfated structures built off type 2 LacNAc core are potential biomarkers of pancreatic cancer.

## Materials and Methods

### *Human plasma specimens*

The study was conducted under protocols approved by the Institutional Review Boards at the Van Andel Research Institute and the University of Pittsburgh Medical Center. All subjects provided written, informed consent. The donors consisted of patients with pancreatic cancer or a benign condition involving the pancreas and healthy subjects (Table 6). The healthy subjects had no evidence of pancreatic, biliary or liver disease. All blood samples were collected prior to any surgical or medical procedures and according to the standard operating procedure from the Early Detection Research Network. The samples were frozen at -70°C or colder within 4 h of time of collection. Aliquots were shipped on dry ice and thawed no more than three times prior to analysis.

***Antibody lectin sandwich assay***

The antibody array methods followed those presented earlier with slight modifications [66, 171, 172]. I printed 48 identical arrays in a 12 x 4 grid onto glass microscope slides coated with ultra-thin nitrocellulose (PATH Slides, Grace BioLabs). The contact printer (Aushon 2470, Aushon BioSystems) was equipped with 110 μm diameter pins that deposit about 0.3 nL per spot. Each array contained six replicate spots of each antibody in randomized positions within the array. Information on the antibodies and detection reagents is in Table 5. After printing, hydrophobic borders were imprinted onto the slides (SlideImprinter, The Gel Company, San Francisco, CA) to segregate the arrays and to allow for individual sample incubations on each array. The arrays were blocked in 1X PBS containing 1% BSA and 0.5% Tween-20 for 1 h at RT. The slides were rinsed in 1X PBS plus 0.5% Tween-20, washed in the same buffer for 15 min, and dried by centrifugation (Eppendorf 5810R, rotor A-4-62, 1500 x g for 3 min).

To prepare the plasma or media, the samples were diluted two-fold or 25-fold into 1X PBS with Tween-20 and Brij-35 (both at 0.05% final concentration); an IgG blocking cocktail (100 μg/mL mouse and rabbit IgG and 50 μg/mL goat and sheep IgG (Jackson ImmunoResearch)); and protease inhibitor (Complete Mini EDTA-free Tablet, Roche Applied Science). I applied 6 μL of each sample to each array and let the samples incubate overnight at 4°C. Each unique sample was applied to three separate arrays. The arrays were washed in three changes of PBST0.1 for 3 min each and dried by centrifugation as above. The arrays to be detected for the sTRA glycan were treated with α2-3 sialidase (P0728L, New England Biolabs, Ipswich, MA) at 250 U/mL in the supplied reaction buffer overnight at 37°C.

The following day, the arrays were washed in three changes of PBST0.1 Tween-20 for 3 min each and dried by centrifugation. I then incubated each array with a biotinylated antibody or lectin (Table 5) prepared at 3 µg/mL in 1X PBS with 0.1% BSA and 0.1% Tween-20. The conjugation of biotin and affinity reagents was performed using a conjugation reagent (EZ-Link Sulfo-NHS-Biotin, Thermo Fisher) according to the manufacturer guidelines. For the arrays incubated with antibodies, the arrays were washed and dried as above, incubated for 1 h at RT with Cy5-conjugated streptavidin (Roche Applied Science, Penzberg, Germany) prepared at 2 µg/mL in 1X PBS with 0.1% BSA and 0.1% Tween-20, and given a final wash and dry. For detection with the Siglec-F lectin, the lectin was precomplexed with Cy5-conjugated streptavidin (2 µg/mL) at a 1:4 molar ratio for one hour prior to incubation. For detection with the Siglec-7 lectin, the arrays were incubated with the lectin as above, and then incubated with biotinylated, anti-polyhistadine antibody (MAB050, R&D Systems, Minneapolis, MN) that had been precomplexed for 1 h with Cy5-conjugated streptavidin.

I scanned the slides for fluorescence (Innopsys InnoScan 1100 AL) using 633 nm excitation. To quantify the signals, I used in-house software called SignalFinder (available upon request) to locate pixels containing signal in each spot [173]. I used a custom script to remove any outliers from the six replicate spots according to the Grubbs' test. The script performs the Grubbs' test for the spot with the greatest deviation from the mean and rejects the spot if the Grubbs' statistic has $p \geq 0.1$. The script repeats until either no outliers or only four spots remain and outputs the mean of the non-excluded replicate spots for each array. The script then averages values between replicate arrays.

### *On-chip gmap glycan profiling*

The methods for on-chip glycan analysis were demonstrated in this laboratory [167, 168] and are briefly described here. I printed 96 identical arrays onto glass microscope slides coated with ultra-thin nitrocellulose, PATH Slides, Grace BioLabs, Billerica, MA). I printed microarrays using a contact printer (Aushon 2470, Aushon BioSystems) equipped with 110 µm diameter pins that deposit about 0.3 nL per spot. Each array contained three replicate spots of each antibody in randomized positions within the array. The printed antibodies were CA19-9 (clone 1116-NS-19-9, Biorbyt, Atlanta, GA), anti-MUC5AC (clone 45M1, Thermo Scientific, Waltham, MA), and anti-MUC16 (clone X325, abcam, Cambridge, UK) (Table 5). By pre-warming up the wax machine at 80°C, 192 format wax-based hydrophobic borders were imprinted onto the antibodies printed slides (SlideImprinter, The Gel Company, San Francisco, CA) to segregate the arrays and allow for individual sample incubations on each array. The plasma sample preparation was identical as ALSA method described above.

The next day, the antibodies printed slides were rinsed in PBST0.5, washed in the same buffer for 15 min, and dried by brief centrifugation at 1000 rpm, with printed arrays facing outside. The arrays were individually blocked using 1% BSA in PBST0.5 for 1 h at RT. I applied 1.2 µL of each plasma samples or conditioned media to each post-blocked array and let the samples incubate overnight at 4°C. Each unique sample was applied to three separate arrays. The arrays were rinsed twice and washed in three changes of PBST0.1 for 3 min each and dried by centrifugation (Eppendorf 5810R, rotor A-4-62, 900 rpm for 3 min).

For assays which attempted to probe is the underlying glycans by removing α2-3 linked sialic acid, I prepared α2-3 neuraminidase (P0728L, New England Biolabs, Ipswich, MA) at 250 U/mL in the supplied G1 reaction buffer and incubated each separately on arrays containing the capture antibodies or spotted glycoprotein controls for 2 h at 37°C. To remove α1-3,4 linked fucoses, we prepared α1-3,4 fucosidase (P0769S, New England Biolabs, Ipswich, MA) at 1:20 dilution in the supplied G1 reaction buffer each separately on arrays containing the capture antibodies or spotted glycoprotein controls for 2 h at 37°C. To remove β1-4 linked galactose, we prepared β1-4 galactosidase (GKX-5014, ProZyme, Hayward, CA) at 1:100 dilution in the supplied reaction, and to remove N-linked glycans, we prepared PNGase F (P0704S, New England Biolabs, Ipswich, MA) at 1:200 dilution in G2 reaction buffer. Each enzyme was incubated separately on arrays containing the capture antibodies or spotted glycoprotein controls for 2 h at 37°C.

The arrays were then washed in three changes of PBST0.1 for 5 min each and dried by centrifugation. I incubated each array with a biotinylated antibody or lectin. Anti-sLeX and the biotinylated lectins, ECL, BPL, RCA-I, ConA, GSL II, SRL, and RSL were prepared at 3 µg/mL in 1X PBS with 0.1% BSA and 0.1% Tween-20 (Table 5). The secondary reagent was Cy5-conjugated streptavidin (Roche Applied Science, Penzberg, Germany) prepared at 2 µg/mL in 1X PBS with 0.1% BSA and 0.1% Tween-20 and incubated for 1 h at RT. The biotinylated CCL2 and Siglec-F lectins, were precomplexed with Cy5-conjugated streptavidin (2 µg/mL, Roche Applied Science, Penzberg, Germany) 1:4 ratio for 1 h prior to incubation. The Siglec-7, Siglec-8, Siglec-9 histadine-tagged fusion lectins from Dr. Schnaar group were prepared at 3 µg/mL in 1X PBS with 0.1% BSA and 0.1% Tween-20; and the secondary reagent was

biotinylated anti-polyhistadine antibody (MAB050, R&D Systems, Minneapolis, MN) precomplexed with Cy5-conjugated streptavidin (2 µg/mL, Roche Applied Science, Penzberg, Germany) for 1 h prior to incubation. The arrays were washed and dried as above, and I scanned the slides for fluorescence using 633 nm excitation (Innopsys InnoScan 1100 AL, Innospys, Carbonne, France).

To quantify the signals, I used in-house software called SignalFinder to locate pixels containing signal in each spot. The program uses the SFT algorithm without user intervention or adjustment of settings [173].

***Cell culture and immunofluorescence***

All cell lines were cultured in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen). For three-dimensional cell culture, cells were trypsinized and washed with Dulbecco's Phosphate-Buffered Saline, and then suspended in culture medium ($1\times10^7$ cells per mL). The cell suspensions were mixed with Matrigel (Corning) in a 1:3 volume ratio and 50 µL of the Matrigel cell suspension were loaded into each well. The cells were feed with 50 µL culture medium on top of the Matrigel and cultured for 2-3 days prior to collection of the media for biomarker analysis using the antibody array methods described above.

To prepare the cultures for immunofluorescence analysis, the following method was used. The cells were diluted into 80% Matrigel in media with a cell density of $2 \times 10^6$ cells/mL. The slurry was loaded into wells formed out of polydimethylsiloxane (PDMS, Ellsworth Adhesives) on a microscope slide treated with silane to inhibit cell adhesion. After two weeks incubation with periodic replenishing of the media, the cultures were

transferred to a fixation cassette (Tissue-Tek) and allowed to fix for 48 h in 10% neutral-buffered formalin (Leica Biosystems, Wetzlar, Germany). I embedded the fixed cultures in paraffin and prepared 5 µm sections on microscope slides.

To perform multimarker-immunofluorescence method [163], I removed paraffin by three citrosol washes followed by ethanol/$H_2O$ rehydration (twice each at 100%, 95%, and 70%) and two washes in 1X PBS. We performed antigen retrieval by incubating the slides in citrate buffer at 100°C for 20 min and blocked the slides in 1X PBS containing 0.05% Tween-20 and 3% BSA for 1 h at RT. Each round of immunofluorescence was incubated in 1X PBS containing 0.05% Tween-20 with 3% BSA containing two different antibodies or lectins (10 µg/mL each), one each labeled with sulfo-Cyanine5 (13320, Lumiprobe) or sulfo-Cyanine3 (11320, Lumiprobe) according to the supplier protocol. I incubated the antibody/lectin solution on a tissue section overnight at 4°C in a humidified chamber. Next, I decanted the antibody solution and washed the slide three times for 3 min each, twice in PBST0.05 and once in 1X PBS. The slide was blotted dry and incubated with Hoechst 33258 (1:1000 dilution in 1X PBS) for 10 min at RT. I washed the slides in 1X PBS twice for 5 min, added a coverslip, and scanned the slides using a scanning-fluorescence microscope (Vectra, PerkinElmer).

I stored the slides in a humidified chamber until removing the coverslip by slide immersion in deionized water at 37°C for 30-60 min. I quenched the fluorescence by incubating the slide in 6% $H_2O_2$ in 250 mM sodium bicarbonate (pH 9.5-10) twice for 20 min each at RT. The subsequent incubations and scanning steps were as described above.

To treat the slide with sialidase, I incubated a 1:200 dilution (from a 50,000 U/mL stock) of the enzyme (α2-3,6,8 Neuraminidase, P0720L, New England Biolabs) in 1X enzyme buffer (5 mM $CaCl_2$, 50 mM pH 5.5 sodium acetate) overnight at 37°C. I washed the slides as above prior to the following antibody incubations. The hematoxylin and eosin (H&E) staining followed a standard protocol with a 5.5 - 6 min hematoxylin incubation and a 3 min eosin incubation.

I used SignalFinder algorithm to locate the pixels containing signal in each image. For each image, SignalFinder creates a map of the locations of pixels containing signal and computes the percentage of tissue-containing pixels that have signal. To arrive at a final number for each core, we averaged over all images for a core.

***Statistical analysis and data preparation***

The relationship between the biomarkers and pancreatic cancer relapse risk was evaluated using the Kaplan-Meier method and Cox proportional hazards regression model as implemented in the “survival” R package. Proportional hazard for cancer relapse was modeled as a univariate response to each of the individual biomarkers. Data for cancer relapse is collected at the time of relapse. Therefore, the dataset does not include any patients who have yet to relapse or have been lost to follow-up.

I graphed the data using Microsoft Office Excel and GraphPad Pro, and I prepared the figures using Canvas 14 and Canvas Draw (ACD Systems). For the Kaplan-Meier curves, I prepared the figures using the ggplot2 R package [174].

## Results

### *Novel approach to decipher sulfated and sialylated glycans*

The structures I targeted are built off the sialylated type 2 LacNAc core (Fig. 10A) and are variously sulfated and fucosylated. The experimental process is to probe the glycans with a panel of lectins after rounds of glycosidase treatment (Fig. 10B), a method called on-chip glycan modification and profiling (on-chip gmap). I assembled lectins and antibodies that would bind sialylated/sulfated/fucosylated variants of the type 2 LacNAc core either before or after enzymatic modification (Fig. 11A). Some are highly specific for just one structure, and others have secondary binding motifs. For example, CCL2 lectin primarily binds to fucose that is linked β1-3 to GlcNAc, but it seems to have weaker binding to sulfated, sialylated, non-fucosylated, type 2 LacNAc [166, 175]. The Siglec family of lectins looked to be useful, as they bind sialylated, type 2 LacNAc with diverse preferences for sulfation and fucosylation [115, 121]. Several details of their specificities, though, have yet to be determined. Based on the glycan-array data provided by the Consortium for Functional Glycomics and previous published data, they showed Siglec-9 binds 6-sulfo-sialyl-type 2 LacNAc with or without fucose; Siglec-F binds 6'-sulfo-sialyl-type 2 LacNAc and, less-so, 6-sulfo-sialyl-type 2 LacNAc, both with or without fucose; and Siglec-8 binds 6'-sulfo-sialyl-type 2 LacNAc with or without fucose [121, 176].

To analyze the data acquired through on-chip gmap, I used software called GlycanSolver (Fig. 11B) [168]. GlycanSolver simulates the patterns of lectin binding to model glycans that potentially are in the sample. The simulated patterns best matching the observed patterns indicate the glycans most likely to be in the sample. The

simulated binding of the lectins is based on glycan-array analyses for the lectins. I validated this approach using comparisons of the predicted glycans to results from conventional analyses of the glycans [168].

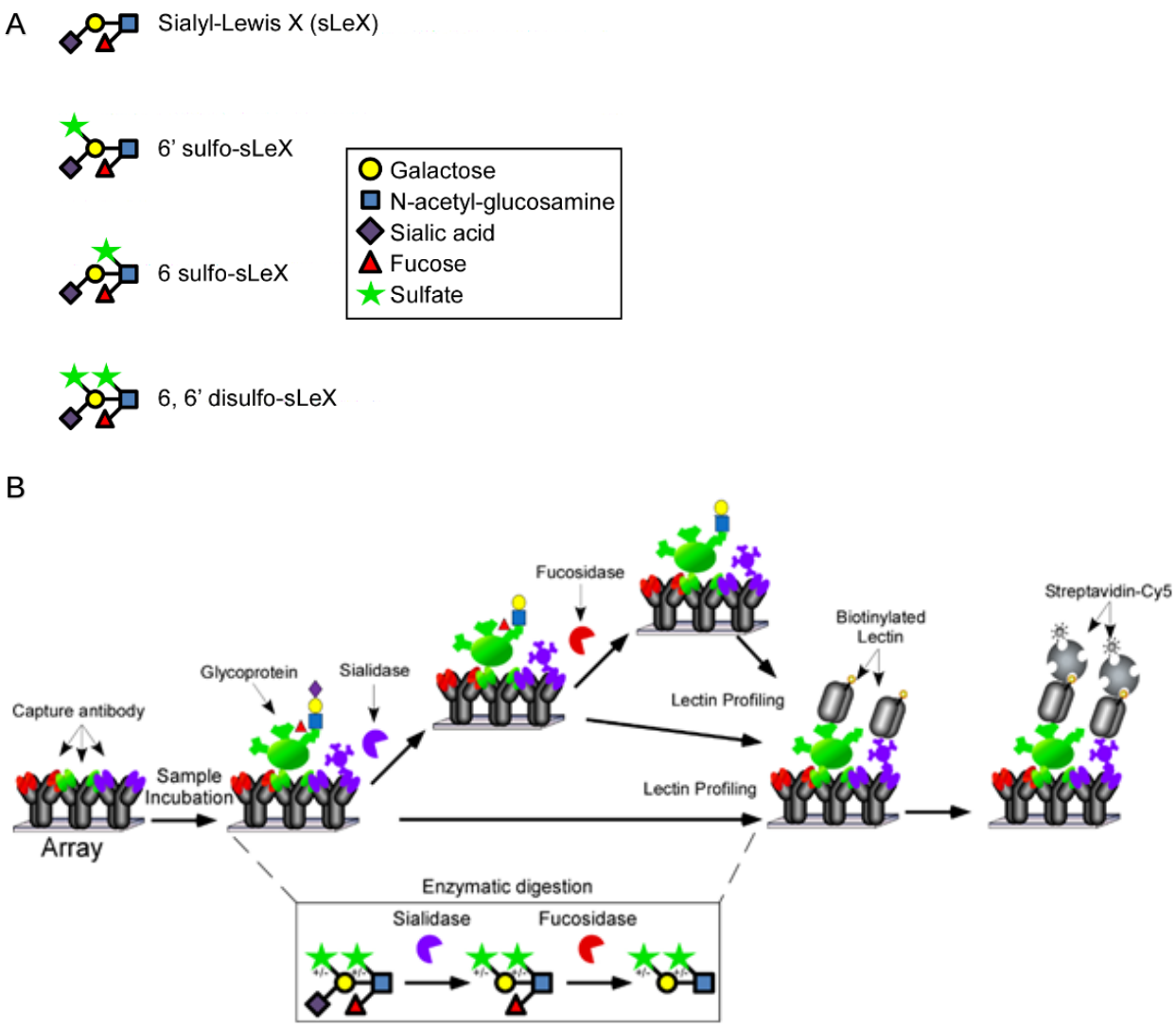



**Figure 10. Analyzing sulfated glycans using on-chip glycan profiling.** (A) The sulfated, and sialylated type 2 LacNAc motifs targeted in this study. (B) Experimental scheme. Biological samples are incubated on printed antibody arrays to allow glycan or glycoprotein capture by the immobilized antibodies. The glycans on the glycoproteins are probed by a panel of antibodies or lectins, either with or without modification by glycosidases. The binding of each antibody or lectin is quantified by fluorescence scanning.

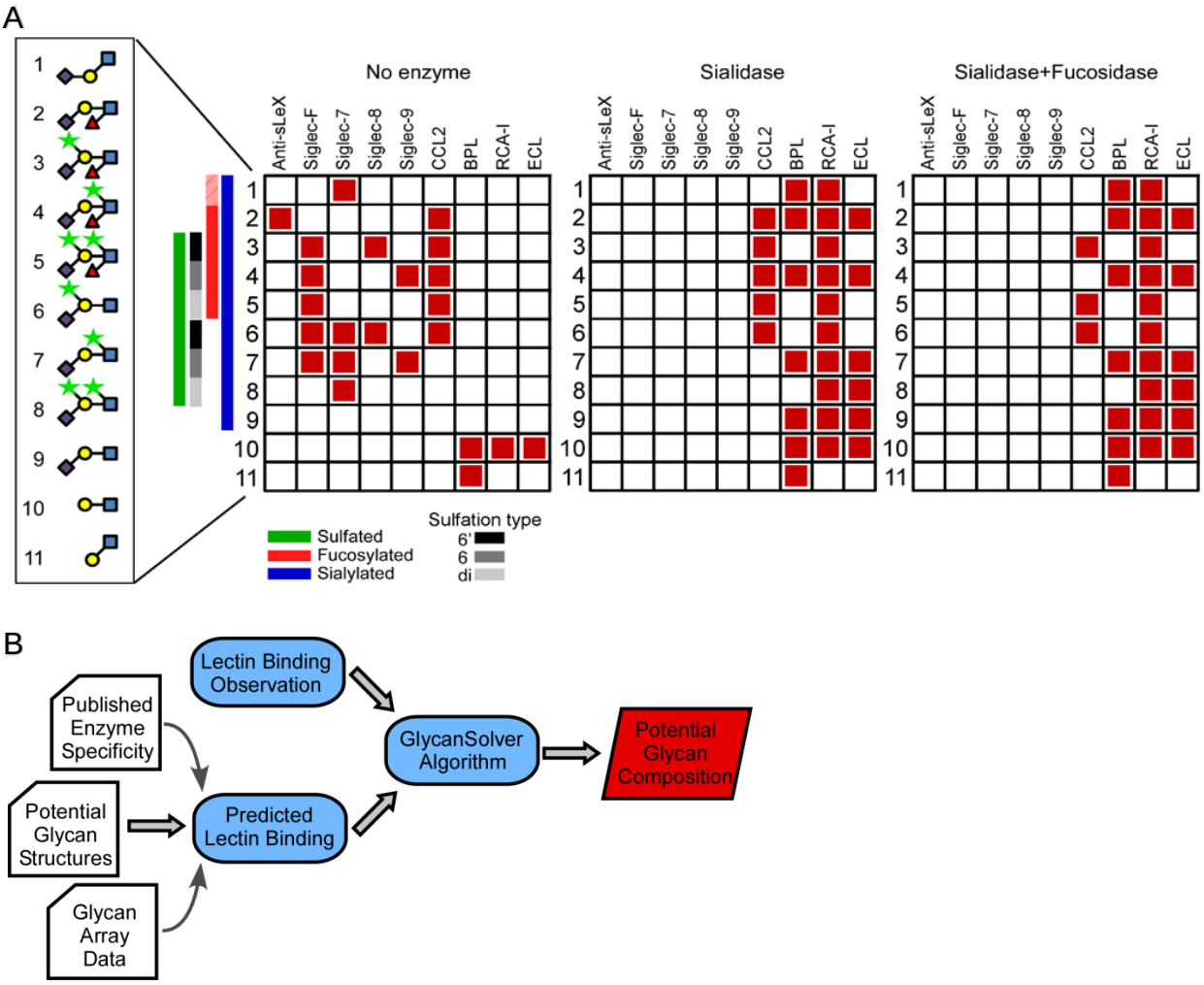



**Figure 11. Glycan motif prediction.** A) The primary binding specificities of the detection reagents, both without and with the prior application of a α2-3 sialidase and a α1-3,4 fucosidase. B) Flowchart for the data analysis. The GlycanSolver algorithm compares the actual (observed) patterns of lectin binding to patterns that are predicted in-silico for model glycans that potentially are in the sample. The predicted binding is derived from three sources of information: glycan array data for each of the lectins, the structures of the model glycans, and the specificities of the enzymes applied to the sample.

## Table 5. Biological reagent list.

| Name | Reagent Type | Source | Catalog # |
|---|---|---|---|
| Anti-MUC5AC | Monoclonal Antibody | Thermo Scientific | MS-145-P1ABX |
| Anti-MUC16 | Monoclonal Antibody | AbCam | X325 |
| CA19-9 | Monoclonal Antibody | Biorbyt | orb116252 |
| Anti-sLeX | Monoclonal Antibody | BD Pharmingen | 551344 |
| TRA-1-60 | Monoclonal Antibody | Novus Biologicals | NB100-730 |
| Anti-polyhistadine | Monoclonal Antibody | R&D Systems | MAB050 |
| Coprinopsis cinerea lectin 2 (CCL2) | Lectin | Dr. Markus Kuenzler | N/A |
| Recombinant Mouse Siglec-F Fc Chimera Protein (Siglec-F) | Lectin | R&D Systems | 1706-SF |
| Siglec-7comp (Siglec-7) | Lectin | Dr. Ronald Schnaar | N/A |
| Siglec-8comp (Siglec-8) | Lectin | Dr. Ronald Schnaar | N/A |
| Siglec-9comp (Siglec-9) | Lectin | Dr. Ronald Schnaar | N/A |
| Erythrina cristagalli lectin (ECL) | Lectin | Vector Laboratories | L-1140 |
| Bauhinea purpurea lectin (BPL) | Lectin | Vector Laboratories | B-1285 |
| Ricinus communis agglutinin I (RCA-I) | Lectin | Vector Laboratories | B-1085-5 |
| Concanavalin A (ConA) | Lectin | Vector Laboratories | B-1005 |
| Griffonia Simplicifolia lectin II (GSL II) | Lectin | Vector Laboratories | B-1215 |
| Sclerotium rolfsii lectin (SRL) | Lectin | Wako | 199-17271 |
| Ralstonia solanacearum lectin (RSL) | Lectin | Dr. Anne Imberty | N/A |
| Fetuin, bovine | Purified Glycoprotein | Sigma Aldrich | F2379 |
| Haptoglobin | Purified Glycoprotein | Calbiochem | 372022 |
| α2-3 Neuraminidase (Sialidase) | Enzyme | New England BioLabs | P0728L |
| α1-3,4 Fucosidase | Enzyme | New England BioLabs | P0769S |
| β1-4 Galactosidase | Enzyme | Proezyme | GKX-5014 |
| PNGase F | Enzyme | New England BioLabs | P0704S |
| Streptavidin-Cy5 | $2^{o}$ detection reagent | Invitrogen/ThermoFisher | 43-4316 |

### *Analysis of glycans in selected plasma samples*

I was particularly interested in identifying glycans that are complementary to sLeA (detected by CA19-9 antibody), or that are produced by tumors not producing sLeA. I first determined the level of the sLeA on various glycoproteins captured by immobilized antibodies (CA19-9, anti-MUC16 and anti-MUC5AC) (Fig. 12). I selected four samples from PDAC patients with a wide range of CA19-9 levels and two samples from healthy subjects with undetectable sLeA level (Fig. 12). I profiled the glycans on the captured proteins using a panel of eight lectins and one anti-glycan antibody, applied with or without enzyme treatment (Fig. 13A). The quantified fluorescence signals (Fig. 13B & 14A) showed major differences between the samples in the patterns of lectin binding, suggesting differences in the glycan structures.

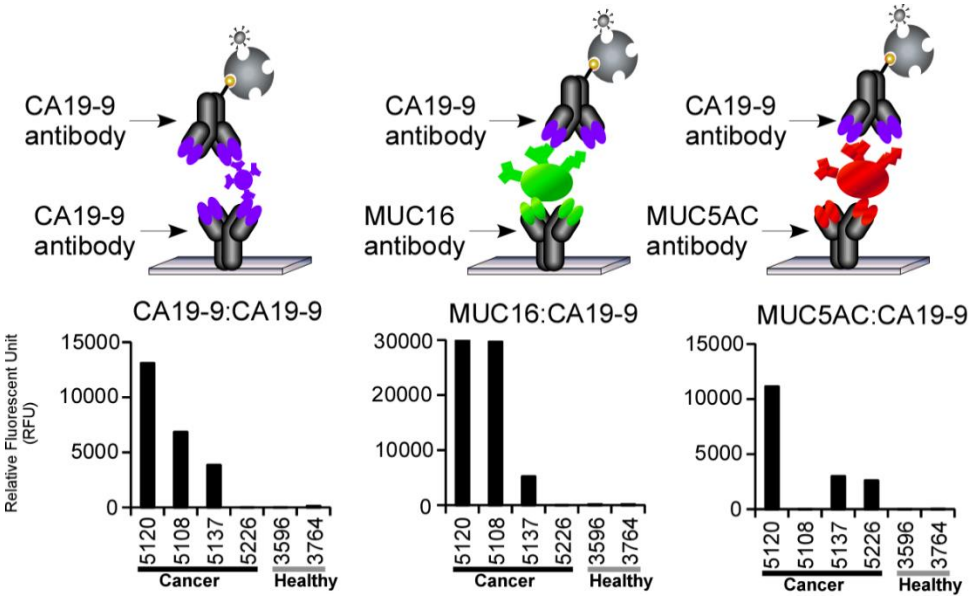



**Figure 12. Profiling of plasma sLeA level by CA19-9 assay.** Measurements of CA19-9 reactive epitope on structure captured by three different protein-specific and glycan-specific antibodies.

The application of GlycanSolver produces a normalized score for each glycan structure, reflecting the correspondence of each structure with the experimental data. From the list of glycans in the output of GlycanSolver, I tallied the occurrences of the targeted motifs across the glycans in each sample. The accuracy of the method was tested by calculating the occurrences of sialyl-type 1 LacNAc and checking for correspondence to the CA19-9 assay (Fig. 12). The sialyl-type 1 LacNAc motif (Fig. 14B), calculated from measurements that did not include the CA19-9 antibody, agreed remarkably well with the CA19-9 assay, thus providing strong validation. Furthermore, motif calculations for the control proteins fetuin and transferrin agree well with previous findings that used a different set of lectins [168]. One of the surprising observations was that CCL2 binding in sample 5120, it was increased after fucosidase treatment (condition 2) (Fig. 14B). The CCL2 lectin has a primary binding toward the α1-3 fucose motif, but it has a less known secondary specificity toward the sulfated type 2 LacNAc. The most likely cause for the increase in CCL2 binding is increased exposure of the secondary, sulfated motif after removal of fucose by fucosidase. The GlycanSolver program effectively incorporated the information about secondary specificity.

I then asked whether any motifs were highly-represented in the two sLeA-low (as detected by CA19-9 assay) cancer samples (5137 and 5226) and not in the healthy subjects (Fig. 14B). I found that sulfated and sialylated type 2 LacNAc without fucose (motifs 5, 6, and 7) were highly represented (relatively scales) on both mucins and on the proteins captured by CA19-9 antibody. Sialyl-type 2 LacNAc (motif 8), which is a common motif found throughout biology, was high in healthy subjects, and other motifs were scattered across the samples (Fig. 14B).

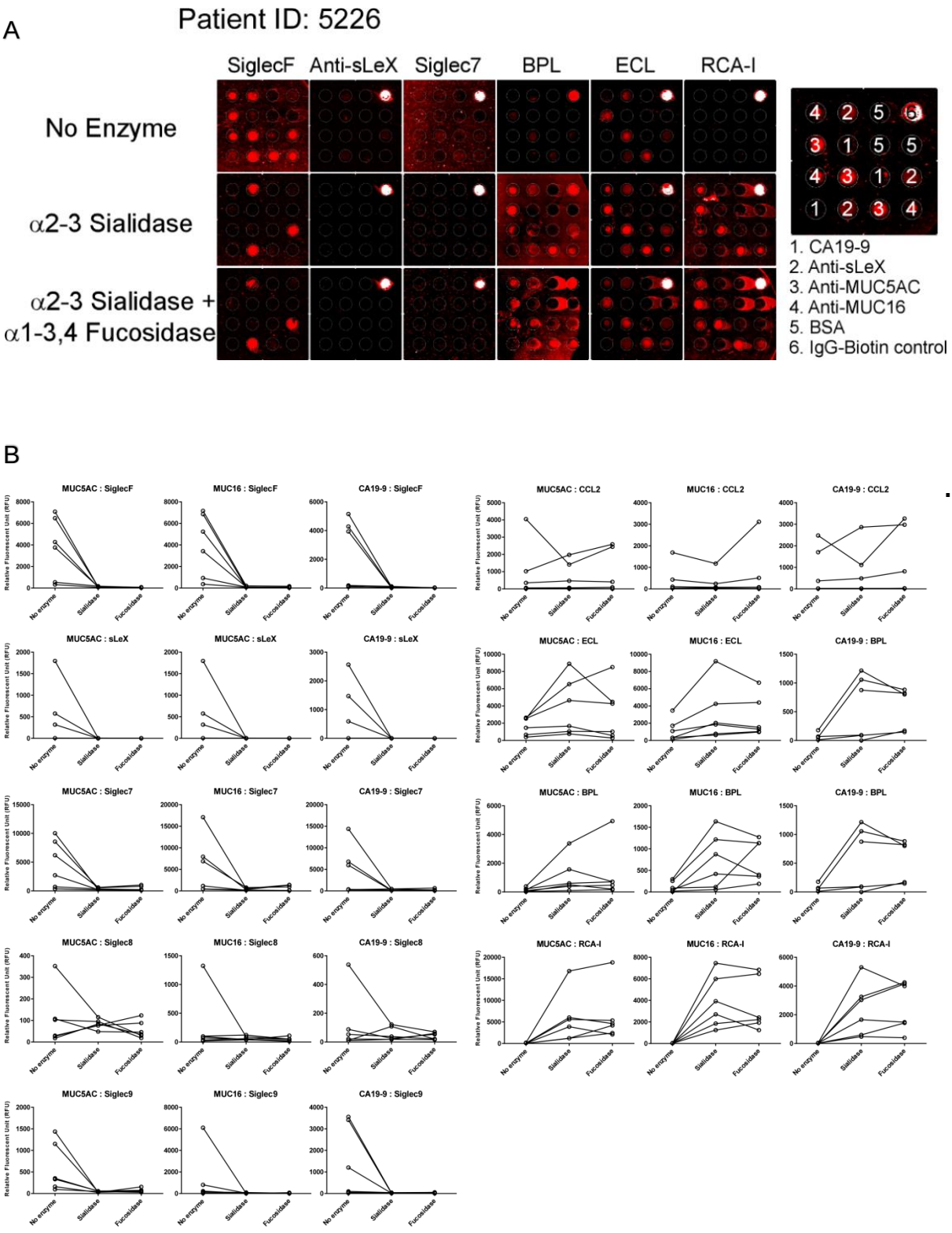



**Figure 13. On-chip gmap**. A) Representative raw microarray images. Each array contained one to three replicates of capture antibodies, IgG-Biotin (positive) and BSA (negative) controls. The three enzymatic conditions (left) and six affinity reagents used

Figure 13 (cont'd)

in this study are shown here. The red fluorescent (Cy5) signals indicated binding of each reagent and white dot indicated overexposure (above detectable level) signals. The results of replicated arrays were well reproduced before and after enzyme treatments. B) The quantitative fluorescence results of each sample across three conditions and nine detections and three captures were average and plotted. Each line connects each sample across the three conditions.

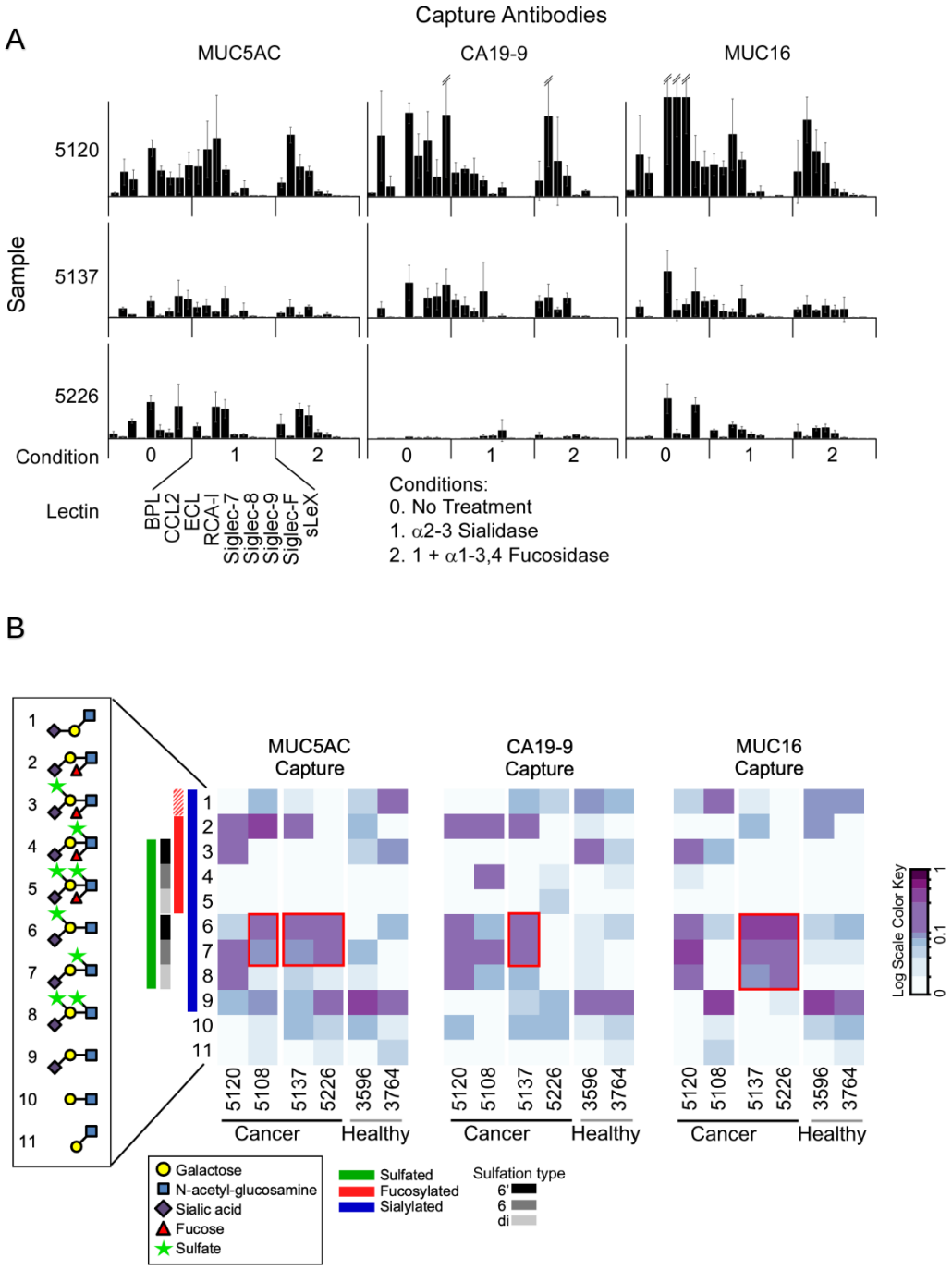



**Figure 14. Elevation of sulfated sialyl-type 2 LacNAc in PDAC plasma.** A) Patterns of lectin binding for three different plasma samples and three different capture antibodies. The values are the quantified fluorescence data, plotted on a normalized scale of 0-1. The error bars represent the standard deviations over the replicate spots. B) Relative abundance of various motifs in each of the samples was calculated by comparing all glycan motifs. The graph presents the normalized abundances of the indicated motifs, based on analysis of the glycans predicted by GlycanSolver to be present in each of the samples.

### *Analysis of glycans in cell culture models*

I next sought to determine whether the sulfated sialyl-type 2 LacNAc motifs observed in sera can be directly produced by cancer cells, as opposed to production by secondary and inflammatory processes. I analyzed conditioned media from PDAC cell lines because the glycans would be presented in such media only if produced by the cancer cells. In a panel of 12 cell lines, four secreted high levels of CA19-9 (Fig. 15A). I observed that some of the cell lines not secreting CA19-9 showed high levels of the glycoform of MUC16 detected by Siglec-F or Siglec-7 (Fig. 15A), lectins with a strong preference for sulfated sialyl-type 2 LacNAc. Such cell lines did not show elevations in other glycoforms, and the remaining cell lines did not show elevations in any of the glycoforms tested here (Fig. 15B).

I investigated the cellular production of the glycan detected by Siglec-F using immunofluorescence staining of 3D cultures of selected cell lines. The 3D cultures produced clusters of cells, with glycan staining occurring at the clusters (Fig. 16A). In some cases, the staining spread to the regions around the cells, indicating secretion of the glycosylated proteins. The quantification of the staining showed highest levels of Siglec-F ligands for the L3.6pl and Panc10.05 cell lines (Fig. 16B), which corresponded to the high levels in the conditioned media (Fig. 15). These analyses showed that pancreatic cancer cell lines, including some that do not produce CA19-9, can produce and secrete the Siglec-F and Siglec-7 ligands.

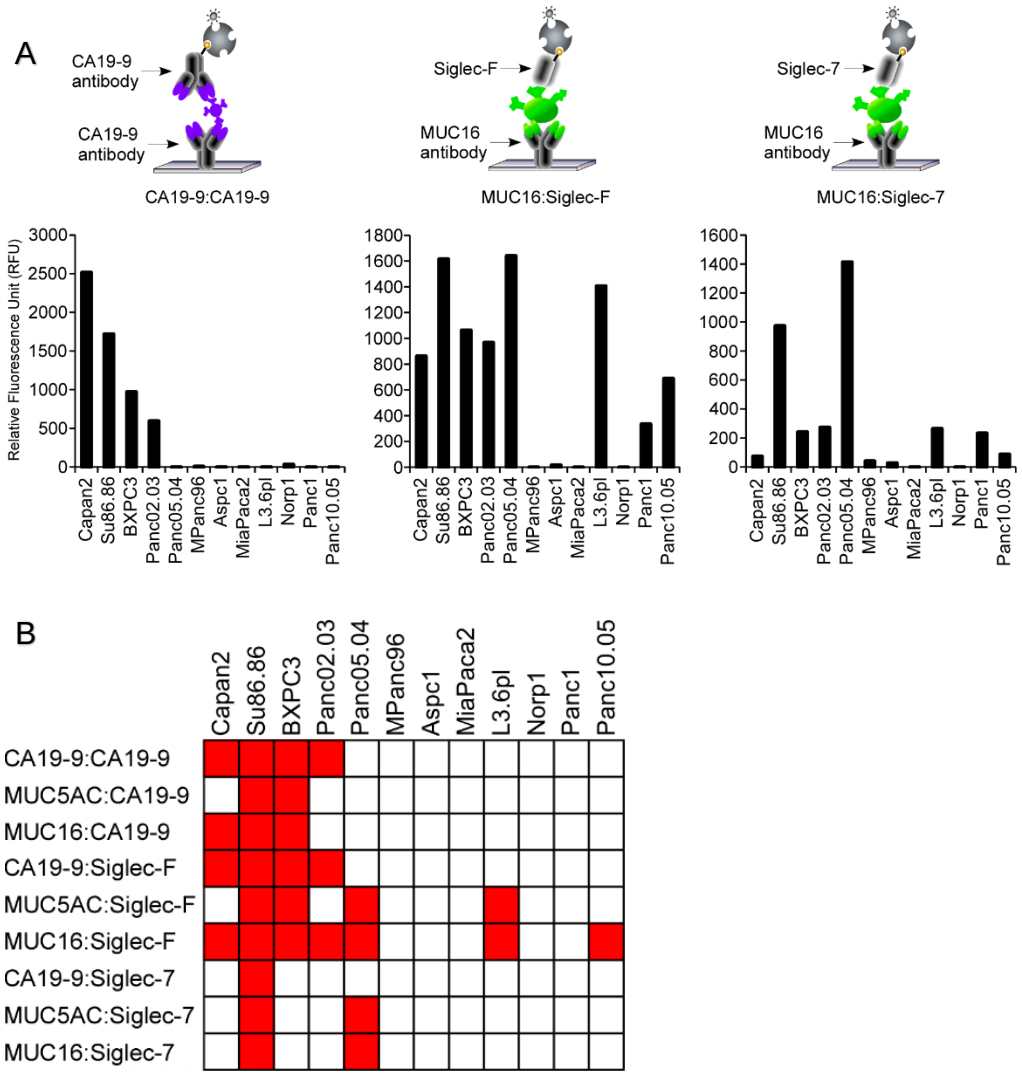



**Figure 15. Glycan profiling of *in vitro* 3D PDAC cell conditioned media.** A) Levels in conditioned media. The sandwich assays depicted above each graph were applied to the conditioned media from various PDAC cell lines. Some media had no CA19-9 but had the Siglec-F or Siglec-7 ligands on the MUC16 carrier protein. B) Summary of assay results for conditioned media. Each red square indicates an assay value above the threshold set for that assay.

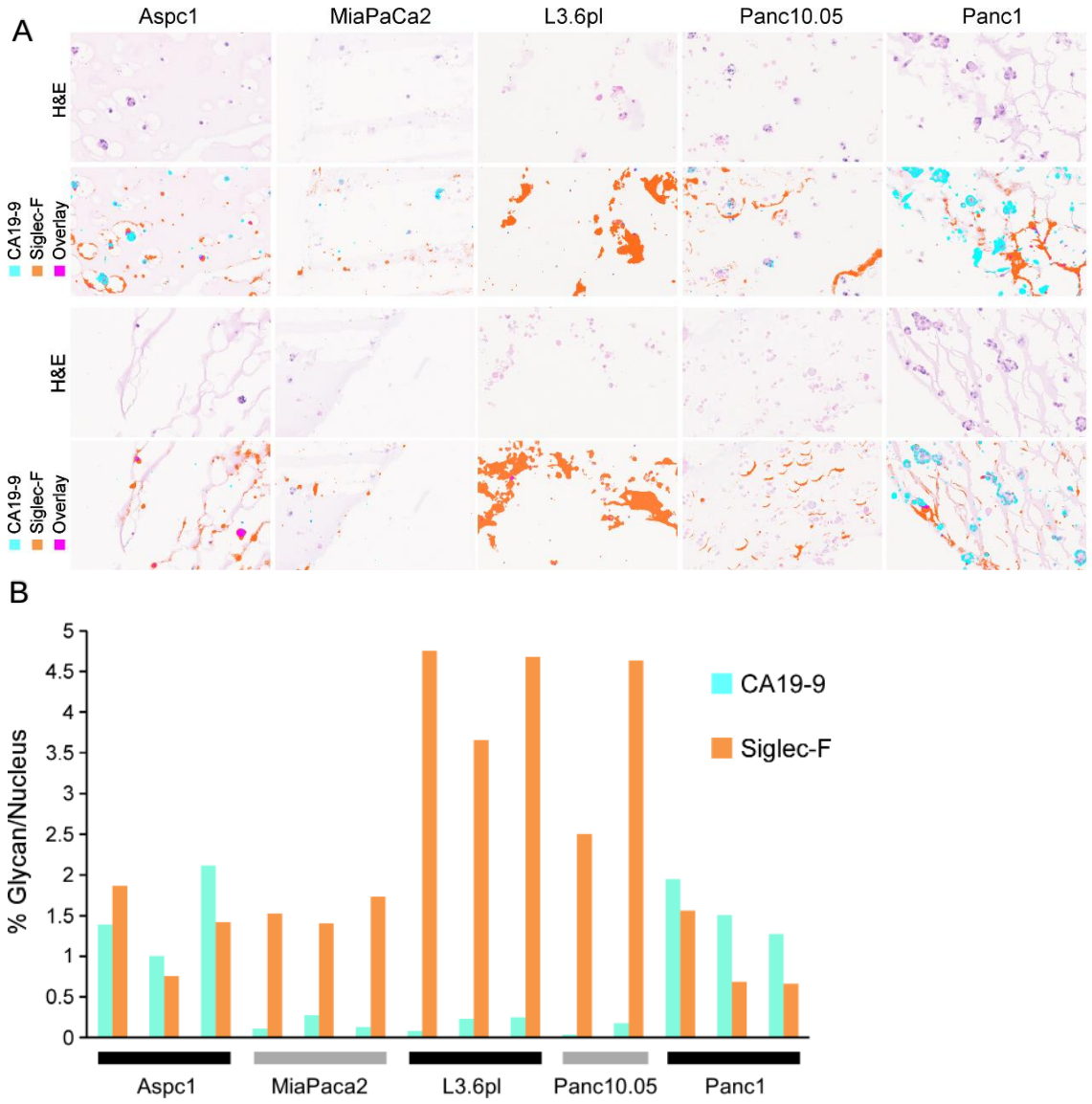



**Figure 16. Production and secretion of the Siglec-F and Siglec-7 ligands by CA19-9-negative cell lines.** A) Immunofluorescent staining results. For the selected cell lines, the H&E image is shown for two regions, along with the H&E image overlaid with the immunofluorescence data from CA19-9 and Siglec-F. The colored regions indicate where signals were detected by SignalFinder. B) Quantification of the immunofluorescence data. The columns indicate the amount of fluorescence from CA19-9 or Siglec-F divided by the amount of Hoechst dye (nucleus stains).

### *Analysis of glycan motifs in cell culture models*

I next asked whether the glycan motifs detected in the conditioned media by Siglec-7 and Siglec-F (Fig. 17) are the same as in the serum samples (Fig. 14). Such a correspondence would support the concept of the production of sulfated sialyl-type 2 LacNAc by certain pancreatic cancers that do not produce CA19-9. I therefore performed on-chip gmap on the glycans attached to the proteins captured out of the cell line conditioned media.

I used an expanded set of lectins and enzyme conditions (Fig. 17A) to more reliably and deeply probe the motifs. The quantified and normalized signals (Fig. 17B) showed major differences in lectin-binding patterns between Aspc1, L3.6pl, and Panc10.05 using the CA19-9 and MUC16 capture antibodies. The MUC5AC capture antibody captured very little material from these media samples (not shown).

The output of GlycanSolver showed that sulfated sialy-type 2 LacNAc motifs were high in L3.6pl and Panc10.05, minimally presented in Panc1, and not presented in the others. This result agreed with the Siglec-F and Siglec-7 results described above. The presence of motifs 6 and 7 (non-fucosylated) agrees with the motifs found in plasma, but the fucosylated and sulfated sialyl-type 2 LacNAc (motifs 2 and 3) was different from the plasma data (Fig. 14B and 17C). The difference could be due to natural variation between cancers, or it could result from the culturing of the cancer cells. Nevertheless, the data confirmed that sulfated sialyl-type 2 LacNAc is produced by certain PDAC cell lines.

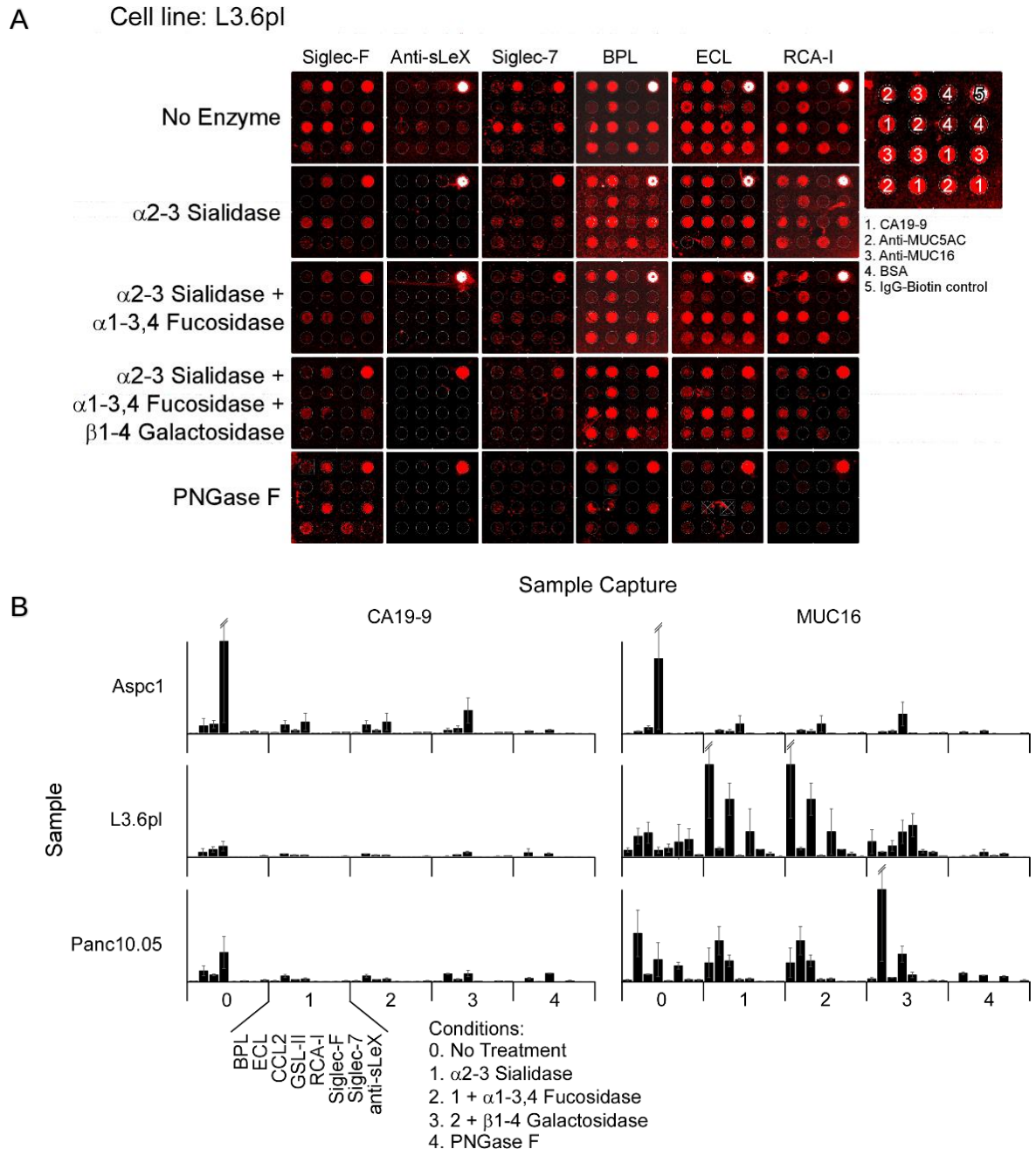
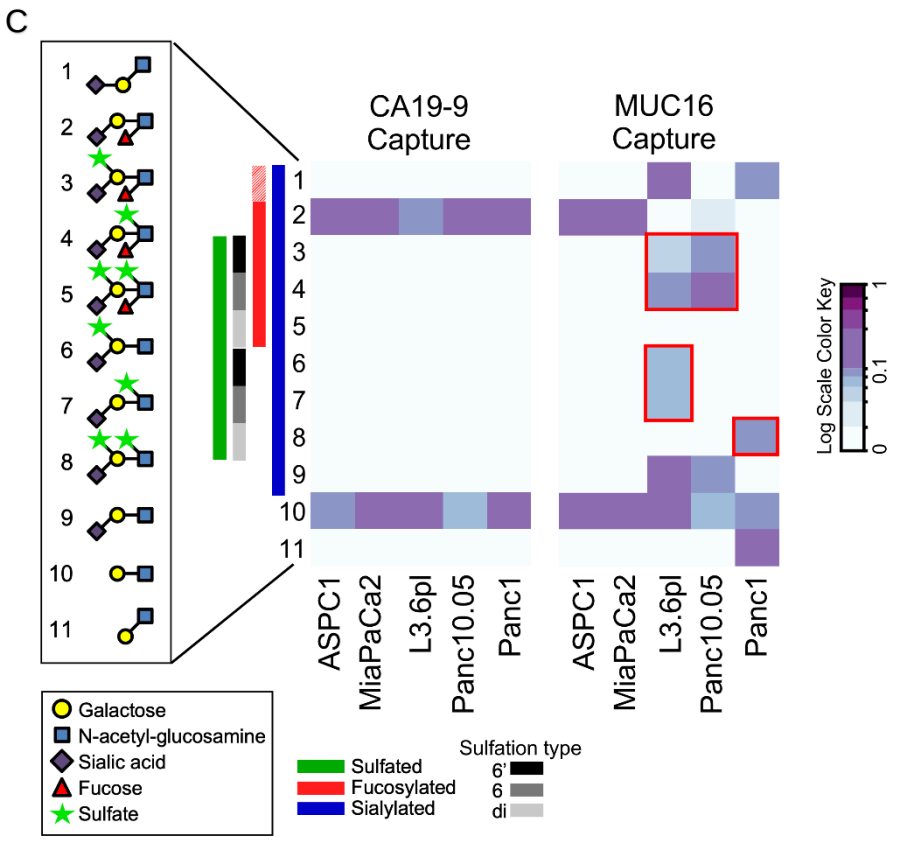



**Figure 17. Secretion of sulfated glycan motifs by CA19-9-negative cell lines.** A) Raw image data for the conditioned media from the L3.6pl cell line. The red fluorescent signals indicated by Siglec-F binding didn't completely diminish after several rounds of enzyme treatments because Siglec-F has affinity to N-linked glycans. In contrast, red fluorescent signals indicated by Siglec-7, which has affinity to N-linked glycans, diminished after rounds of enzyme treatments. B) Quantification of the image data. Each column the average over the replicate spots for either the CA19-9 (left) or the MUC16 (right) capture antibody. The error bars are the standard deviations over the replicates. Data from three cell lines are presented.

Figure 17 (cont'd)




C) Relative abundance of various motifs in the indicated conditioned media. Each square gives the normalized abundance of the indicated motifs, based on analysis of the glycans predicted by GlycanSolver to be presented at the CA19-9 (left) or MUC16 (right) capture antibodies.

### *Profiling sulfated sialyl-type 2 LacNAc in cohorts of PDAC patients and controls*

The above experiments provided in-depth analyses in a small number of samples, but a remaining question was whether specific sulfated sialyl-type 2 LacNAc motifs are statistically associated with PDAC, particularly with PDACs that do not have sLeA elevation. To advance this project, I probed plasma samples from 96 patients with pancreatic cancer and 100 subjects with benign pancreatic conditions (Table 6) for the glycans detected by CA19-9 antibody, Siglec-F, and Siglec-7. I saw statistically-significant elevations in cancer of specific assays, particularly CA19-9, MUC5AC detected with Siglec-F (referred to as MUC5AC:Siglec-F), and MUC16 detected with Siglec-7 (referred to as MUC16:Siglec-7) (Fig. 18A). Using thresholds defined by the second-highest value in the controls, 23 PDAC patients (24%) were elevated in MUC5AC:Siglec-F and 14 (15%) in MUC16:Siglec-7. None of the mucin glycoforms showed a statistical association with age, gender or type of benign disease (not shown). Thus, I saw significant elevations among a subset of PDAC patients for both markers.

In order to determine whether the elevations occurred in some patients that did not have sLeA elevations, I examined the relationships between the markers in scatter plots (Fig. 18B). Using the thresholds defined above and a threshold for CA19-9 assay giving maximum accuracy to discriminate cases from controls, seven and five patients low in sLeA showed MUC5AC:Siglec-F and MUC16:Siglec-7 elevations, respectively (Fig. 18B). Substantial groups of patients also were elevated in both markers, or only sLeA, or neither. A matrix of the elevations show that specific assays were able to detect many of the CA19-9-low cancer patients with only minimal false-positive detection of subjects with benign conditions (Fig. 18C), demonstrating the separate subsets of

patients. All of the markers showed a statistical association with stage of disease, consistent with previous observations for sLeA, but each marker showed about equal elevations in high-sLeA and low-sLeA patients, regardless of stage (Table 7) [177].

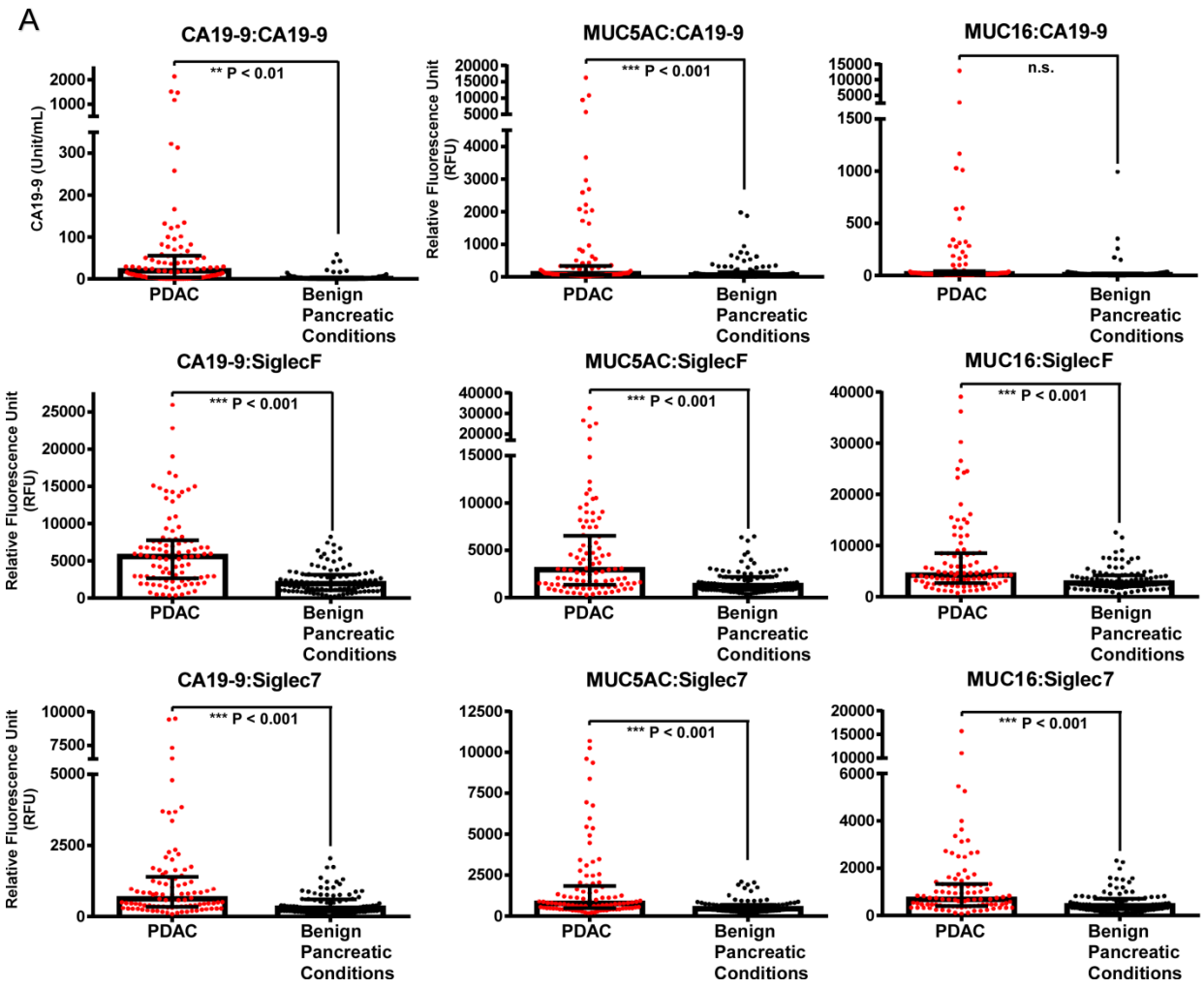
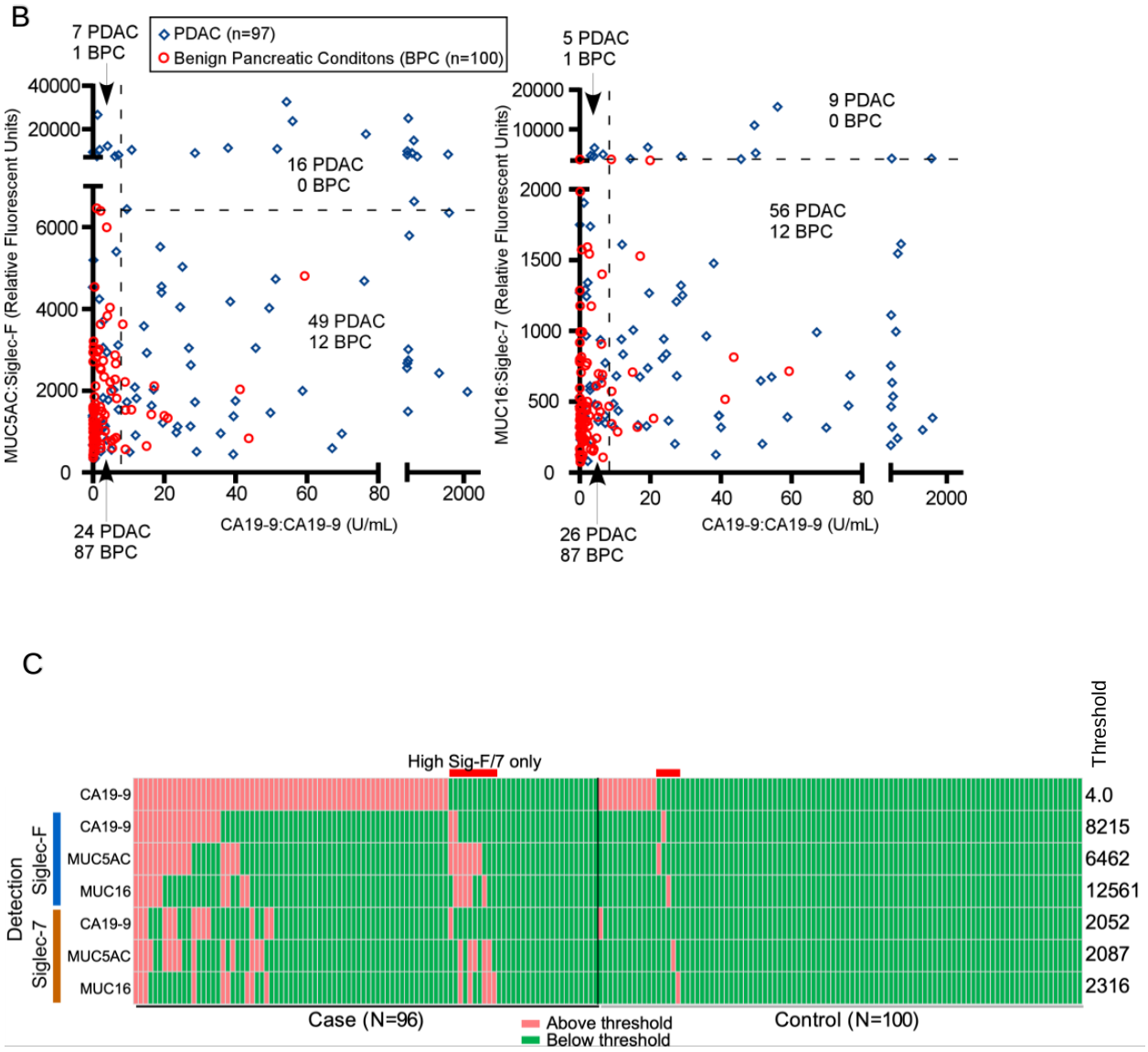



**Figure 18. Elevation of the Siglec-F and Siglec-7 glycoforms of MUC5AC and MUC16 in a subset of PDACs.** A) Assay results for detected with CA19-9, Siglec-F, and Siglec-7.

Figure 18 (cont'd)




B) Relationships between the assays. The MUC5AC:Siglec-F (left, y axis) or MUC16:Siglec-7 (right, y axis) value es were plotted with respect to the CA19-9 values (x axes) over all patients. The dashed lines are the thresholds indicated in panel A. C) Summary of elevations. Each column represents data from a single subject, and each row is an assay. A red box indicates a value above the threshold given at right.

**Table 6. Composition of the sample set.** The asterisk (*) indicates a significant difference ($p < 0.05$, Fisher's exact test) between cases and controls.

| | **TRAINING SET** |
|---|---|
| SITE | UPMC |
| TOTAL SAMPLES, N | 196 |
| CANCER, N | 96 |
| AVERAGE AGE, Y (SD) | *67.3 (10.6) |
| PERCENT MALE | 51.5% |
| CONTROL, N | 100 |
| AVERAGE AGE, Y (SD) | *58.7 (15.5) |
| PERCENT MALE | 53.0% |
| CANCER STAGES | |
| STAGE I, N | 3 |
| STAGE II, N | 58 |
| STAGE III, N | 20 |
| STAGE IV, N | 16 |
| CONTROL TYPES | |
| CHRONIC PANCREATITIS, N | 46 |
| BENIGN STRICTURE, N | 23 |
| ABNORMAL IMAGING, N | 27 |
| CHRONIC DIABETIC, N | 0 |
| HEALTHY CONTROL, N | 0 |
| PANCREATIC CYST, N | 4 |

**Table 7. 95% specificity table.** The table showed sample distribution (number and percentage) and expression of individual markers in the samples.

| 95% Specificity Table | | Assays and measurements | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CA19-9 | MUC5AC:CA19-9 | MUC16:CA19-9 | MUC5AC:Siglec-F | CA19-9:Siglec-F | MUC16:Siglec-F | MUC5Ac:Siglec-7 | CA19-9:Siglec-7 | MUC16:Siglec-7 |
| Number # | Early stage (I & II) (n=61) | 7 | 1 | 2 | 7 | 5 | 6 | 6 | 3 | 5 |
| | Stage III (n=20) | 7 | 6 | 1 | 6 | 9 | 5 | 5 | 5 | 4 |
| | Stage IV (n=15) | 7 | 5 | 2 | 10 | 6 | 4 | 8 | 6 | 5 |
| All cancer detected (#) | | **21** | **12** | **5** | **23** | **20** | **15** | **19** | **14** | **14** |
| Percentage of individual stage | Early stage (I & II) | 11.48% | 1.64% | 3.28% | 11.48% | 8.20% | 9.84% | 9.84% | 4.92% | 8.20% |
| | Stage III | 35.00% | 30.00% | 5.00% | 30.00% | 45.00% | 25.00% | 25.00% | 25.00% | 20.00% |
| | Stage IV | 46.67% | 33.33% | 13.33% | 66.67% | 40.00% | 26.67% | 53.33% | 40.00% | 33.33% |
| All cancer detected (%) | | **21.88%** | **12.50%** | **5.21%** | **23.96%** | **20.83%** | **15.63%** | **19.79%** | **14.58%** | **14.58%** |

### *Sulfated and sialylated type 2 LacNAc expression associated with early cancer recurrence*

The possibility exists that the cancers elevated in the sulfated sialyl-type 2 LacNAc structures are different from the others in their aggressiveness. I had information on time-to-progression (TTP) for 40 of the 96 PDAC patients. CA19-9 did not show an association with TTP, as assessed by Kaplan-Meier analysis (Fig. 19A), Cox regression modeling (Table 8), or direct comparisons between groups (Fig. 19B). The glycoform of MUC5AC detected by the CA19-9 antibody (MUC5AC:CA19-9) showed a weak association with survival by each analysis (Fig. 19 and Table 8). In contrast, the glycoform of MUC5AC detected by Siglec-7 showed a strong association with TTP (Fig. 19). By Cox regression modeling, the hazard ratio was 4.8 (95% C.I. 2.4-9.5, $p = 3.0 \times 10^{-6}$ by log-rank test), but the hazard ratio for MUC5AC:CA19-9 was just 1.8 (95% C.I. 1.2-2.8, $p = 0.0027$ by log-rank test) (Table 8). The MUC5AC:Siglec-F assay also showed a relationship with survival, but with less significance, and the MUC16

glycoforms showed no relationship with survival (Table 8). Thus, the distinct subset of cancers detected by the sulfated sialyl-type 2 LacNAc glycoform of MUC5AC could be a subtype of PDAC that is particularly aggressive.

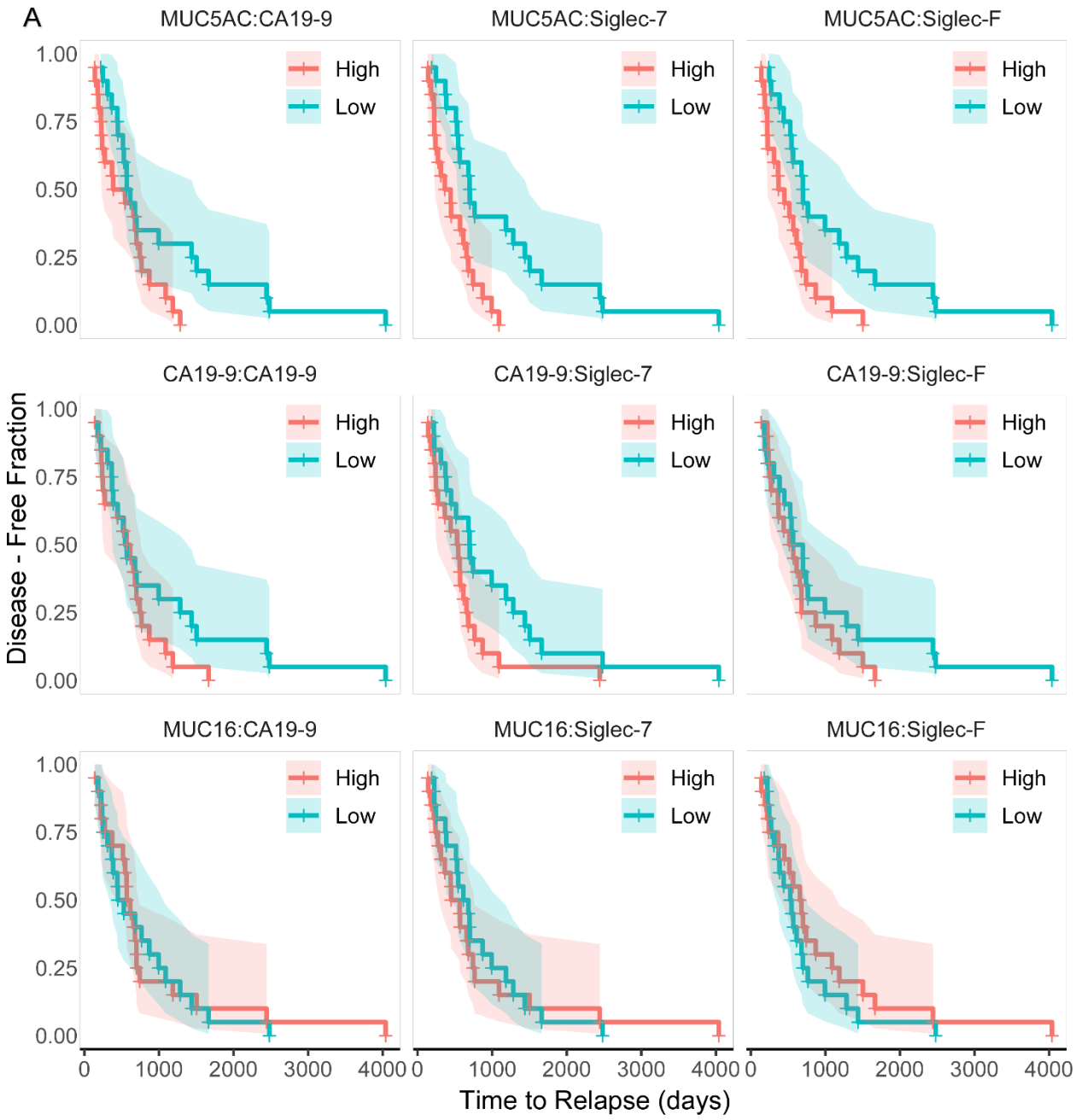
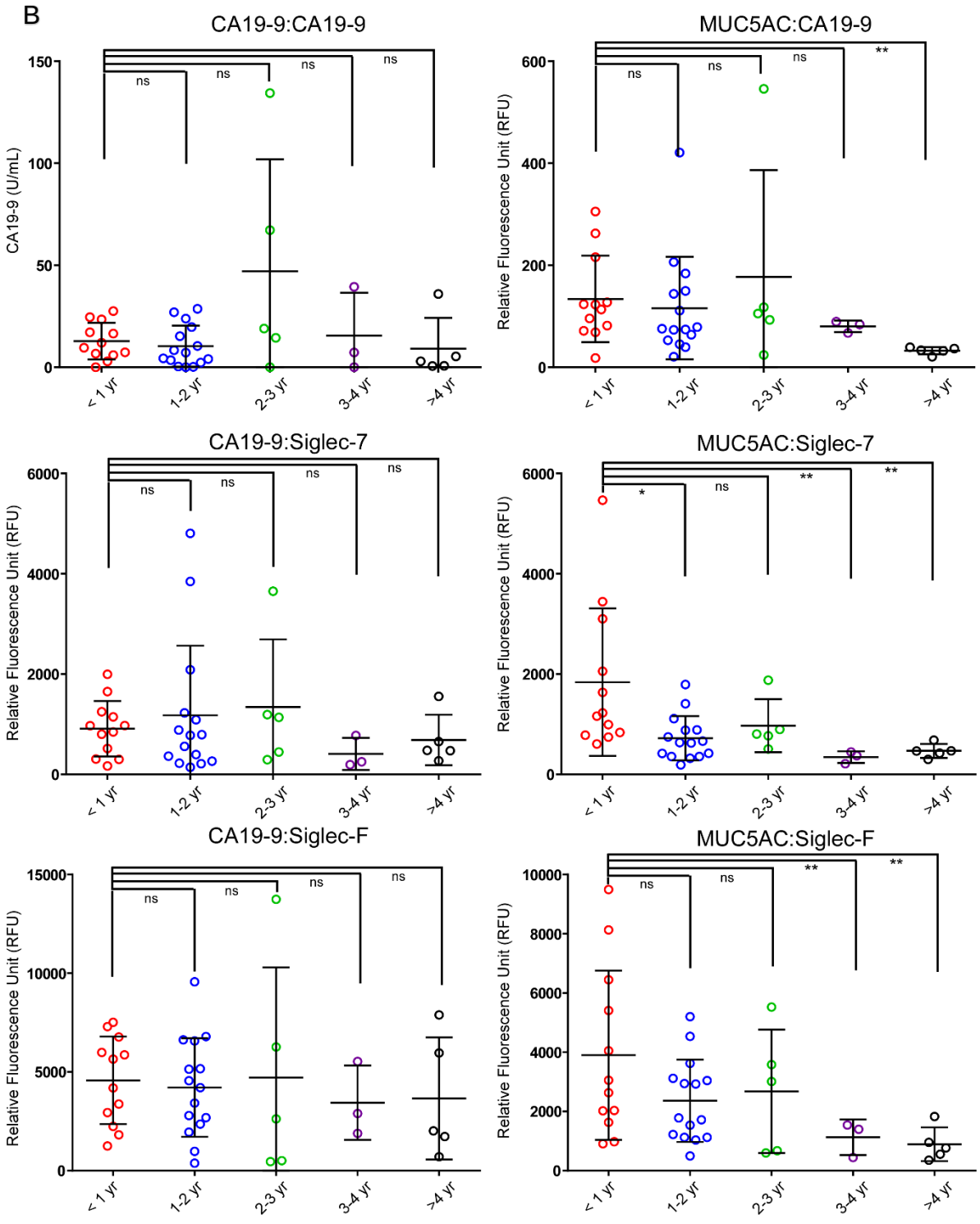



**Figure 19. Associations with time-to-progression.** A) Kaplan-Meier plots. The patients were grouped using the 50th percentile in each marker.

Figure 19 (cont'd)




B) Values in each range. NS, not significant; * indicates $p < 0.05$, ** indicates $p < 0.01$ (Student's t-test).

**Table 8. Cox regression modeling.** The hazard ratio (HR) is for the per-unit increase in the log-transformed biomarker. The p-value is based on the log-rank test.

| Marker | HR | 0.95 CI | | p-value |
|---|---|---|---|---|
| CA19-9 | 1.06 | 0.92 | 1.22 | 0.45 |
| CA19-9:Siglec7 | 1.17 | 0.79 | 1.73 | 0.42 |
| CA19-9:SiglecF | 1.28 | 0.86 | 1.92 | 0.22 |
| MUC16:CA19-9 | 1.09 | 0.79 | 1.49 | 0.61 |
| MUC16:Siglec7 | 0.99 | 0.66 | 1.47 | 0.96 |
| MUC16:SiglecF | 0.92 | 0.58 | 1.44 | 0.70 |
| MUC5AC:CA19-9 | 1.84 | 1.23 | 2.75 | 0.0027 |
| MUC5AC:Siglec7 | 4.81 | 2.44 | 9.48 | 3.00E-06 |
| MUC5AC:SiglecF | 1.72 | 1.21 | 2.42 | 0.0017 |

## Discussion

The heterogeneity between pancreatic tumors causes huge difficulties in developing effective biomarkers and treatments. Molecular markers that define the distinct subpopulations of cancers could enable the detection of the various subgroups and potentially give guidance on the best course of treatment. Using a new technology to analyze protein glycosylation, including certain sulfated glycans, I found that sulfated and sialylated type 2 LacNAc is a biomarker of pancreatic cancer. I found it is attached to MUC5AC and MUC16 and secreted by some PDACs that do not produce the CA19-9 antigen. As such, it could be a useful complement to CA19-9. Furthermore, it may be an indicator of a subgroup with distinct biology and rapid progression.

Sulfated sialyl-type 2 LacNAc has been well-studied in the context of the immune system. Such glycans are generally immunosuppressive, as shown by pro-apoptotic effects on NK cells, neutrophils and eosinophils [118, 119]. On the other hand, pro-inflammatory phenomenon could be introduced through the engagement of Siglec-7 to trigger pro-inflammatory cytokines and chemokines, and by interacting with L-selectin to mediate lymphocyte homing [107, 117]. The type 2 LacNAc glycans seem to be much more involved in immune regulation than the type 1 LacNAc glycans. For example, sLeA, a type 1 LacNAc glycan, is primarily expressed in the pancreas on centroacinar cells and intralobular ducts; seminal fluid, bile, and saliva; and as free glycans in milk, whereas sLeX, its type 2 LacNAc isomer, is on cells of myeloid lineage in normal blood [59-62, 69, 70].

In agreement with my findings, the up-regulated expression of variants of sulfated sialyl-type 2 LacNAc was shown in several other cancers. Sulfated variants sLeX was

upregulated in mucinous adenocarcinomas of the colon and ovary, and 6-sulfo-type 2 LacNAc on extended core-1 O-glycans, as detected with the MECA-79 antibody, is on some bladder urothelial cancer cells and gastric cancer cells [108, 127, 128]. A gene-expression study also showed strong upregulation of sulfation-associated genes in subsets of pancreatic-cancer cells which can be an additional evidence in supporting the upregulation of sulfated type 2 LacNAc glycan found in this study [178].

The current study complements the previous studies by demonstrating elevations of sulfated sialyl-type 2 LacNAc in pancreatic cancers. This study also provides more details about the glycan motifs than probing with a single lectin or antibody. Any individual lectin or antibody does not have perfect specificity for one glycan, and some glycan motifs are not targeted by any known lectin or antibody. But by bringing together information from multiple lectins and conditions, I could reliably calculate relative abundances of a wide-range of motifs that previously were difficult to measure. The analyses from multiple lectins and enzymes unequivocally confirms two major points about the glycans in pancreatic cancer, specific type 2 LacNAc(s) are elevated where the type 1 LacNAc(s) (such as CA19-9) are not; and sulfated type 2 LacNAc(s) showed more significance than the non-sulfated versions. The potential for pancreatic cancers to upregulate type 2 LacNAc was previously known, but only for fucosylated, non-sulfated motifs (e.g. sialyl-Lewis X). The findings here show that sulfation on the 6-carbon of the Gal and potentially also the GlcNAc of sialyl-type 2 LacNAc is a prevalent feature in pancreatic cancers, and that in such motifs, the fucosylation of the type 2 LacNAc is not critical.

A role for the Siglec receptors in cancer immunosuppression was supported in previous studies, for example by impairing NK cell immunosurveillance and cell destruction [47, 125, 126]. In one such study, an *in vivo* model of immunodeficient mice with reconstituted human NK cells and human cancer cells showed that subsets of Siglec-9+ NK cells and their 6-sulfo-sLeX ligands are responsible for inhibition of NK cell mediated tumor cell killing [125]. Thus, the shorter survival observed in association with the high MUC5AC:Siglec-7 marker could result from immunosuppression. Because only a subset of PDAC patients with short survival expressed MUC5AC:Siglec-7 marker, other mechanisms of rapid progression are likely to be at work in certain PDACs, for example resistance to treatment. If such subtypes in fact exist, markers to identify the subtypes of PDAC would have immense value for guiding treatment and research. My results indicate that future research should aim to further determine the characteristics of the PDAC expressing sulfated sialyl-type 2 LacNAc(s) and to assess the value of the new biomarker for patient care.

# CHAPTER FOUR: PRELIMINARY RESULTS AND FUTURE WORK

## Summary

Pancreatic cancer remains the worst among epithelial cancers in 5-year survival rate. The identification and implementation of the CA19-9 biomarker as a blood test for pancreatic cancer occurred over three decades ago. Modern molecular technologies and methods have been incapable of improving or complementing CA19-9 to produce a better diagnostic marker. The goal of present study was to determine whether other glycans within the Lewis blood group family besides sLeA are aberrantly increased in the subpopulation of PDAC patients who do not secrete sLeA. We identified additional glycan markers, including sLeX variants and sialylated type 1 LacNAc, were upregulated in subgroups of PDAC patients who do not produce sLeA. Consequently, a biomarker panel consisting of these glycan markers plus CA19-9 served as a better diagnostic test with improved accuracy by 16-18% relative to the CA19-9 biomarker alone. In addition, I found that sulfated and sialylated type 2 LacNAc glycans were also biomarkers for pancreatic cancer by using a recently developed on-chip gmap method. These sulfated glycans complemented and improved CA19-9 test as a better diagnostic marker. Furthermore, they may be indicators of a subgroup of PDAC patients with rapid progression.

### *Current work: defining sulfated and fucosylated glycans in pancreatic cancer cell models*

I also found that 10% of the PDAC patients remained that could not be correctly diagnosed by best biomarker panel. I also demonstrated that fucosylated glycans built off the type 2 LacNAc core were upregulated in PDACs. The fucosylated glycan motifs are commonly associated with the ABO blood group and the cancer-associated Lewis

blood group as I previously demonstrated (chapters 2 and 3). However, there are other potential cancer-related sulfated and fucosylated glycans that were not fully identified in my current studies. I now have a completely developed platform capable of integrating new probes and enzymes to improve structural characterization of glycan motifs. Through a collaboration with the New England Biolabs, I obtained four fucosidases and one endoglycosidase with known specificity: α1-2 fucosidase from *Xanthomonas manibotis*, α1-2,3,4,6 fucosidase from bovine kidney, α1-2,4,6 fucosidase from *Omnithropica*, α1-3,4 fucosidase from *Prunus duicis*, and Endo F3 from *Elizabethkingia miricola*. In addition, I also incorporated in this study two fucosidases from *Bacteroide Stecoris* and *Bacteroide Fragilis* whose fine specificities remain to be defined. Using conditioned media from a panel of 12 pancreatic cancer cell lines I performed the on-chip gmap experiments with seven enzymes and 12 lectins in attempting to characterize the α1-2, α1-3, α1-4, and α1-6 fucosylation levels in pancreatic cancer cells. Currently, I am awaiting the glycan array data for these enzymes, which is been performed by Dr. Geert-Jan Boon's group in University of Georgia. Once I have the specificity information of these enzymes, I will be able analyze the raw on-chip gmap results and better characterize other novel sulfated and/or fucosylated glycans as potential markers for PDAC diagnosis and prognosis.

## Future Studies

### *Clinical application of sulfated glycans as prognostic indicator*

The glycoforms of MUC5AC detected with the Siglec-7 and Siglec-F showed potential as prognostic markers for indicating early cancer recurrence. To further build upon this finding, I will attempt to confirm the preliminary result with larger numbers of plasma

samples from PDAC patients with clinical information, including cancer-free survival and overall-survival. My research group has established collaborations with the University of Pittsburgh Medical Center, the Medical University of South Carolina and Mercy Health in Grand Rapids, MI. If the next studies are confirmatory, I will examine these markers in a blinded study to determine of the sulfated glycans present on MUC5AC are useful prognostic markers for indicating early pancreatic cancer recurrence. I also plan to examine this question using immunofluorescence assays in the tissue microarray (TMA) platform. There are commercially available pancreatic cancer TMAs with individual patient's survival and tumor progression information from resources such as US Biomax. These studies will aim to confirm specific sulfated glycan markers as reliable prognostic indicators, potentially leading to improved outcomes of PDAC patients and better immunotherapeutic targets.

***Subtyping pancreatic cancer cells with sulfated glycan markers***

If the sulfated and sialylated type 2 LacNAc present on MUC5AC is found to be associated with poorer prognostic subtype of pancreatic cancers, it will set a proper foundation for testing the hypothesis that sulfated and sialylated type 2 LacNAc(s) are markers for immunosuppressive subtype of pancreatic cancers. The literature suggestes that interactions between ligands (sulfated and sialylated type 2 LacNAc(s)) and receptors (Siglec-7 and Siglec-9) on NK cells and tumor-associated macrophages (TAMs) played multiple roles in tumor immunosurveillance mechanisms, which these findings that can serve as bases for this hypothesis [126, 127]. The hypothesis can be tested using immunofluorescence staining of cytotoxic subset of NK cells, TAMs and CD8+ T cells, together with the sulfated-glycan binders, Siglec-7 and Siglec-9, in

primary pancreatic tumor specimens. I initially speculate that PDAC tumors with positive staining in sulfated and sialylated type LacNAc glycans will have also have high amounts of TAM staining but low amounts of NK cells or CD8+ T cells staining. I will use a multimarker-immunofluorescence method to quantify the individual sulfated glycans and immune cell markers to shed light on the hypothesis that sulfated and sialylated type 2 LacNAc(s) are markers for immunosuppressive subtype of pancreatic cancer.

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