PANCREATIC CANCER MAKERS BASED ON ABERRANT GLYCOSYLATION OF SERUM PROTEINS

Meritxell Balmaña Esteban

DOCTORAL THESIS

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Doctoral Programme in Molecular Biology, Biomedicine and Health

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PhD thesis submitted in fulfilment of the requirements to obtain the doctoral degree from the Universitat de Girona

This PhD thesis contains 2 annexes

Certificate of thesis direction

Hereby, Dr. Rosa Peracaula and Dr. Sílvia Barrabés from the Universitat de Girona

CERTIFY:

That this doctoral thesis entitled "***Pancreatic cancer markers based on aberrant glycosylation of serum proteins***", that Meritxell Balmaña Esteban submitted to obtain the doctoral degree from Universitat de Girona has been completed under their supervision, and meets the requirements to opt for the *International Doctor* mention.

For all intents and purposes, the following certification is signed:

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Girona, 2016

“If we knew what it was we were doing, it would not be called research, would it?”

— Albert Einstein

Agraïments (Personal thanks)

I ha arribat aquell moment en què PER FI la tesi està llesta per imprimir i és quan m'adono que no he escrit els agraïments... M'agradaria no deixar-me a ningú, però ens fem grans i la memòria comença a fallar... (si us plau, no m'ho tingueu en compte), però, per si de cas: MOLTES GRÀCIES A TOTS!

Primer de tot, voldria començar donant les gràcies a la Rosa i en Rafa per haver-me donat l'oportunitat de formar part del grup Bioquímica del Càncer on he crescut personalment i professional. De manera especial vull agrair a les meves supervisores, la Rosa i la Sílvia, per tot el que m'han ensenyat. Especialment, moltes gràcies Sílvia pels bons consells i la paciència que has tingut: quan jo veia el got mig buit (i tu m'ajudaves a veure'l mig ple!). També, vull donar les gràcies a la "*minijefa*" Esther pel suport científic i moral, i a tots els membres del "105": Alba, Anna, Ariadna B, Ariadna S, Ari G, Clara, Cristina, Dani (el meu "*pupilo*!"), Dolors, Eva, Imma, Mª Ángeles, Maria José, Marina, Marta PG, Pedro i Sònia. I, especialment, moltíssimes gràcies amb qui he compartit aquesta aventura de principi a fi, Marta "petita" ("*pistaxín")* i Montse, sense vosaltres no hauria estat tan divertit!

També vull donar les gràcies a tota la gent que ha col·laborat i ha fet possible aquesta tesi. L'Estela, amb qui es podria dir que hem tingut una relació telefònica/mailing. Gràcies per haver tingut sempre temps per explicar-me això tan complicat que fas! Además, no quiero olvidarme de ¡mis amigos de la capital! ¡Muchas gracias Mercedes y Ángel por acogerme en vuestro laboratorio y por tratarme tan bien cuando estuve de visita! También a Agustín, Juan Carlos, Mónica, Noemí y Olga (¡qué risas y buenos ratos!). Moltes gràcies també a tota la gent de l'Hospital Universitari Dr. Josep Trueta per fer possible el treball amb mostres de pacients.

During my thesis I had the opportunity to stay in the Glycobiology in Cancer Group in Ipatimup, Porto. I want to express my gratitude to Dr. Celso for allowing me to join the group. Thank you Catarina for guiding me! All the members of the group (and others of the Institute) made my "Portuguese experience" unforgettable! Ana D, Ana M, Claudia, Daniela, Joana, Henrique, Salomé, Sandra, Sara, Stefan, Teresa, Tiago O, Tiago S, *muito obrigada por tudo!* And specially thanks, Márcia and Cíntia, for being my confidants! ;) And of course, my runner girls: Estefania and Veronica! Looking forward to start again!

Gràcies també a tots els becaris del Departament de Biologia pel bon ambient i per tants moments que hem compartit (dins i fora la uni): Àlex "Veí", Anna V, Ari V, David D, Elena, Gerard, Glòria, Imma, Jess, Laia, Luis ("Maño"), Mariona, Mireia F, Mireia L, Olga, Pau B, Pau C, Sandra F,

Roger V, Vero, Vicky. En especial, vull donar les gràcies a la Sara i en Santi, dos grans amics amb qui a més, he tingut el gran plaer de patir junts la tesi! Vam començar el màster i després la tesi junts, acabem junts i estic segura que seguirem junts! Molta sort! (espero que no defensem el mateix dia!)

Igual d'important ha sigut el suport que he rebut fora de l'entorn professional. Diuen que tot allò que té un inici també té un final; i això segur que us fa molt contents a uns quants! Finalment, s'haurà acabat escoltar-me parlar de la tesi!!!! Anna! No sé com agrair-te el teu suport incondicional! Tiraré de tòpic i diré que en aquest cas, les paraules sobren! En aquesta tesi sempre hi haurà una part de tu! (m'encanta la portada!) Gràcies al "*grupillo"* Mar, David (no més tesi en les excursions!, però alguna reparació electrònica segur que seguirà requerint del teu servei), Geni, Mariona, Rubén i Zaïra. Aquesta tesi també ha arribat al final gràcies al suport de tots vosaltres! Gràcies família i amics (*kartoffeln kollegen*, vilatanes, *primos*...) per estar en els bons i els mal moments! Albert, Fina, Xevi i Joan, vosaltres també n'heu format part.

I can't finish my thesis acknowledgements without a special mention to you, Stefan. Thank you for all your support. You have suffered the nervous and stress of the writing part… but you will also be with me to celebrate the end! :) Vielen Dank! I remember and keep my promise: Ich werde mein Deutsch nach der Promotion verbessern!!

De manera molt especial vull agrair a la meva mare per ajudar-me en tot, procurar sempre pel meu benestar i en definitiva, per ajudar-me a ser qui sóc, sense tu no ho hauria aconseguit!

Moltíssimes gràcies a tots!

This thesis was financially supported by the University of Girona (BR2012, pre-doctoral grant and MOB2014, short-term mobility grant, awarded to M. B.); the Government of Catalonia (grant 2014 SGR 229) and the Spanish Ministry of Science and Innovation (grant BIO 2010-16922, awarded to R.P.).

Publications derived from this PhD thesis

This doctoral thesis has been written as published peer reviewed articles compendium based on specific regulations of the PhD program of the University of Girona.

Peer reviewed paper scientific publications presented as **chapters 1** and **2** of this PhD thesis are listed below, whose impact factor and position within category are detailed according to the last update (2014) of the Journal Citation Report:

- **Balmaña M**, Giménez E, Puerta A, Llop E, Figueras J, Fort E, Sanz-Nebot V, de Bolós C, Rizzi A, Barrabés S, de Frutos M, Peracaula R.
  Increased α1-3 fucosylation of α-1-acid glycoprotein (AGP) in pancreatic cancer.
  J Proteomics. 2016 Jan 30;132:144-54. doi: 10.1016/j.jprot.2015.11.006.

  Impact factor of the *Journal of Proteomics* (ISSN 1874-3919) in 2014: 3.888; 5-year Journal Impact Factor: 4.029. Ranks 16/79 in the Biochemical research methods category (Q1).

- **Balmaña M**, Sarrats A, Llop E, Barrabés S, Saldova R, Ferri MJ, Figueras J, Fort E, de Llorens R, Rudd PM, Peracaula R.
  Identification of potential pancreatic cancer serum markers: Increased sialyl-Lewis X on ceruloplasmin.
  Clin Chim Acta. 2015 Mar 10;442:56-62; doi: 10.1016/j.cca.2015.01.007.

  Impact factor of the *Clinica Chimica Acta* journal (ISSN 0009-8981) in 2014: 2.824; 5-year Journal Impact Factor: 2.772. Ranks 5/30 in the Medical Laboratory Technology category (Q1).

The **chapter 3** is in preparation to be submitted in an international scientific journal:

- **Balmaña M**, Gomes C, López-Martos R, Barrabés S, Reis CA, Peracaula R.
  Identification of potential pancreatic cancer biomarkers based on aberrant mucin glycoforms by in situ proximity ligation assay.

Peer reviewed paper scientific publications presented as **annexes** of this PhD thesis are listed below, whose impact factor and position within category are detailed according to the last update (2014) of the Journal Citation Report:

- Giménez E, **Balmaña M**, Figueras J, Fort E, de Bolós C Sanz-Nebot V, Peracaula R, Rizzi A.
  Quantitative analysis of n-glycans from human alfa-acid-glycoprotein using stable isotope labeling and ZIC-HILIC-ESI-MS as tool for pancreatic disease diagnosis.
  Anal Chim Acta. 2015 Mar 25;866:59-68; doi: 10.1016/j.aca.2015.02.008.

  Impact factor of the *Analytica Chimica Acta* journal (ISSN 0003-2670) in 2014: 4.513; 5-year Journal Impact Factor: 4.667. Ranks 5/74 in the Analytical Chemistry category (Q1).

- Mereiter S, **Balmaña M**, Gomes J, Magalhaes A, Reis CA.
  Glycomic Approaches for the Discovery of Targets in Gastrointestinal Cancer.
  Front Oncol. 2016 Mar 9;6:55; doi: 10.3389/fonc.2016.00055.

  Impact factor of the *Frontiers in Oncology* journal (ISSN 2234-943X) in 2014: not available

Abbreviations

The following table contains the most commonly used abbreviations thorough this thesis.

AAL	*Aleuria aurantia* lectin	**HILIC**	Hydrophilic interaction liquid chromatography
AGP	Alpha-1 acid glycoprotein or orosomucoid	**HPAEC**	High-performance anion-exchange chromatography
APP	Acute phase protein	**HPLC**	High-performance liquid chromatography
CA19-9	Carbohydrate antigen 19-9	**IdoA**	L-iduronic acid
CE	Capillary electrophoresis	**IHC**	Immunohistochemistry
CEA	Carcinoembryonic antigen	**IPMN**	Intraductal papillary mucinous neoplasm
ChrP	Chronic pancreatitis	**IUPAC**	International Union of Pure and Applied Chemistry
CP	Ceruloplasmin	**LC**	Liquid chromatography
CZE-UV or CE	Capillary electrophoresis	**MALDI**	Matrix-assisted laser desorption ionization
EGFR	Epidermal growth factor receptor	**Man**	D-mannose
ELISA	Enzyme-Linked ImmunoSorbent Assay	**MCN**	Mucinous cystic neoplasm
ELLA	Enzyme-linked lectin assay	**MGAT5**	*N*-acetylglucosaminyl-transferase V
ER	Endoplasmic reticulum	**MRI**	Magnetic resonance imaging
ESI	Electrospray ionization	**MS**	Mass-spectrometry
FDA	Food and Drug Administration	**MUC1**	Mucin 1
Fuc	L-fucose	**MUC5AC**	Mucin 5AC
Gal	D-galactose	**Neu5Ac**	N-acetylneuraminic acid
GalNAc	N-acetyl-D-galactosamine	**Neu5Gc**	N-glycolylneuraminic acid
Glc	D-glucose	**NMR**	Nuclear magnetic resonance spectroscopy
GlcA	D-glucuronic acid	**NP-HPLC**	Normal phase high-performance liquid chromatography
GlcNAc	N-acetyl-D-glucosamine	**OST**	Oligosaccharyltransferase enzyme
GPI-anchor	Anchored glycosylphosphatidylinositol	**PanIN**	Pancreatic intraepithelial neoplasia
HC	Healthy control	**PDAC**	Pancreatic ductal adenocarcinoma
HCC	Hepatocellular carcinoma	**PhoSL**	*Pholiota squarrosa* lectin
HER2	Human epidermal growth factor receptor 2	**PLA**	Proximity ligation assay

PTM	Post-translational modifications	**STn**	sialyl-Tn
SAX	Strong anion exchange chromatography	**TACA**	Tumour-associated carbohydrate antigen
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis	**TF or T antigen**	Thomsen-Friedenreich
SELDI	Surface enhanced laser desorption/ionization	**TNM**	Tumour-node-metastasis system
shRNA	Short hairpin RNA	**TOF**	Time of flight
SLe^a	Sialyl Lewis A	**UPLC**	Ultra-performance liquid chromatography
SLe^x	Sialyl Lewis X	**VNTR**	Variable number of tandem repeats
SNA	*Sambucus nigra agglutinin*	**vWF**	Von Willebrand factor
SSEA-1	Stage-specific embryonic antigen-1	**Xyl**	D-xylose
SSEA-3	Stage-specific embryonic antigen-3	**ZIC-HILIC**	Zwitterionic hydrophilic interaction capillary liquid chromatography

List of figures

List of figures included in the Results section

CHAPTER 1

CHAPTER 2

CHAPTER 3

List of tables

List of tables included in the Results section

CHAPTER 1

CHAPTER 2

CHAPTER 3

Contents

Summary

Cancer is one of the leading causes of death worldwide. The survival rate of cancer patients increases when they are diagnosed in the early stages of the pathology; therefore, it is necessary to have specific and indicative markers of the early stages.

Typically, pancreatic ductal adenocarcinoma (PDAC) is characterized by high intrinsic aggressiveness and late diagnosis, causing poor prognosis and resulting in the lowest five-year survival rate among all cancers (around 7%).

Nowadays, the only biomarker for this cancer is CA19-9, but due to its inability to differentiate PDAC from other benign pancreatic disorders, such as chronic pancreatitis, its clinical use has only been approved for monitoring. For this reason, the research of new biomarkers is of great interest.

Tumour cells present aberrant glycosylation on their cell surface and also in the secreted glycoconjugates. It is known that these alterations correlate with the greater invasive and metastatic capacities of these cells. Hence, a strategy for new tumour biomarker discovery is based on the identification of specific glycoforms in secreted proteins by tumour cells in the bloodstream.

Based on previous findings of the group, this work has explored the glycosylation of two serum glycoproteins, the α-1-acid glycoprotein (AGP) and the ceruloplasmin (CP), both acute phase proteins.

AGP has been immunopurified from a cohort including healthy individuals, PDAC patients and chronic pancreatitis patients. Using the purified AGP, the distribution of the different AGP isoforms has been analysed using capillary electrophoresis and glycan composition by mass spectrometry. In addition, an assay with *Aleuria aurantia* lectin has been established to easily quantify AGP fucosylation. Patients with PDAC present an increase in AGP fucosylation, and this increase is even higher in advanced PDAC patients.

CP has also been identified as a putative candidate bearing differential expression of sialyl-Lewis X (SLe^x) after depleting the twelve most abundant serum proteins in healthy

individuals, PDAC patients and chronic pancreatitis patients. To verify this hypothesis, CP has been purified from the serum of a cohort including patients and healthy individuals. The resulting ratio SLe^x/CP has been determined to be higher in PDAC patients.

These results suggest that acute phase proteins secreted mainly by the liver are good carriers of pathological information in response to tumour presence. However, their suitability as PDAC biomarkers should be assessed in a larger cohort of patients also containing different neoplasias and other pancreas-related diseases to determine their role in the diagnosis, monitoring or detection of recurrences.

Serum levels of many acute phase proteins, such as AGP and CP, range from 0.1-2mg/mL hindering the detection of minor proteins that could be secreted by pancreatic tumour cells. Therefore, another approach has been developed to discover a more specific biomarker for PDAC diagnosis using biopsies of healthy tissues and PDAC tissues. The presence of the tumour-associated antigens SLe^x and sialyl-Tn (STn) on the epithelial mucins MUC1 and MUC5AC, two proteins that are over- and neo-expressed, respectively, during malignant transformation, have been analysed by *in situ* proximity ligation assay (PLA) in healthy and PDAC tissues. The results suggest that the appearance of SLe^x on these mucins could be a feature present in many PDAC tissues and, consequently, the study of these mucin glycoforms in serum, or other fluids, would improve the current PDAC diagnosis.

Resum

El càncer és una de les principals causes de mort en el món. La taxa de supervivència dels pacients d'aquesta malaltia augmenta quan són diagnosticats en els estadis inicials de la patologia, per tant, és necessari disposar de marcadors específics i indicatius d'aquests estadis primerencs.

Concretament, el càncer de pàncrees (PDAC) es caracteritza per una gran agressivitat intrínseca i un diagnòstic tardà causant un pronòstic desolador i sent el càncer amb la taxa relativa de supervivència als 5 anys més baixa (al voltant d'un 7%).

Actualment, l'únic marcador per a aquest càncer és el CA19-9, però el seu ús només ha estat aprovat per a la monitorització ja que no permet diferenciar el PDAC d'alteracions pancreatobiliars benignes, com la pancreatitis crònica. Per aquest motiu, es considera de vital importància la recerca de nous marcadors.

Les cèl·lules tumorals presenten una glicosilació alterada en la seva superfície cel·lular i en els glicoconjugats que secreten. S'ha descrit que aquests canvis correlacionen amb una major capacitat invasiva i metastàtica de les mateixes. Per tant, una estratègia per al descobriment de nous marcadors tumorals es basa en la identificació de glicoformes específiques en proteïnes secretades per les cèl·lules tumorals presents en el torrent sanguini.

Basant-nos en estudis previs del nostre grup, en aquest treball s'ha investigat la glicosilació de dos glicoproteïnes sèriques: la α-1 glicoproteïna àcida (AGP) i la ceruloplasmina (CP), ambdues proteïnes de fase aguda.

La glicoproteïna AGP s'ha immunopurificat del sèrum d'una cohort d'individus sans, pacients amb càncer de pàncrees i pacients amb pancreatitis crònica. A partir de l'AGP purificada s'ha analitzat com es distribueixen les diferents isoformes per electroforesi capil·lar, s'han analitzat els glicans per espectrometria de masses i s'ha establert un assaig amb la lectina *Aleuria aurantia* que permet una fàcil quantificació de la fucosilació de l'AGP. Hem pogut determinar que els pacients amb PDAC presenten una major

fucosilació en l'AGP, essent aquest increment major en els estadis més avançats de la patologia.

També s'ha identificat la CP com una candidata amb una expressió diferencial de l'epítop sialil-Lewis X (SLe^x) després d'eliminar les dotze proteïnes majoritàries del sèrum en individus sans, pacients amb càncer de pàncrees i pacients amb pancreatitis crònica. Per tal de verificar aquesta hipòtesi, s'ha purificat la CP del sèrum d'una cohort de pacients i controls sans i s'ha determinat que la ratio SLe^x/CP és més elevada en pacients amb PDAC.

Aquests resultats suggereixen que les glicoproteïnes sèriques secretades majoritàriament pel fetge són portadores d'informació com a resposta a la presència del tumor. No obstant, la seva utilitat com a marcadors per al PDAC s'hauria d'avaluar en una cohort major de pacients que alhora contingués diferents neoplàsies i altres patologies del pàncrees per tal de determinar el seu paper en el diagnòstic, monitorització o detecció de recurrències.

Els nivells sèrics de moltes proteïnes de fase aguda, com són l'AGP i la CP, es troben a 0.1-2mg/mL de manera que dificulten la detecció de proteïnes minoritàries que podrien ser secretades per les cèl·lules tumorals del pàncrees. Per tant, en una altra aproximació, s'ha desenvolupat una nova estratègia per al descobriment d'un marcador més específic per al diagnòstic del PDAC utilitzant biòpsies de teixit de PDAC i teixit pancreàtic sa. En aquests teixits de pàncrees sans i tumorals, s'ha analitzat mitjançant la tècnica de *in situ proximity ligation assay* (PLA), la presència dels antígens associats a tumor SLe^x i sialil-Tn (STn) en les mucines epitelials MUC1 i MUC5AC, que estan sobre- i neo-expressades respectivament en la transformació tumoral del pàncrees. Els resultats obtinguts suggereixen que l'aparició de l'epítop SLe^x en aquestes mucines seria una característica present en molts teixits de PDAC i conseqüentment, considerem que l'estudi d'aquestes glicoformes de les mucines en sèrum, o altres fluids, permetria millorar el diagnòstic actual del PDAC.

Resumen

El cáncer es una de las principales causas de muerte en el mundo. La tasa de supervivencia de los pacientes de esta enfermedad aumenta cuando son diagnosticados en los estadios iniciales de la patología, por lo tanto, es necesario disponer de marcadores específicos e indicativos de estos estadios tempranos.

Concretamente, el cáncer de páncreas (PDAC) se caracteriza por una gran agresividad intrínseca y un diagnóstico tardío causando un pronóstico desolador y siendo el cáncer con una tasa relativa de supervivencia a los 5 años más baja (alrededor de un 7%).

Actualmente, el único marcador para este cáncer es el CA19-9, pero su uso sólo ha sido aprobado para la monitorización ya que no permite diferenciar el PDAC de otras alteraciones pancreatobiliares benignas, como la pancreatitis crónica. Por este motivo, se considera de vital importancia la investigación de nuevos marcadores.

Las células tumorales presentan una glicosilación alterada en su superficie celular y en los glicoconjugados que secretan. Se ha descrito que estos cambios correlacionan con una mayor capacidad invasiva y metastásica de éstas. Por lo tanto, una estrategia para el descubrimiento de nuevos marcadores tumorales se basa en la identificación de glicoformas específicas en proteínas secretadas por las células tumorales presentes en el torrente sanguíneo.

Basándonos en estudios previos de nuestro grupo, en este trabajo se ha investigado la glicosilación de dos glicoproteínas séricas: la α-1 glicoproteína ácida (AGP) y la ceruloplasmina (CP), ambas proteínas de fase aguda.

La glicoproteína AGP se ha inmunopurificado del suero de una cohorte de individuos sanos, pacientes con cáncer de páncreas y pacientes con pancreatitis crónica. A partir de la AGP purificada se ha analizado como se distribuyen las diferentes isoformas por electroforesis capilar, se han analizado los glicanos por espectrometría de masas y se ha establecido un ensayo con la lectina *Aleuria aurantia* que permite una fácil cuantificación de la fucosilación de la AGP. Hemos podido determinar que los pacientes

con PDAC presentan una mayor fucosilación en la AGP, siendo este incremento mayor en los estadios más avanzados de la patología.

También se ha identificado la CP como una candidata con una expresión diferencial del epítopo sialil-Lewis X (SLe^x) después de eliminar las doce proteínas mayoritarias del suero en individuos sanos, pacientes con cáncer de páncreas y pacientes con pancreatitis crónica. Con el fin de verificar esta hipótesis, se ha purificado la CP del suero de una cohorte de pacientes y controles sanos y se ha determinado que la ratio SLe^x/CP es más elevada en pacientes con PDAC.

Por lo tanto, estos resultados sugieren que las glicoproteínas séricas secretadas mayoritariamente por el hígado son portadoras de información como respuesta a la presencia del tumor. No obstante, su utilidad como marcadores para el PDAC debería ser evaluada en una cohorte mayor de pacientes que a su vez contuviese diferentes neoplasias y otras patologías del páncreas para determinar su papel en el diagnóstico, monitorización o detección de recurrencias.

Los niveles séricos de muchas proteínas de fase aguda, como son la AGP y la CP, se encuentran a 0.1-2mg/mL de manera que dificultan la detección de proteínas minoritarias que podrían ser secretadas por las células tumorales del páncreas. Por lo tanto, en otra aproximación, se ha desarrollado una nueva estrategia para el descubrimiento de un marcador más específico para el diagnóstico del PDAC usando biopsias de tejido de PDAC y tejido pancreático sano. En estos tejidos de páncreas sanos y tumorales se ha analizado mediante la técnica de *in situ proximity ligation assay* (PLA), la presencia de los antígenos asociados a tumor SLe^x y sialil-Tn (STn) en las mucinas epiteliales MUC1 y MUC5AC, que están sobre- y neo-expresadas respectivamente en la transformación tumoral del páncreas. Los resultados obtenidos sugieren que la aparición del epítopo SLe^x en estas mucinas sería una característica presente en muchos tejidos de PDAC y consecuentemente, consideramos que el estudio de estas glicoformas de las mucinas en el suero, u otros fluidos, permitiría mejorar el diagnóstico actual del PDAC.

Scientific background

1. Cancer

Cancer is a group of diseases characterized by uncontrolled cell growth with the potential to invade or spread to other parts of the body. The process by which a cell ultimately becomes malignant is called carcinogenesis, a multistep process resulting from the accumulation of errors in vital regulatory circuits that govern normal cell proliferation and homeostasis (Figure 1). At first, cancer cells with an altered phenotype form the primary tumour, a localized accumulation of cells that are considered benign and resectable. Secondary tumours appear when cells acquire the ability to invade the surrounding tissues through blood or lymphatic vessels and produce metastasis [1, 2].

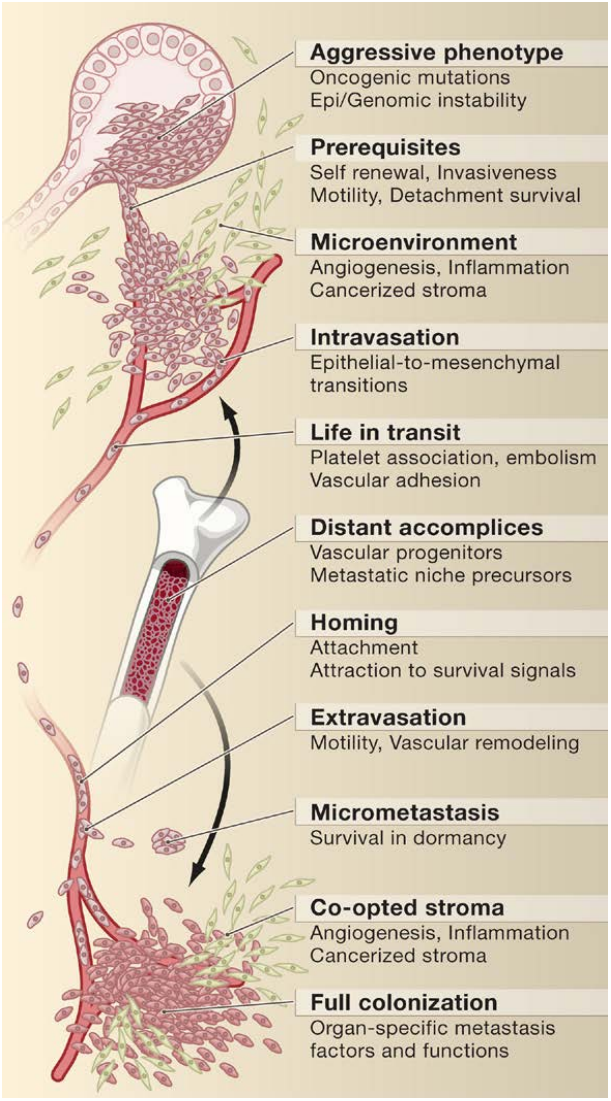


Figure 1: Stages of tumour progression. Metastasis occurs through the gradual acquisition of traits that allow malignant cells to spread from one organ to a secondary site. Although these features are presented as part of a continuous biological sequence, their acquisition during metastatic progression does not necessarily follow this order. (Extracted from Gupta and Massagué, 2006 [2]).

Nowadays, cancer is one of the leading causes of death worldwide. The World Health Organization states that approximately 14 million new cases appeared and 8.2 million people died from cancer in 2012 [3]. It is expected that annual cancer cases will rise to 22 million within the next two decades [3]. These data reflect the importance of cancer in public health and the need to develop efficient methods and therapies for diagnosis and treatment.

There are more than 100 distinct types and subtypes of human cancers depending on the affected organ or tissue. However, Hanahan and Weinberg have suggested ten essential alterations or skills that allow cells to become malignant (Figure 2). These abilities include genomic instability, the promotion of inflammatory microenvironments, and the reprogramming of cellular metabolism that enables cancer cells to sustain chronic proliferation and avoid recognition by the immune system. Studies are currently underway to understand these processes and order to design therapies to prevent or stop tumorigenesis [4].

Figure 2: Hallmarks of cancer. The fundamental characteristics that a cell acquires to become malignant are: self-sufficiency in growth signals, insensitivity to factors that inhibit growth, apoptosis evasion, limitless replicative potential, ability to promote angiogenesis, invasion of other tissues (extravasation and intravasation, a phenomenon known as metastasis), immune system evasion, promoting inflammation to create an inflammatory microenvironment that stimulates the survival of tumour cells, acquisition of genomic instability that favours the production of mutations, and finally, dysregulation of cellular energy metabolism. (Extracted and modified from Hanahan and Weinberg, 2011 [4]).

1.1. Diagnosis and treatments of cancer

The earlier cancer is diagnosed and treated, the better the chance for the patient to be cured. In general terms, cancer is diagnosed based on a combination of a person's symptoms, the results of physical and imaging examinations, abnormalities found in screening tests of blood, urine, and/or stool, and biopsies whenever necessary. Tumour location and staging are determined by imaging procedures such as X-rays, computed tomography (CT), magnetic resonance imaging (MRI), ultrasound, fibre-optic endoscopy examinations and tissue or cytological examinations. The tumour stage describes how advanced the cancer has become, including criteria such as the size and whether it has spread to neighbouring tissues or more distantly to lymph nodes or other organs. Also,

after corroborating the presence of the tumour cells, other tests can provide specific information about the cancer [5, 6].

Cancer treatment aims to eradicate all primary tumour cells from the body and thereby permanently cure the patient. When a cure is not possible, treatment is also used to relieve the disease symptoms and to preserve the patient's quality of life. Currently, cancer treatment is based mainly on different approaches that can be used alone or in combination to establish the best strategy for each patient: cancer surgery, oncologic radiation, antineoplastic pharmacologic therapy and palliative care [7].

1.2. Pancreatic cancer

The pancreas is a gland of the digestive system located in the abdominal cavity, behind the stomach. It is a retroperitoneal organ that has both endocrine functions, such as insulin production, and exocrine functions, such as digestive enzyme secretion to facilitate digestion in the small intestine.

Pancreatic ductal adenocarcinoma (PDAC) is the most common neoplasia of the exocrine pancreas [8]. As one of the most aggressive human malignancies, it presents an exceptionally rapid progression [9-11]. It is usually diagnosed late when metastases have already occurred [12], since patients with PDAC are often asymptomatic [13]. As a result, PDAC patients have an extremely poor prognosis and the five-year survival rate is only 7% [14]. Although PDAC is the fourth leading cause of cancer death in Europe and the United States, it is not found among the most common cancers (new cases of PDAC represent only 3% of all cancers diagnosed)(Figure 3) [14, 15]. PDAC has been projected to become the second leading cause of cancer-related deaths by 2030 [16]. Besides, PDAC is the only neoplasm with increasing incidence and mortality in both genders in Europe [17].

Risk factors for this malignant disease include smoking, family history of chronic pancreatitis, advancing age, male sex, diabetes mellitus, obesity, non-O blood group and occupational exposures [5]. According to the American Cancer Society, 10% of PDAC may be caused by an inherited DNA alteration, meaning a genetic predisposition to cancer due to family lineage.

Figure 3: Estimated new cancer cases and deaths worldwide by gender and level of economic development. (Excluding non-melanoma skin cancers). (Extracted and modified from Torre *et al*., 2015 [15]).

Genetic, clinical and pathological studies have identified different PDAC precursor lesions that affect the pancreatic duct [18, 19]. These different lesions are the intraductal papillary mucinous neoplasm (IPMN), the mucinous cystic neoplasm (MCN) and the pancreatic intraepithelial neoplasia (PanIN), which is the most common PDAC precursor lesion (Figure 4) [5, 20].

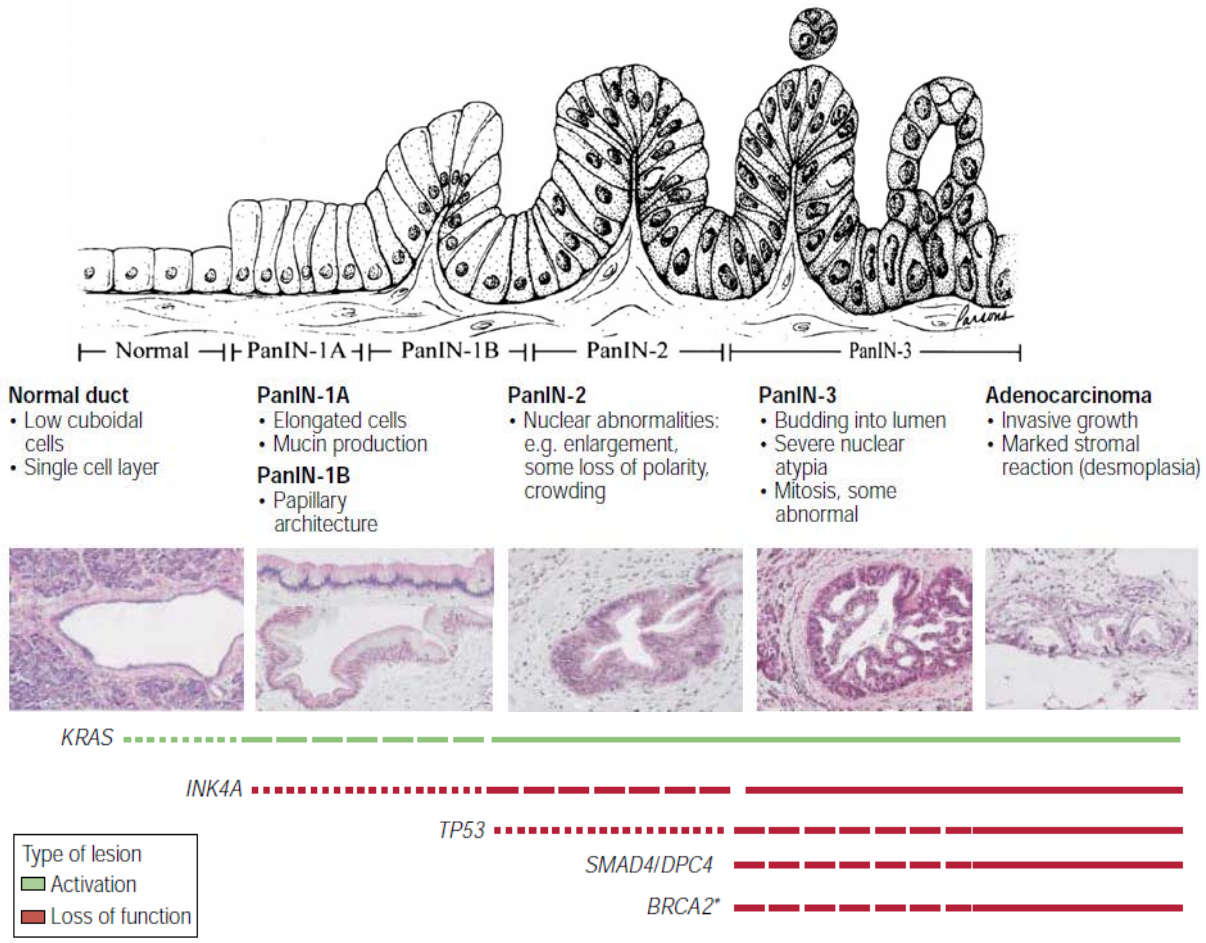


Figure 4: Progression of pancreatic cancer. Normal duct pancreatic epithelium progresses to infiltrating cancer (left to right) through a series of histologically defined precursor lesions (PanINs). (Extracted and modified from Hruban *et al*., 2000 [18], and Bardeesy and DePinho, 2002 [10]).

Some of the major molecular alterations involved in PDAC carcinogenesis are known (Figure 4). They include activation of *KRAS*, *HER-2/neu*, *BRCA2, AKT2*, and *BRAF* oncogenes and inactivation of *p16/CDKN2A*, *TP53*, and *SMAD4/DPC4* tumour suppressor genes [21-23]. *KRAS* mutation (75-100% of PDACs [24]) and *p16/CDKN2A* inactivation (80-95% of PDACs [25]) are early events in PanIN; IPMN and MCN and are related to the grade of dysplasia. *TP53* mutation (50-75% of PDACs [24]) and *SMAD4/DPC4* inactivation (55% of PDACs [26]) are late events that can be detected in PanIN-3 and

IPMN, but are mainly evident in carcinomas. *HER-2/neu* is overexpressed in approximately 70% of infiltrating ductal carcinomas of the pancreas [27]. *BRCA2* is only inactivated in 7-10% of PDACs and is therefore considered to be a rare event in pancreatic carcinogenesis [28]. Likewise, the mutations in *BRAF* [29] and *AKT2* [30, 31] genes occur in 5% and 10-20% of PDAC tumours respectively. Recently, an integrated genomic analysis of 456 PDACs identified 32 recurrently mutated genes that aggregate into 10 pathways: KRAS, TGF-β, WNT, NOTCH, ROBO/SLIT signalling, G1/S transition, SWI-SNF, chromatin modification, DNA repair and RNA processing [32].

PDAC has an intense desmoplastic stroma, which comprises an extracellular matrix, together with a number of different host cell types, including fibroblasts, pancreatic stellate cells, small endothelial-lined vessels, residual normal epithelia and a variety of inflammatory cells [19, 33, 34]. These stromal components have been described as constituting a microenvironment that is actively involved in pancreatic tumorigenesis, promoting tumour growth and invasion [35-37].

1.2.1 Diagnosis and treatment of pancreatic cancer

PDAC diagnosis is based on a combination of the following steps: (i) the patient experiences weeks or months of symptoms such as abdominal pain, weight loss, itching or jaundice; (ii) during a physical exam, the doctor might feel a mass in the abdomen and notice swollen lymph nodes in the neck; (iii) some laboratory tests may show abnormalities in the function of the liver and other organs that can be affected by the presence of a tumour in the pancreas (for instance, the bilirubin blood test may show that the bile flow is being blocked or the presence of tumour markers such as CA19-9); (iv) several imaging assessments allow strong suspicion of PDAC when a pancreatic mass is invading surrounding organs; and (v) a biopsy can then confirm PDAC diagnosis, and also rule out benign disorders such as autoimmune pancreatitis [5, 6].

PDAC patients are classified into different groups: resectable, borderline resectable, locally advanced and metastatic disease. Unfortunately, the vast majority of PDAC patients (80-85%) are diagnosed with advanced unresectable disease [5]. The most common system currently used to specify the PDAC staging is the TNM system, which classifies pancreatic cancer according to its anatomic extent (Table 1) [38].

Table 1: Clinical classification of pancreatic cancer staging: Tumour-node-metastasis system (TNM). LN, lymph node; CA, celiac axis; SMA, superior mesenteric artery; CHA, hepatic artery; PV, portal vein; SMV, superior mesenteric vein. (Extracted and modified from Katz *et al.,* 2008 [38], and Vincent *et al.,* 2011 [5]).

Definitions		Description
Primary tumour (T)		
Tx	Cannot be assessed	
T0	No evidence of primary	
Tis	Carcinoma i*n situ*	Includes the PanIN3
T1	Limited to pancreas, ≤ 2cm	
T2	Limited to pancreas, ≤ 2cm	
T3	Extends beyond pancreas, no involvement of CA or SMA	Emphasis on anatomic factors that determine resectability; extrapancreatic extension to CA or SMA is T4; all other extrapancreatic extension is T3
T4	Involves CA or SMA	
Regional lymph nodes (N)		
Nx	Cannot be assessed	
N0	No nodal metastasis	
N1	Regional lymph node metastasis	No distinction between the number of involved nodes
Distant metastasis (M)		
M0	No distant metastasis	
M1	Distant metastasis	

Stage groupings

Stage 0	Tis N0 M0	*In situ* disease	
Stage I	IA - T1 N0 M0	Potentially resectable disease that is confined to the pancreas	***Local or resectable***
	IB - T2 N0M0		about 10%, median survival
Stage II	IIA - T3 N0 M0	Usually potentially resectable; may involve venous structures, adjacent organs, N, or CHA, but not CA or SMA	17-23 months
	IIB - T1-3 N1 M0		
Stage III	T4 N0-1 M0	Locally advanced; unresectable due to CA or SMA involvement	***Borderline resectable*** 10%, median survival up to 20 months ***Locally advanced or unresectable*** ~30%, median survival 8-14 months
Stage IV	T1-4 N0-1 M1	Metastatic; unresectable due to distant metastatic disease	***Metastatic*** ~60%, median survival 4-6 months

Pancreatic cancer, like other cancers such as breast and lung cancer, results from the accumulation of gene mutations, as previously described. Thus, the study of oncogene activation (KRAS) and tumour suppressor gene silencing (P16INK4, CDKN2A) is also useful in the diagnosis and classification of PDAC [39-42].

Finally, it must be pointed out that not a single tumour marker has been approved for PDAC diagnosis. However, CA19-9 (carbohydrate antigen 19-9) and CEA (carcinoembryonic antigen) are used in clinical settings to distinguish PDAC from other cancers or to monitor for cancer recurrence. Ongoing research to identify novel tumour markers that are specific and sensitive enough to diagnose PDAC will be described in section 3 of this introduction.

PDAC is a heterogeneous disease at the molecular, pathological and clinical levels. The patient's response to treatment and the outcome depend on many factors, including the biology of the cancer and the patient's performance status and pattern of disease progression [5, 7]. So far, the only treatment that cures the disease is surgery; however, only 15–20% of PDAC cases can be expected to be resectable. Despite advances in surgical approaches, post-operative survival rates have not improved significantly over the past decades. Recent studies have suggested that surgical resection followed by adjuvant therapy with gemcitabine increases survival rates [43].

Due to the frequently late diagnosis of PDAC (approximately 40% of patients show distant metastases and between 30–40% have locally advanced neoplasms at the time of diagnosis), surgery is not always feasible. Under these circumstances, chemotherapy and radiotherapy are the most suitable options [42]. Even when surgery is performed, chemotherapy is applied to eradicate remnant tumour cells.

Among all chemotherapeutic agents, gemcitabine is the only one approved by the Food and Drug Administration (FDA) for locally advanced or metastatic PDAC. Some clinical benefits of chemotherapy are the reduction of the primary tumour size, the relief of symptoms, the prolongation of survival time and the improvement of patients' quality of life [42]. Current studies in chemotherapy are based on a combination of different chemical agents to determine the best response. Nowadays, there are clinical trials combining gemcitabine with other chemicals such as capecitabine or nab-paclitaxel [44, 45].

Radiotherapy efforts are aimed at treating the pathological area in a minimally invasive way. Two methods currently used are three-dimensional conformal radiation therapy (3D-CRT) and SGS-I Super Gamma Knife radiotherapy [42].

Greater knowledge of PDAC molecular alterations has led to the development of targeting therapies [46, 47]. For example, nimotuzumab a humanized monoclonal antibody that binds to the EGFR, and erlotinib, an inhibitor of the tyrosine kinase activity of EGFR, have shown improved efficacy when administered together with gemcitabine [48, 49]. Moreover, attaching the therapeutic agents to monoclonal antibodies can be used to enhance the efficacy of radiotherapy or chemotherapy. Evidence of this is trastuzumab-DM1, which is already approved for metastatic breast cancer, and is formed by coupling the cytotoxic agent DM1 (emtansine) to the monoclonal antibody trastuzumab (against the human epidermal growth factor receptor 2, HER2) [50, 51]. Another targeted strategy is the use of short hairpin RNAs (shRNAs) that can selectively silence the expression of certain differently expressed genes in PDAC such as the ATP-binding cassette sub-family C member 4 (ABCC4) [52]. Similarly, the design of specific inhibitors like erlotinib, which inhibits the autophosphorylation of EGFR and avoids downstream signalling cascades, could improve the therapeutic outcome [42].

2. Glycobiology

2.1 General overview

The human genome contains approximately 20,000 protein coding genes [53]. This relatively small number suggests that the complexity and diversity characteristic of different cell types may be found in post-transcriptional events [54]. Thus, different protein profiles of tissues or cells are the result of not only an altered gene expression, but also different post-translational modifications (PTMs). The PTMs play an indispensable role in many biological systems and, among those taking place in eukaryotes, glycosylation is considered to be the most complex. Nowadays, genes that regulate glycosylation in humans are estimated to represent at least 2% of the total genome [54, 55].

Glycobiology is the study of the structure, biosynthesis, biology and evolution of saccharides (carbohydrate or oligosaccharide chains, or glycans) that are widely distributed in nature, and the proteins that recognize them [56]. Glycans are mostly found on the cell surface and extracellular matrix, but also in organelles such as the Golgi, the endoplasmic reticulum (ER), lysosome, cytosol and the nucleus. In mammals, the major glycans comprise ten monosaccharide building blocks (Figure 5), all derived from glucose, which can be classified in [57, 58]:

- Pentoses: five-carbon neutral sugars, e.g., D-xylose (Xyl).
- Hexoses: six-carbon neutral sugars, including D-glucose (Glc), D-galactose (Gal) and D-mannose (Man).
- Hexosamines: hexose with an amino group at the C2-position that can be either free or, more commonly, N-acetylated: N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-D-galactosamine (GalNAc).
- Deoxyhexoses: six-carbon neutral sugars without the hydroxyl group at the C6-position: L-fucose (Fuc).
- Hexuronic acids: six-carbon negatively charged sugars with a carboxyl group at the C6-position: D-glucuronic acid (GlcA), L-iduronic acid (IdoA).
- Sialic acids: family of nine-carbon acidic sugars. The most common is N-acetylneuraminic acid (Neu5Ac). The 5-N-acetyl group can also be hydroxylated, giving N-glycolylneuraminic acid (Neu5Gc).

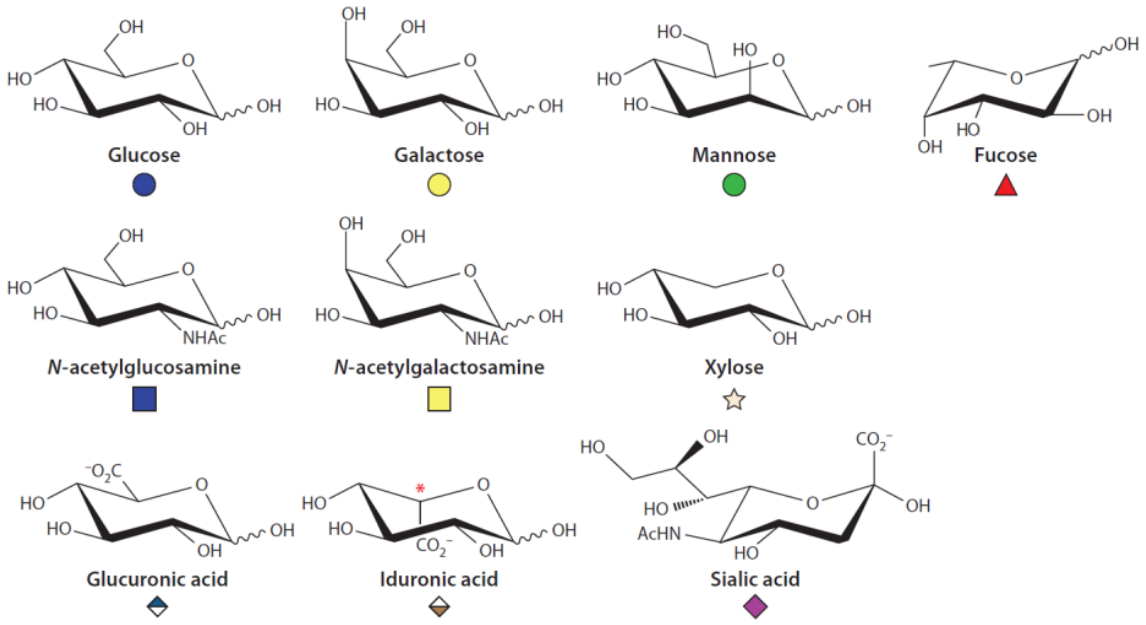


Figure 5: Chemical structure of human monosaccharides and the corresponding depiction in Consortium for Functional Glycomics notation. (Extracted from Rakus and Mahal, 2011 [58]).

The glycosidic linkage is fundamental among carbohydrates to form oligosaccharide chains, and also to link carbohydrates with other molecules, such as proteins or lipids, to form glycoconjugates.

Glycosylation is the addition of glycans to proteins or lipids (or other organic molecules). It is a complex form of modification that provides functional diversity and allows many phenotypes to be generated from a limited genotype. The enzymes necessary for this process are transmembrane proteins located along the secretory ER and Golgi apparatus pathways, and their location corresponds to the sequence in which they act. The glycosyltransferases build the oligosaccharide structure, whereas the glycosidases trim it [54].

2.2 Glycoconjugates

Glycoconjugate is the general term used to classify a group of structures that are formed by carbohydrates covalently linked to an aglycon (non-glycan part). The main glycoconjugates found in animals are glycoproteins and proteoglycans, where the aglycon is a protein/peptide molecule, and glycosphingolipids and glycosylphosphatidylinositol (GPI) anchors, which are attached to lipids (Figure 6) [56, 59].

Regarding their biological functions, numerous natural bioactive molecules are glycoconjugates, and the attached glycans can have dramatic effects on their biosynthesis, function, stability and turnover in intact organisms. In some cases, the pattern of glycosylation may be of functional significance to the destination of the glycoprotein, such as the transport of the incorrectly folded proteins to proteasomes in order to be degraded [56].

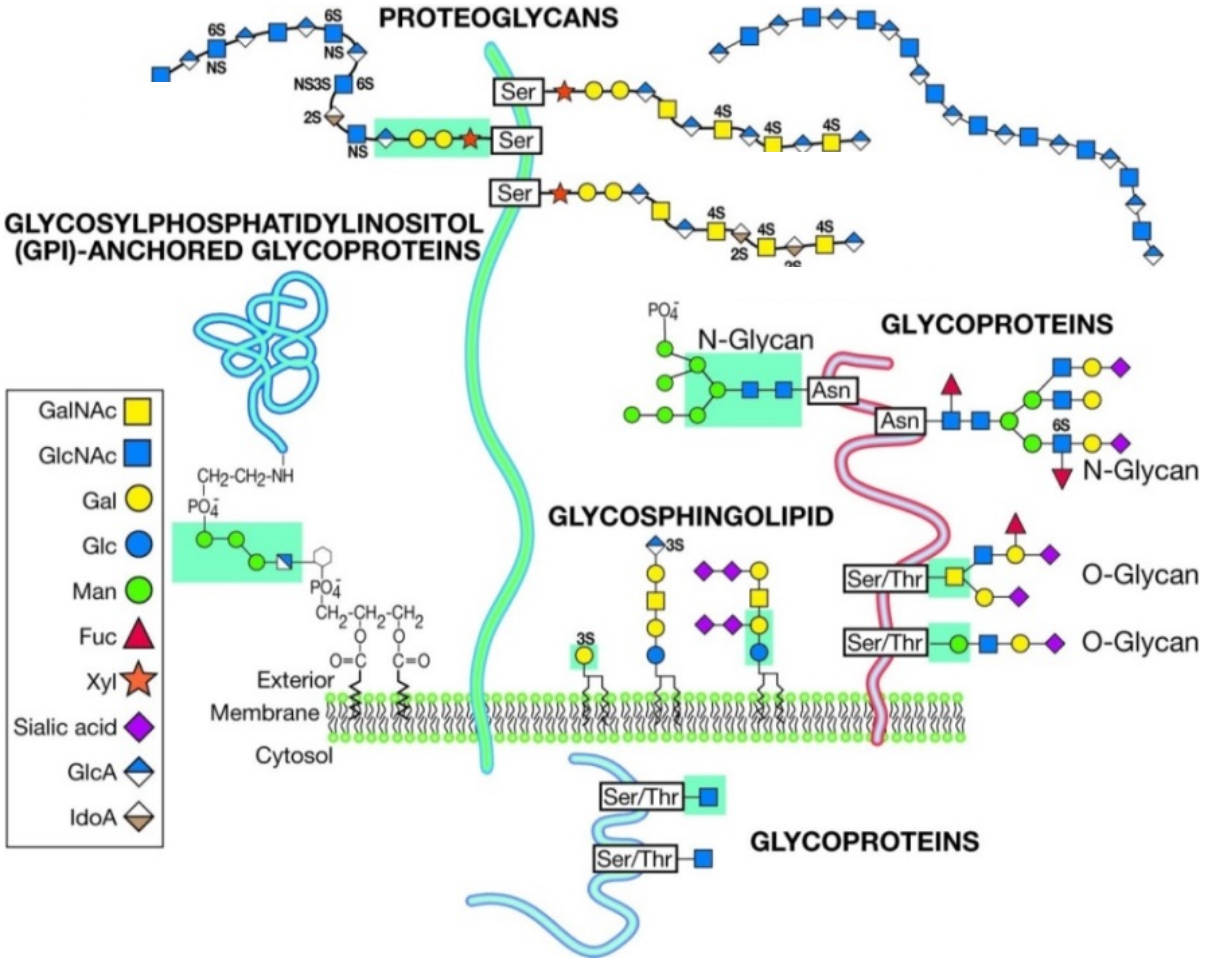


Figure 6: Common glycoconjugates in animals. Glycoproteins, whose glycans can be *N*- or *O*-linked to the protein backbone, glycosphingolipid, glycosylphosphatidylinositol. (GPI)-anchored glycoproteins and proteoglycans. (Extracted and modified from Varki and Sharon, 2009 [56]).

2.3 Types of protein glycosylation

Glycosylation is one of the most frequently occurring co- or post-translational modifications on proteins following the secretion pathway of the cell. (It is estimated that more than 50% of all proteins are glycosylated) [60]. Glycosylation affects many physicochemical properties of glycoproteins, such as conformation, flexibility, charge, and hydrophobicity. Thus, oligosaccharide modification also affects biological processes, including receptor activation, signal transduction, endocytosis and cell adhesion, and leads to the regulation of many physiological and pathological events, including cell growth, migration, differentiation, tumour metastasis and host-pathogen interactions [61].

Unlike proteins, which are generally encoded by a single gene, a glycan determinant is produced by the concerted action of several related genes, making it difficult to elucidate the genetic regulatory mechanism for the expression of some cell-surface glycans. Glycosylation is a modification that does not occur at every potential glycosylation site on a given protein or even among different molecules of the same protein. This variation in glycan site occupancy is referred to as macroheterogeneity, and is a function of site availability, enzyme kinetics and substrate concentrations in the ER. On the other hand, microheterogeneity indicates that at any given glycosylation site on a specific protein synthesised by a particular cell type, a range of variations in the precise structure of the glycan can be found [56]. Even the extent of this heterogeneity can vary considerably from glycosylation site to glycosylation site, from protein to protein, and from cell type to cell type. Glycoproteins with a common polypeptide chain but bearing different glycans are called glycoforms [62]. The occurrence of microheterogeneity is associated with the processing reactions that take place primarily in the Golgi apparatus.

Regarding proteins, glycans can be attached to an asparagine (*N*-linked glycans), to a serine or threonine (*O*-linked glycans) (Figure 6) or, in rare instances, to a tryptophan residue (*C*-linked glycans). *C*-linked glycosylation is poorly understood and is not further described in this thesis.

2.3.1 N-glycosylation

In *N*-glycosylation, the glycan chain is *N*-linked to the amide group of the Asn via a GlcNAc molecule with a β-N-glycosidic bond. The polypeptide consensus sequence for *N*-glycosylation is Asn-X-Ser/Thr, in which X may be any amino acid other than Pro [63]. However, recent work has demonstrated other possible consensus sequences [64].

N-linked glycosylation of proteins is a co-translational event initiated during protein synthesis into the ER and is further processed in the ER and in the Golgi apparatus where the enzymes are located (Figure 7).

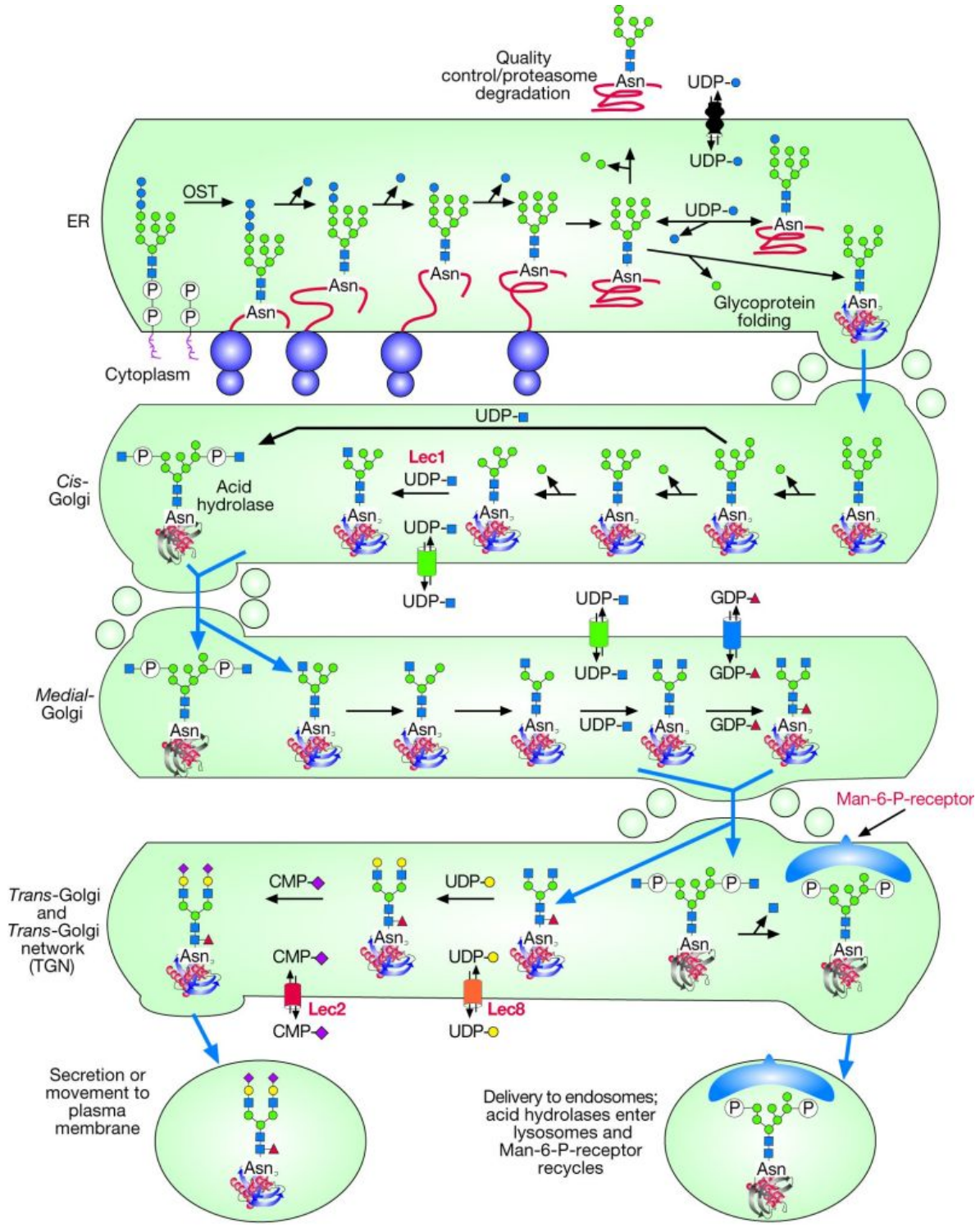


Figure 7: Processing and maturation of *N*-glycans. The mature Dol-P-P-glycan Glc3Man9GlcNAc2 is transferred to Asn-X-Ser/Thr sequence during protein synthesis as proteins are being translocated into the ER. Then, ER glucosidases and ER mannosidase remove the three glucose residues and a mannose residue, respectively. For most glycoproteins, additional mannose residues are removed in the cis compartment of the Golgi until Man5GlcNAc2 is generated. The action of GlcNAcT-1 on Man5GlcNAc2 in the medial-Golgi initiates the first branch of an *N*-glycan. The resulting bi-antennary *N*-glycan is extended by the addition of fucose, galactose, and sialic acid to generate a complex *N*-glycan with two branches. Complex *N*-glycans can have many more sugars than shown in this figure, including additional residues attached to the core, additional branches, branches extended with poly-N-acetyllactosamine units, and different "capping" structures. (Extracted from Stanley *et al.*, 2008 [65]).

The nascent polypeptide chain emerging from the ribosome contains an endoplasmic reticulum signal sequence and is guided to the membrane of the rough ER where the signal sequence is recognised by a receptor. Protein synthesis continues with the nascent polypeptide chain being fed through a translocation channel in the rough ER membrane into the lumen of the organelle. The addition and processing of *N*-linked oligosaccharides begins almost immediately on the nascent polypeptide chain with the transfer of a 14 residue oligosaccharide structure (Glc3Man9GlcNAc2) from a dolichol on the consensus Asn, by the oligosaccharyltransferase enzyme (OST). Afterwards, different enzymes (glycosyltransferases and glycosidases) in the ER and Golgi apparatus generate the large glycan diversity, including core structures (high-mannose, hybrid and complex), different branching and capping, and it is completed by the time the glycoprotein leaves the trans-Golgi network [65]. It is important to highlight that several check points exist, given that the correct glycosylation is relevant for the correct protein folding [66, 67].

Glycosylation is cell-, tissue- and site-specific and environmentally sensitive. Only ~30% of putative N-linked glycosylation sites are glycosylated and are often a heterogeneous mixture of the three types of *N*-linked oligosaccharides: high mannose, complex and hybrid (Figure 8). Within each of the classes, there is considerable micro-heterogeneity in glycan composition [68].

N-linked oligosaccharides have been shown to have roles in: (i) recognition events such as protein folding, enzyme function, serum clearance of proteins, protein signalling and cell adhesion; and (ii) physical functions such as protease protection/stability, prevention of non-specific interaction and orientation of cell-surface molecules. The functions of *N*-linked oligosaccharides on serum proteins include correct folding and conformation of the protein, maintaining protein stability and metabolic turnover [65, 67].

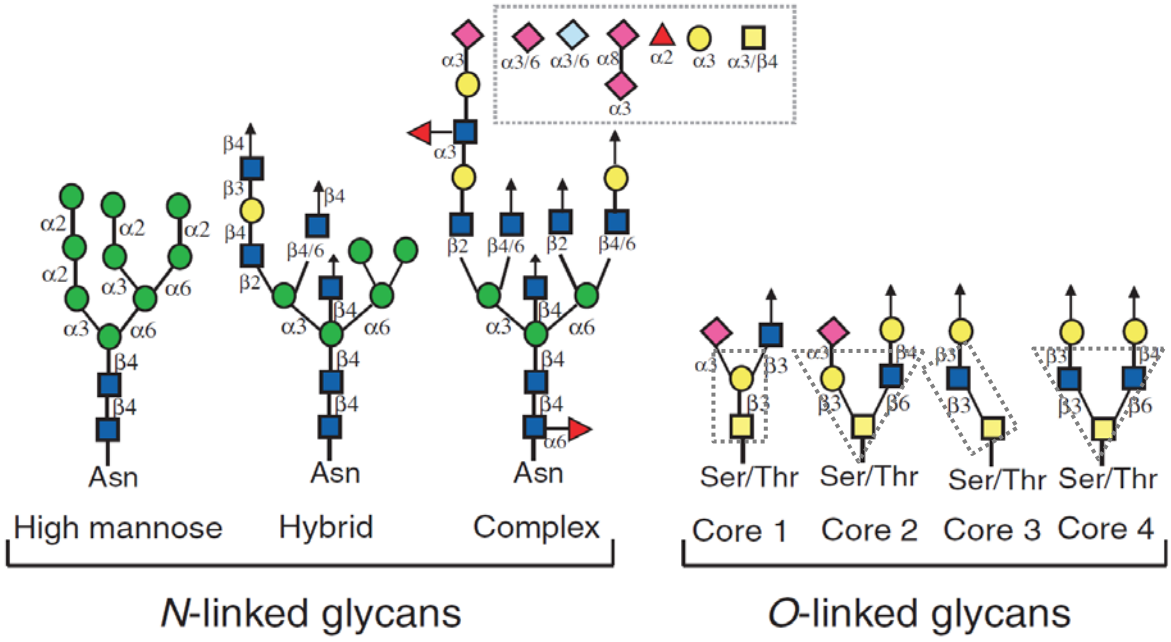


Figure 8: Main *N*-glycan and *O*-glycan structures. Directionality is from non-reducing end at the top to the reducing end at the bottom with the arrows indicating the extension at the non-reducing end. Linkages between monosaccharides contain the anomeric configuration of the monosaccharide (α or β) and the carbon number in the reducing end monosaccharide containing the hydroxyl group to which it is linked to. "/" is used to represent either-or case (β3/4 means β3 or β4). In the case of complex *N*-linked glycans, the common terminal motifs attached to Gal are shown in a dotted box. For *O*-glycans, the most common core structures are framed in dotted boxes. (Extracted and modified from Raman *et al.,* 2005 [68]).

2.3.2 O-Glycosylation

An oligosaccharide is *O*-linked to a protein when it is linked to a hydroxyl group of an amino acid residue, usually serine or threonine, on the polypeptide chain of a protein. One of the most common *O*-glycosylation types in animals is the linkage via a GalNAc molecule. It can be also referred to as mucin-type O-glycosylation due to the abundant *O*-GalNAc glycosylation of mucins. Nevertheless, there are several types of *O*-glycans, including α-linked *O*-fucose, β-linked *O*-xylose, α-linked *O*-mannose, β-linked *O*-GlcNAc (N-acetylglucosamine), α- or β-linked *O*-galactose and α- or β-linked *O*-glucose glycans [57, 69]. In this thesis, however, the term *O*-glycan will refer to mucin-type *O*-GalNAc glycans, unless otherwise specified.

Mucin type *O*-linked glycosylation is not solely found on mucins, but also on a variety of glycoproteins and proteoglycans. It is a post-translational event, which unlike *N*-linked glycosylation, begins after completion of the protein synthesis. *O*-glycosylation is initiated by ppGalNAc-transferases in the cis-Golgi by the addition of a single GalNAc

residue, and further elongation is also executed by the stepwise addition of one monosaccharide residue at a time along the Golgi apparatus [70, 71].

The completed O-glycans are mainly linear or bi-antennary, and may be as simple as a single monosaccharide (forming Tn antigen) or longer oligosaccharides based on core structures giving rise to poly-N-acetyllactosamine sequences ($[Gal\beta1\text{-}4GlcNAc]_n$) terminated by sialic acid or fucose. These reactions depend on the activity of specific glycosyltransferases and the availability of precursor substances. Although hundreds of different *O*-glycan chains of varying degrees of complexity are possible, they can be classified according to their core structures (Figure 8). Most *O*-glycan structures found in glycoproteins are based on the core 1 and core 2 structures [69].

2.4. Altered glycosylation in cancer

Alterations in glycosylation are associated with different diseases [72]. These alterations can have a genetic origin, known as congenital disorders of glycosylation (CDGs), or can involve chemical reactions of sugars and immunological responses to them. In addition, diseases in which glycosylation changes are correlated with the disease phenotype. In this latter group, cancer is the most studied case, as alterations in glycosylation are a feature of malignant transformation and tumour progression [57, 73, 74]. Glycan analysis of cancer is of interest for three main reasons: (i) the changes in glycosylation may explain some of the phenotype changes in tumour cells; (ii) these changes can be exploited to design new treatments for cancer; and (iii) specific changes in glycosylation could be used as diagnostic markers [57], which is the objective of this thesis.

Glycan alterations in cancer can be summarised as loss or excessive expression of certain structures, the accumulation of precursors and the appearance of novel structures (Figure 9).

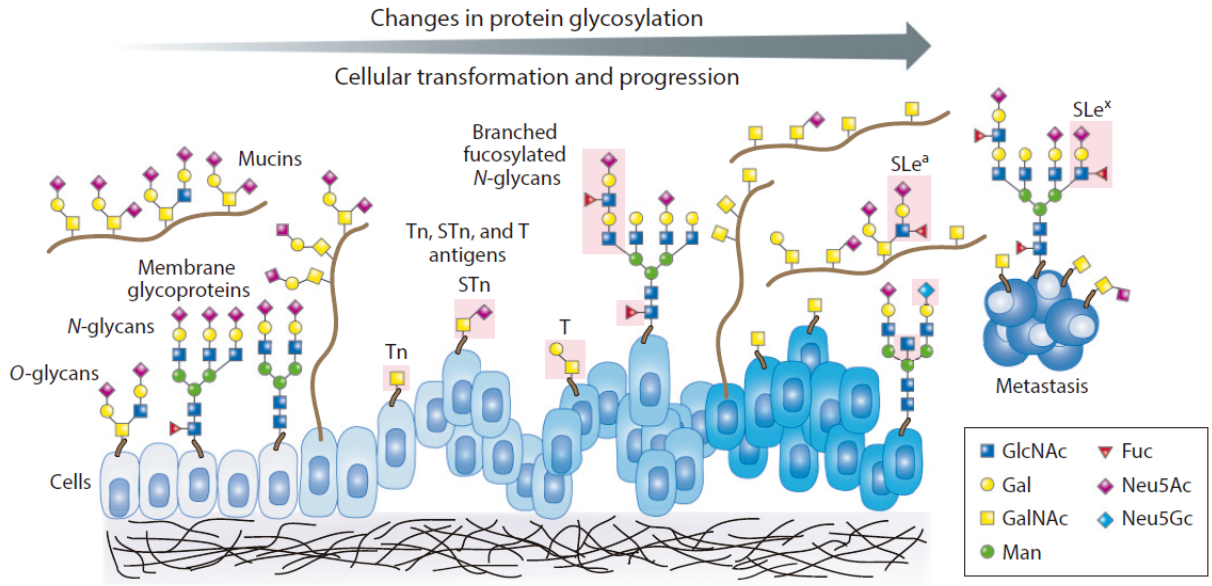


Figure 9: Changes in protein glycosylation during cancer transformation. During cellular transformation, changes in protein glycosylation on membrane and soluble glycoproteins are typical and may occur early and/or late in cancer progression. Different types of changes are shown in the pink-boxed areas, highlighting changes in *O*-glycans epitopes (T, Tn, and STn) and in branching and Lewis antigens (SLe^x and SLe^a) in *N*-glycans. (Extracted from Stowell *et al.*, 2015 [57]).

Namely, malignant cells can present an increase in β1-6 branching of *N*-glycans, which results from enhanced expression of *N*-acetylglucosaminyl-transferase V (MGAT5). Also, changes in sialic acid expression are common in malignancy. In particular, tumour cells show an overall increase in cell-surface sialic acid content, which reduces attachment of metastatic tumour cells to the matrix, and may help to prevent them from being recognised by the immune system. Another modification in tumorigenesis is the appearance of tumour-associated carbohydrate antigens (TACAs). TACAs are molecular markers overexpressed on human tumour cells resulting from the combined action of glycotransferases and glycosidases abnormally expressed in tumour cells that can be used to distinguish them from normal cells [75]. The expression of TACAs on cancer cells is associated with signal transduction, survival, extravasation and metastases, because of their interaction with glycan receptors on tissues [76, 77]. Signalling cascades are intimately interconnected with TACA expression and interaction with the microenvironment. TACAs can regulate, for example, the interaction between integrin and focal adhesion kinase (FAK), which in turn, regulates cancer cell adhesion and invasion [78, 79].

The transition in glycosylation patterns of cancer cells reflects a myriad of processes that correlate with a poor prognosis of cancer, affecting cell signalling and communication, cell motility and adhesion, and angiogenesis. The carbohydrate antigens that have been found to be tumour-associated (Table 2) include the mucin-related Tn, sialyl Tn (STn) and Thomsen-Friedenreich (TF/T) antigens, the blood group Lewis-related Lewis Y, Sialyl Lewis X (SLe^x) and Sialyl Lewis A (SLe^a), and Lewis X (also known as stage-specific embryonic antigen-1, SSEA-1), the glycosphingolipids, Globo H, and stage-specific embryonic antigen-3 (SSEA-3), the sialic acid containing glycosphingolipids, the gangliosides, GD2, GD3, GM2, fucosyl GM1 and Neu5GcGM3 and polysialic acid [77, 80].

Table 2: Common carbohydrate antigens targets from tumour biopsy specimens (Extracted from Monzavi-Karbassi *et al.*, 2013)

Carbohydrate antigens	**Structure**
Polysialic acid	α2,8-NeuAc
Tn	GalNAcSer/Thr
sialyl Tn (STn)	Neu5Acα2-6GalNAcαSer/Thr
T antigen	Galβ1-3GalNAcαSer/Thr
Globo-H	Fucα1-2Galβ1-3GalNAcβ1-3Gaαl1-4Galβ1-4Glc
Lewis Y	Fucα1-2Galβ1-4(Fucα1-3)GlcNAc
Sialyl Lewis X (SLe^x)	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc
Sialyl Lewis A (SLe^a)	Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc

The overexpression of certain Lewis antigens, in particular SLe^x and SLe^a, are carbohydrate molecules that mediate the adhesion between tumour cells and the endothelium [81]. The interaction of the antigen SLe^x with E-selectin on endothelial cells facilitates tumour cell invasion in blood microvessels, extravasation and migration into tissue [82]. A positive correlation between SLe^x and metastasis and poor survival has been proven in patients for several types of cancer, such as colon cancer [83, 84], gastric carcinoma [85, 86], breast cancer [81, 87], and PDAC [88-90]. Tumour cells might benefit from this new SLe^x expression to mimic the mechanisms used by leukocytes to leave the blood vessels and enter the surrounding tissue. SLe^x has been described as commonly expressed over 80% in malignant pancreatic tissue and is associated with

tumour progression and metastasis, but it is not detected in healthy pancreatic specimens [91, 92].

Certain mucin-type *O*-glycans can either facilitate or attenuate cell adhesion to extracellular matrix components and lectins, depending on their core structures and the structures of the non-reducing termini. Several studies revealing the role of core 2 *O*-glycans in immune responses show that core 2 expression is a biologically significant change [93]. Sialylated core 2 branched *O*-glycans without additional modifications exhibit anti-adhesive properties, which might be related to anoikis resistance [77]. Moreover, the truncation of *O*-glycans to short epitopes, such as T, Tn and STn, is also a common alteration in cancer cells [94]. This could be produced by the lack of activity of T-synthase (C1GalT1), which elongates the Tn antigen to form core 1 *O*-GalNAc glycans. In PDAC, *COSMC,* which encodes for a chaperone necessary for the activity of T-synthase has been shown to be hypermethylated [95]. This is the prevalent cause of truncated *O*-glycans, triggering the sialylated epitope STn [96, 97], which is expressed in most gastrointestinal carcinomas, including PDAC, correlating with decreased cancer cell adhesion, increased cancer cell invasion and poor prognosis of the patients [96, 98]. The appearance of the STn in mucins is a late event in PDAC disease progression [99].

Pancreatic tumours can produce glycoproteins bearing abnormal amounts of sialyl-fucosylated epitopes that could be shed into fluids like pancreatic juice or blood and, thus, be useful as PDAC tumour markers. Hence, deciphering the glycan pattern of these aberrantly glycosylated candidates can improve the accuracy of the diagnosis.

The major factors that affect protein glycosylation in tumour cells are the expression levels and the localization of specific glycosyltransferases and glycosidases, the availability of protein substrates and the kinetics of glycoprotein turnover, the activity of sugar nucleotide and monosaccharide transporters, the pH of the ER and Golgi apparatus, the expression of specific molecular chaperones that regulate protein folding, and the quality control of glycoproteins and glycosyltransferases [57, 59].

2.5. Glycan analysis

Glycan studies have become important in clinical and biomedical research, as many biomarkers of health and disease are glycoproteins, although in most cases only the protein backbone is used for the diagnosis. Such biomarkers now encompass many major diseases ranging from diabetes, rheumatoid arthritis or cardiovascular pathologies to various cancers [100].

Both *N*- and *O*-glycosylation are complex post-translational modifications whose characterization represents a major challenge. This complexity can be summarised in four factors. First, glycans are constructed biosynthetically from many reactions that involve redundant and overlapping enzymes, sugar nucleotide transporters and other cellular machinery. Second, different molecules of the same protein from the same tissue sample may have different carbohydrate structures attached to a specific amino acid residue (glycan microheterogeneity), and not each potential glycosylation site is always glycosylated (glycan macroheterogeneity or different site occupancy). Third, many of the monosaccharides that compose oligosaccharide structures can be isomers. And fourth, the branched nature of the structures must be considered [58].

Different strategies are used to analyse glycan structures. The most appropriate approach for each piece of research will depend mainly on two factors: (i) the level of structural detail requested and (ii) the amount of sample available. The methodologies employed throughout this thesis, their advantages and limitations, and also the new emerging methodologies for glycan analysis are summarised in this chapter.

2.5.1. Antibodies, lectins and glycosidases

Glycan analysis can be approached in two different ways. The first is the analysis of glycans of a single protein that has been previously isolated from the biological fluid, tissue or cell. Routine techniques including electrophoresis, immunopurification and chromatography are used to achieve the purification. On the other hand, the interest might be focused not on a single protein glycan but on the analysis of the glycan composition of a complex sample (e.g., blood, tissue, cell or urine), enabled by a general glycan release from proteins or lipids. In both strategies, antibodies, lectins and glycosidases are often required.

2.5.1.1 Antibodies and lectins

Specific glycan binding antibodies and lectins are widely used in glycan analysis because their specificities enable them to discriminate between various glycan structures. Lectins and antibodies have different specificities. While antibodies specifically recognise certain epitopes such as Lewis or sialyl-Lewis antigens, lectins can identify general glycan determinants such as α2-6-linked sialic acid (SNA) or α-fucose (AAL) [101, 102].

Some applications of antibodies against glycan structures and lectins include cell separation and analysis by flow-cytometry, cytochemical characterization/staining of cells and tissues, and purification and characterization of glycan-conjugates in columns and microplates. Using arrays of different lectins and antibodies to probe glycoproteins, it is possible to deduce many aspects of their glycan structure [101, 103].

In this work, lectins and anti-glycan antibodies have been used in Western blotting to detect glycan epitopes on immobilized glycoproteins, as well as in enzyme-linked lectin assays (ELLAs) and in immunohistochemistry (IHC) assays.

2.5.1.2 Glycoanalytical enzymes: glycosidases

One strategy to obtain structural data of glycans is to separate sugar moieties from the protein. Common procedures for glycan removal are enzymatic digestions with endoglycosidases or chemical release. In addition, it is also possible to totally disassemble the polypeptide chain with pronase, which is a commercially available mixture of proteases.

Glycosidases are the enzymes responsible for the hydrolysis of glycosidic bonds in complex sugars. Several different enzymes are routinely used, depending on the required specificity of the cleavage. The most frequently employed endoglycosidase, PNGase F, is used to release *N*-glycans from the polypeptide backbone in samples in solution as well as on proteins on blotting membranes or polyacrylamide gels. On the other hand, for the general liberation of intact *O*-glycans, no enzyme is available and chemical cleavage (β-elimination) must be performed. Nevertheless, the enzyme *O*-glycanase can be used for core 1 *O*-glycan digestion.

Once released, glycans can be labelled in their free reducing termini with fluorescent tags, enabling subsequent analysis by different techniques that apply a fluorescence detector, such as capillary electrophoresis (CE), fluorophore-assisted carbohydrate electrophoresis or high-performance/ultra-performance liquid chromatography (HPLC/UPLC).

Glycan enzymes are also useful to characterise specific moieties. A wide range of exoglycosidases, including sialidases, fucosidases and galactosidases, present linkage-defined specificities that let researchers study the presence or absence of certain epitopes. The result of these digestions can be analysed with different antibodies or lectins as previously described, or by CE, HPLC/UPLC, or mass-spectrometry (MS), as will be discussed in the following sections.

One of the most common strategies in glycoproteomic analysis consists of the characterisation of the peptides and glycopeptides by CE, HPLC, liquid chromatography-electrospray MS (LC-ESI MS) and matrix-assisted laser desorption ionization-time of flight MS (MALDI-TOF MS) after proteolytic degradation. Trypsin, chymotrypsin, Lys-C, Gly-C and Asp-N endopeptidases are the most commonly used enzymes to obtain glycopeptides with single glycosylation sites [104].

2.5.2. Separation technologies for glycan analysis

A great effort has been made to develop new methods and analytical instruments for high-sensitivity glycoprotein and/or glycan analysis.

Capillary electrophoresis (CE) separates the components of a sample depending on the mass/charge ratio using an electrical field. CE provides an isoform profile, influenced by its glycan moieties, of a purified glycoprotein. However, it does not give any information about the nature of the glycan chains attached to the protein. To overcome this drawback, attempts are currently being made to perform MS in conjunction with CE [105]. However, this approach requires resolving isoforms into well separated peaks, which is not always the case. CE can also be applied to glycan analyses, but synthetic standards for every analysed glycan, pre-fractionation steps and glycan labelling processes are required prior to CE. On the other hand, the advantages of capillary electrophoresis are the low sample volumes required, the high throughput

implementation and the wide detection range [58, 106]. In the present work, CE has been performed with α-1-acid-glycoprotein purified from serum samples.

Another technique used primarily to study monosaccharide composition, nucleotide sugars, cellulose, and single-glycoprotein glycosylation is high-performance anion-exchange chromatography (HPAEC). This method is based on the principle that hydroxyl groups of carbohydrates, although neutral at physiological pH, can be ionized in sufficiently basic solutions (pH>12) [58, 106].

Normal phase high-performance liquid chromatography (NP-HPLC) is frequently applied in glycomic research to analyse both neutral and charged carbohydrates. In this method glycans are usually enzymatically liberated from the protein and fluorescently labelled prior to analysis [58, 106]. A group led by Professor P.M. Rudd has developed a method that allows a detailed structural analysis of *N*-glycans using HPLC combined with exoglycosidase array digestions [107, 108]. In this work, *N*-glycans from the SDS-PAGE gel bands of ceruloplasmin have been enzymatically released and fluorescently labelled with 2-aminobenzamide, and then digested using arrays of different enzymes before being analysed by hydrophilic interaction liquid chromatography (HILIC).

2.5.3. Structural analysis

Mass spectrometry (MS) is widely used in the field of glycomics (Figure 10). The most valuable advantages of MS over other methods are its robustness, sensitivity and accuracy. Nevertheless, sample pre-treatments, which usually involve chemical modifications, slow down the identification process [109]. When ionization methods are compared, electrospray ionization (ESI) offers a milder process. It leaves the glycan molecules unfragmented and enables the analysis of unstable glycans, which is an advantage over MALDI. On the other hand, the MALDI spectrum is less complex to interpret because it generates single charged ions [109].

MS and nuclear magnetic resonance spectroscopy (NMR) [110] are the most suitable methods for analysing previously undescribed glycans or when enzymes are not available to define certain linkages. The amount of material required for NMR analyses is substantially larger than for MS.

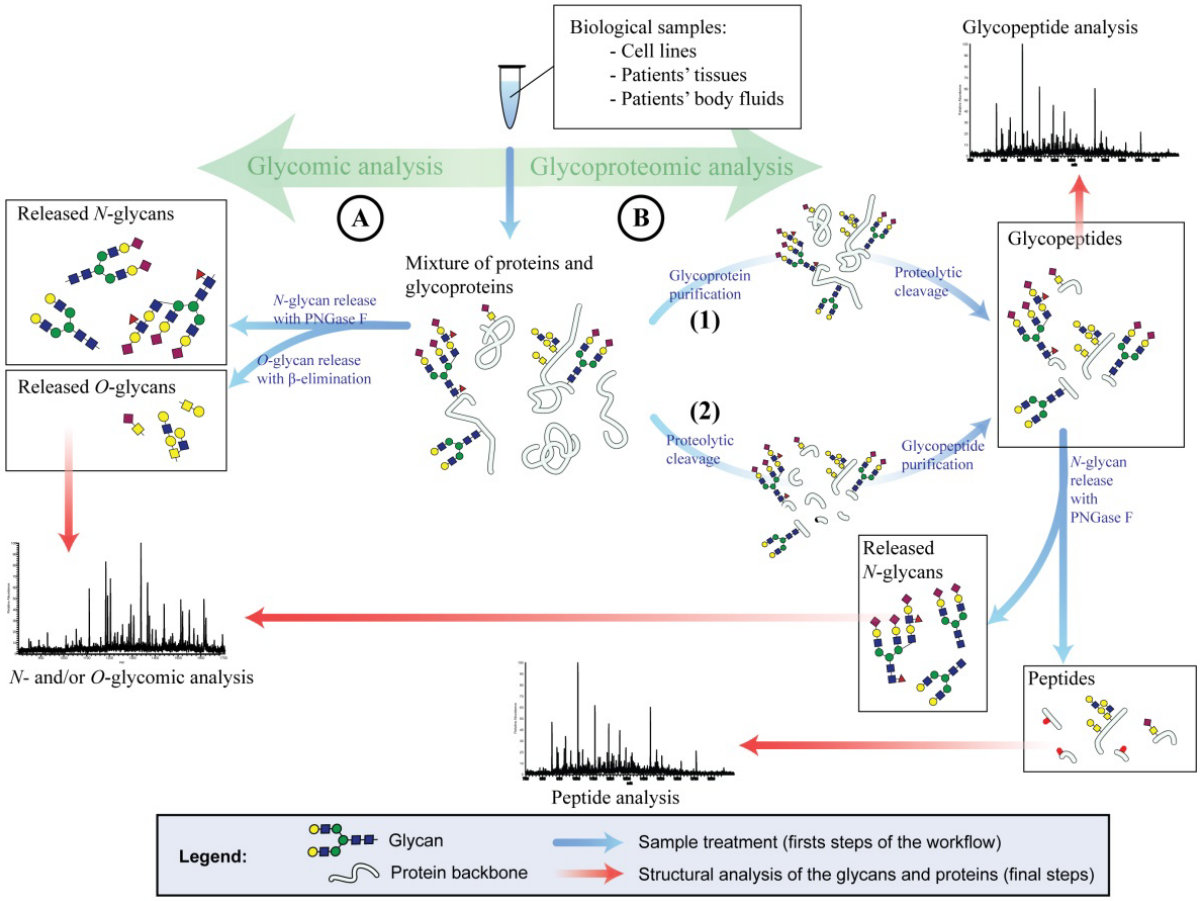


Figure 10: Glycomic and glycoproteomic strategies for glycan analysis. (A) Glycomic analysis. *N*- and *O*-glycans are PNGase F or chemically released, respectively. Once the glycans are released, they are structurally analysed. (B) Glycoproteomic analysis. The glycoproteins are either purified from a complex mixture and then enzymatically digested (1) or first digested and then purified (2). Method 1 will result in glycopeptides and non-glycosylated peptides originated solely from glycoproteins. Method 2 will result in glycopeptides only. These glycopeptides can be directly analysed to generate site-specific glycan structures. Alternatively, the glycans of the glycopeptides can be released as described in (A) and the peptides are analysed. (Extracted from Mereiter *et al.*, 2016 [98]).

In this work, the *N*-glycans of human α-1-acid glycoprotein have been analysed by ESI-TOF-MS, prior to separation of the glycans by zwitterionic hydrophilic interaction capillary liquid chromatography (ZIC-HILIC).

2.5.4. Microarray-based methods

Conceptually, a microarray is a miniaturised format that enables the study of multiple probes covalently attached to a solid substrate (usually a glass slide or silicon thin-film cell) and their recognized partners from large amounts of biological material using high-throughput screening miniaturised, multiplexed and parallel processing and detection methods [98]. In glycan analysis, microarrays can be divided into general subtypes depending on which molecule is deposited on the surface. Hence, there are carbohydrate

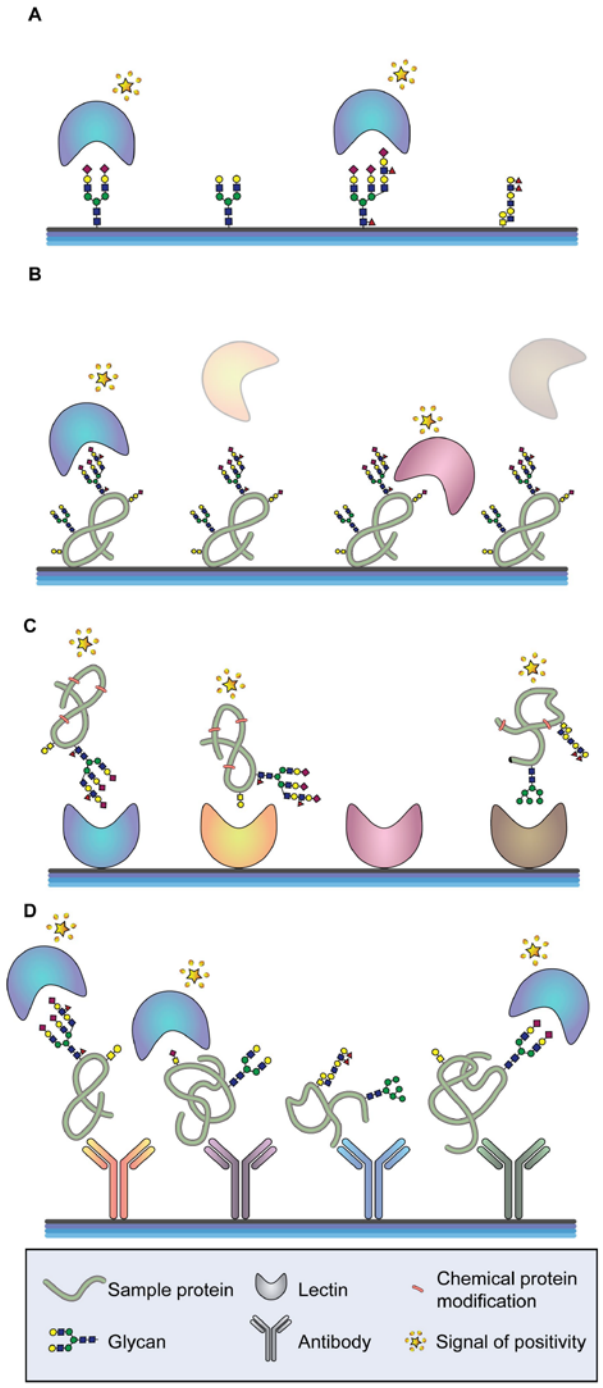


Figure 11: Arrays for glycan analysis. (A) Glycan arrays. Different glycan moieties are spotted onto the array to determine specificities of labelled glycan-binding molecules (typically lectins or antibodies). (B) Glycoprotein arrays. Glycoproteins are enriched from a sample and spotted onto the array. The glycosylation moieties of the spotted glycoproteins are determined by screening with different glycan-binding molecules. (C) Lectin arrays. A series of lectins with well-defined glycan-binding properties are spotted onto the array and different labelled proteins are tested. (D) Antibody-lectin sandwich arrays. Multiple antibodies for a series of proteins of interest are spotted onto the array, and the glycan epitopes of the captured glycoproteins are probed using labelled lectins and glycan-binding antibodies. (Extracted from Mereiter *et al.*, 2016 [98]).

microarrays (11A), lectin microarrays (11C) and glycoprotein microarrays (11B and 11D) [58].

Carbohydrates microarrays (Figure 11A) are used mainly to characterize the binding specificity and affinity of proteins (mostly antibodies and lectins) towards glycans. Current platforms consist of approximately 20,000 microspots of carbohydrate determinants and have the capacity to include most known human microbial pathogens, autoantigens and tumour-associated antigens [111].

Lectin microarrays (Figure 11C) enable rapid and high-sensitivity profiling of complex glycan features without the need to liberate the glycans. Lectin microarrays consist of depositing lectins in a solid support and incubating the array with fluorescently labelled samples to allow lectin-carbohydrate binding. Target samples include an extensive range of glycoconjugates present in cells, tissues and body fluids, as well as synthetic glycans and their mimics [112].

In glycoprotein microarrays (Figure 11B), glycoproteins from a complex sample, such as immunodepleted serum, are fractionated with lectin-affinity

chromatography, followed by HPLC, and the discrete fractions are spotted onto nitrocellulose substrates. Fractions from multiple samples can be included in the same array. Then, each array is incubated with a lectin to identify differences in the composition of fractions. Variants of this method are the antibody-lectin sandwich arrays (Figure 11D), where antibodies against known glycoproteins are arrayed on a solid support, and complex glycoprotein samples, which can be crude or pre-fractionated, are bound to the microarray and probed using labelled lectins and glycan-binding antibodies [58, 113].

2.5.5. Glycoinformatics

High-throughput methods, such as glycan analysis by MS and microarrays, generate a great amount of new data. Consequently, it is fundamental to have databases and tools to store, retrieve and analyse these data in an efficient way [114]. The large variety of storage formats, databases and tools in use make the exchange of knowledge among glycobiologists difficult, and efforts are currently being made to unify all systems. These include normalization of the nomenclature of monosaccharides and oligosaccharide chains following the standardized guidelines of the International Union of Pure and Applied Chemistry (IUPAC) [115] and the unification of the monosaccharide symbols to represent the glycan structures. The most common graphical representations are the Oxford system and the Consortium for Functional Glycomics (CFG) symbolic representation [114]. The present work uses the CFG notation for glycan depiction as shown in Figures 5 and 6.

For experimental data, including carbohydrate-protein interactions, two main resources exist: the Consortium for Functional Glycomics and the Japanese Consortium for Glycobiology and Glycotechnology, which make available a vast amount of experimental data.

Several glycomic databases have been established in recent years. One of the most comprehensive is UniCarbKB, which provides information concerning glycan structures, and publishes glycoprotein information including global and site-specific attachment information [116].

3. Biomarker research

A biomarker, according to the National Cancer Institute, is a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process, or of a condition or disease, such as cancer [117]. The molecular alterations found in diseased patients can be due to a number of factors, including germ-line (inherited) or somatic mutations, transcriptional changes and posttranslational modifications. The enormous variety of biomarkers includes proteins, nucleic acids, antibodies, peptides, etc. A biomarker can also be a collection of alterations, such as gene expression, proteomic, and metabolomic signatures [117]. Biomarkers can be found in the circulatory system, excretions or secretions or can be tissue-derived.

Markers can be classified into two different categories according to their source: tumour-derived markers, which are produced by neoplastic cells; and tumour-associated antigens, which include substances produced by normal tissue in response to the presence of the neoplastic cells.

Cancer biomarkers have different clinical uses. They can be used to estimate the risk of developing cancer, to screen for primary cancer, to differentiate between benign or malignant states or among types of malignancies, to determine the prognosis of the disease, to predict response to therapy and to monitor disease recurrence or the response or progression in metastatic disease [117].

In order to assess the accuracy of a biomarker, two factors are determined in the clinical practice: sensitivity (proportion of cancer patients identified correctly) and specificity (proportion of non-cancer patients identified correctly).

As described in section 2.4, oncogenesis leads to a remarkable alteration of cellular glycosylation and thus, tumour-secreted glycoproteins may reflect an altered glycosylation pattern and are likely to be good candidates for tumour markers. In fact, most of the tumour markers currently approved by the FDA are glycoproteins, although in most cases the protein moiety is used for the analysis [118].

3.1 Tumour markers in pancreatic cancer

The currently available tumour markers for PDAC are not capable of detecting early pancreatic cancer. The best treatment is curative resection, but it is only possible in early stage PDAC, so the search to establish new tumour markers is of major interest. To achieve this goal, strategies based on exploring different body sources and/or analysing types of macromolecules have been developed.

Although the current research is more focused on the discovery of new biomarkers in body fluids obtained in a non- or minimally-invasive manner, especially serum, specimens obtained from surgical resection can help to extend the knowledge of tumorigenesis and to identify novel markers. Since PDAC tissue contains the highest concentration of PDAC-specific markers, it is the most direct source for proteomic cancer biomarker research. Other putative sources for PDAC biomarkers are urine, stool, saliva and pancreatic juice [119].

PDAC markers were discovered as a result of the study of serum substances, and a broad variety of serologic markers has been associated with PDAC. The markers studied can be divided into tumour-associated antigens, enzymatic proteins, oncofetal antigens, and others including a wide range of molecules that are being studied (for example, circulating tumour cells, circulating free DNA and microRNAs) [120]. Among all the studied candidates, only CA19-9 and CEA, which are tumour-associated antigens, currently play a clinical role [121].

Carcinoembryonic antigen (CEA) is a group of cell membrane-attached glycoproteins involved in cell adhesion [122]. They were first described in 1965 by Gold and Freedman after the immunization of rabbits with an extract of human colon carcinoma [123]. The major antigenic epitopes are localized on the protein part, and at least six different epitopes are identified [124]. CEA is normally produced in intestinal tissue during foetal development, and its levels drop significantly just before birth. Therefore, it is normally present at very low levels in the blood of healthy adults. For more than a decade, CEA was the only serum tumour marker used clinically in the diagnosis of PDAC [125]. However, since high CEA levels can be found in patients with other carcinomas (colorectal, gastric, lung, breast and medullary, as well as in some non-neoplastic

conditions) [57, 126-128], in practice it is being replaced by other markers, which have shown a higher sensitivity [125].

Carbohydrate antigen 19-9 (CA19-9) is the most relevant biomarker for PDAC, and the only marker approved by the FDA for monitoring the disease [129]. CA19-9 is characterised by a monoclonal antibody generated by immunizing mice with the human colorectal cancer cell line SW1116 [130]. CA19-9 corresponds to a sialyl Lewis-A antigen that occurs on glycoproteins, such as mucins [131]. It is expressed primarily in pancreatic and biliary tract cancers, but it may also be present in patients with other malignancies, such as breast and lung cancer, as well as in non-malignant diseases such obstructive jaundice, pancreatitis, cirrhosis, etc., but also in some normal pancreas [42, 132-134]. Thus, CA19-9 shows an unsatisfactory sensitivity (79-81%) and specificity (82-90%), which makes it not accurate enough to be used as a screening test but helpful as a prognostic factor and for the assessment of treatment response. CA19-9 serum levels decrease after curative surgery and increase in recurrent disease [132, 135]. As yet, no better alternative has emerged for clinical application.

Commercially monoclonal antibody PAM4, known also as clivatuzumab, is currently in clinical trials for early detection of PDAC. PAM4 identifies a biomarker expressed by 90% of PDAC, as well as by the precursor lesions PanIN and intraductal papillary mucinous neoplasms, but not by a normal pancreas. Immunohistochemistry analysis has reported that PAM4 allows the distinction of PDAC from non-neoplastic pancreatic disease, particularly chronic pancreatitis [136]. In particular, PAM4 recognises the mucin MUC5AC [137].

A lot of research is being conducted to find a good PDAC biomarker. Unfortunately, despite the efforts made, many putative markers have not improved the sensitivities and specificities of CA19-9 [125]. Taking into account that aberrant glycosylation of serum proteins is caused by the presence of the tumour, previous studies looking for potential biomarkers showed that altered glycosylation of acute phase proteins could be useful to develop PDAC biomarkers [138, 139], an issue that will be developed in this thesis.

3.1.1. Acute phase proteins

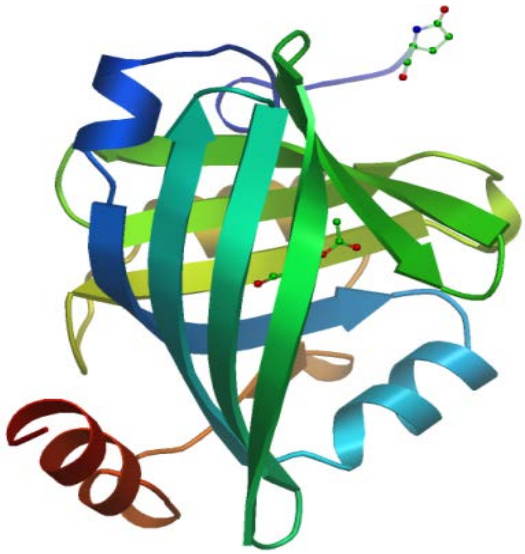
The liver is an essential organ for metabolism, detoxification and immune response. One of the roles of the innate immune response is the production of acute phase proteins (APPs) as a consequence of hepatocyte stimulation by pro-inflammatory cytokines during the acute phase response [140-142].

This reaction takes place in individuals when disturbances in their homeostasis are detected. Causes for this event can be infection, tissue injury, trauma or surgery, stress, neoplastic growth or immunological disorders [141]. The goal of this complex systemic reaction is to re-establish homeostasis and to promote healing [142].

The acute phase response may result in changes in more than 200 proteins. They are classified as either positive APPs or negative APPs depending on whether their concentration in the blood is increased or decreased, respectively [142].

APPs have been used for human diagnostic medicine and are especially important in the diagnosis and prognosis of cardiovascular disease [143, 144], autoimmunity [145], organ transplants [146-148] and cancer treatment [149]. Although synthesised mainly by the liver, APPs have been described as good carriers of pathological information of cancers from a non-liver origin such as lung adenocarcinoma [150], colorectal cancer [151] and ovarian cancer [152].

3.1.1.1 Alpha-1 acid glycoprotein



Alpha-1 acid glycoprotein (AGP), or orosomucoid, is also a positive APP and is considered, after albumin, to be the next most important drug-binding plasma protein in human adults. It plays an important role in the binding of steroids and many basic and neutral drugs [153].

Figure 12: Alpha-1-acid glycoprotein (AGP) structure. Crystal structure of the recombinant unglycosylated human AGP solved using the UV-radiation-damage-induced phasing (UV RIP) method. (Extracted from Schönfeld *et al.*, 2008 [157]).

Although its concentration may change under various physiological and pathological conditions, such as during the acute-phase reaction, values in the range of 55-140 mg/dL are considered normal in healthy young adults. It is estimated that the AGP half-life is approximately five days.

AGP is a negatively charged acidic glycoprotein (pI=2.7) of about 41kDa and the single polypeptide chain is formed by 183 amino acid residues (22KDa) (Figure 12). The carbohydrate content represents 45% of its molecular weight and is composed of five complex-type *N*-linked glycans attached to the polypeptide backbone (Asn15, Asn38, Asn54, Asn75 and Asn85) including di-, tri- and tetra-antennary structures [154-157].

Changes in AGP glycosylation patterns are associated with cancer and chronic inflammation and include mainly an increase in glycan branching and in the expression of the SLe^x epitope [138, 140, 154, 155].

3.1.1.2 Ceruloplasmin

Ceruloplasmin (CP) is a positive acute-phase protein whose function is related to copper transport in blood. It has also a role in homeostasis regulation and it shows iron oxidation, superoxide dismutase and amino-oxidase activities. Although the liver is the predominant source of serum CP, extrahepatic CP gene expression has been demonstrated in many tissues including spleen, lung, testis and brain [158].

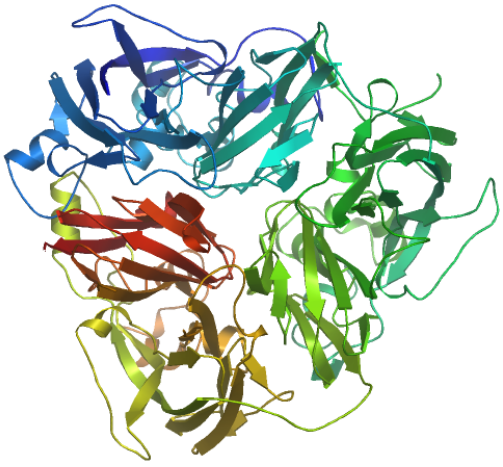


Figure 13: Ceruloplasmin structure. Crystal structure obtained by X-ray diffraction of human serum ceruloplasmin (Extracted from Zaitseva *et al.*, 1996 [160]).

CP has a half-life of 5.5 days in serum and its normal concentration ranges from 20 to 35 mg/dL. An increase in the hepatic copper pool results in a sustained increase in serum CP concentration, whereas a deficiency in nutritional copper uptake results in a marked decrease in serum CP. Physical exercise, pregnancy, ovarian hyperfunction, arteriosclerosis, epilepsy, inflammation and tumours are factors that can lead to an increase in CP transcription by stimulating inflammatory cytokines in hepatocytes[158].

From a structural point of view (Figure 13), CP consists of a single peptide chain (1046 amino acids, 122KDa) with a molecular weight around 132kDa when glycosylated [159]. Even though copper ion (Cu^{2+}) is its main ligand, GlcNAc and oxygen have also been defined as possible ligands [160]. CP has four described *N*-glycosylation sites (Asn119, Asn339, Asn378, and Asn743) with complex bi-, tri-, and tetra-antennary structures both sialylated and fucosylated, containing the SLe^x epitope mainly in tri-antennary structures and to a lesser extent in bi- and tetra-antennary ones [161, 162].

This glycoprotein, which is classified as a member of the multicopper oxidase family of enzymes, has six copper binding sites [158]. As this metal is an essential cofactor for angiogenesis and neovascularisation, increased levels of CP are found in some diseases and it has been proposed as marker for different solid cancers [163].

3.1.2 Mucins

Mucins are high molecular weight glycoproteins, usually over 200kDa, synthesised by epithelial cells in most organisms of the animal kingdom. They are formed with a protein backbone bearing numerous carbohydrate side chains, mainly *O*-glycans attached in high-density in clusters. As a result, mucins are heavily glycosylated molecules with carbohydrates making up as much as 50-80% of their mass. The peptide part of the mucin molecule is referred to as apomucin.

Mucin glycans range in size from a single monosaccharide (a GalNAc residue linked directly to a serine or threonine of the polypeptide backbone of the protein) to complex linear or branched oligosaccharide chains of more than 20 residues [69]. There may be several hundred oligosaccharide chains attached to a single protein molecule. Oligosaccharide chains of mucins are predominantly *O*-linked, but some *N*-linked oligosaccharides may be present on the same molecule [164].

Clustering of the *O*-linked oligosaccharide chains on the polypeptide backbone of the protein usually occurs at sites within sequences with many repeated amino acids. These tandem repeat sequences differ from mucins in precise amino acid composition, but share common features. They contain many serine and/or threonine amino acid residues usually in a proline-rich stretch of the polypeptide chain [164, 165]. These proline-rich regions are sterically favoured so the serine/threonine residue side chains are physically accessible. Typically, the polypeptide also contains cysteine-rich regions,

which are not *O*-glycosylated and are involved in protein folding by means of disulphide bonds. These regions may contain attachment sites for *N*-glycans.

In mammals, the large mucin protein family covers a broad variety of functions with tissue-specific expression profiles. Mucins have a central role in maintaining homeostasis and, therefore, in promoting cell survival in variable conditions [166]. These glycoproteins can be either secreted or bound to the cell-surface membrane [164]. Mucins might serve as cell-surface receptors and sensors, and conduct signals in response to external stimuli that lead to coordinated cellular responses such as proliferation, differentiation, apoptosis or the secretion of specialized cellular products [166]. Secreted mucins form a protective barrier that can capture and hold biologically active molecules that might function as indicators of a molecular or physical breach of the mucin layer and, following their release, might incite an inflammatory repair or healing process [166].

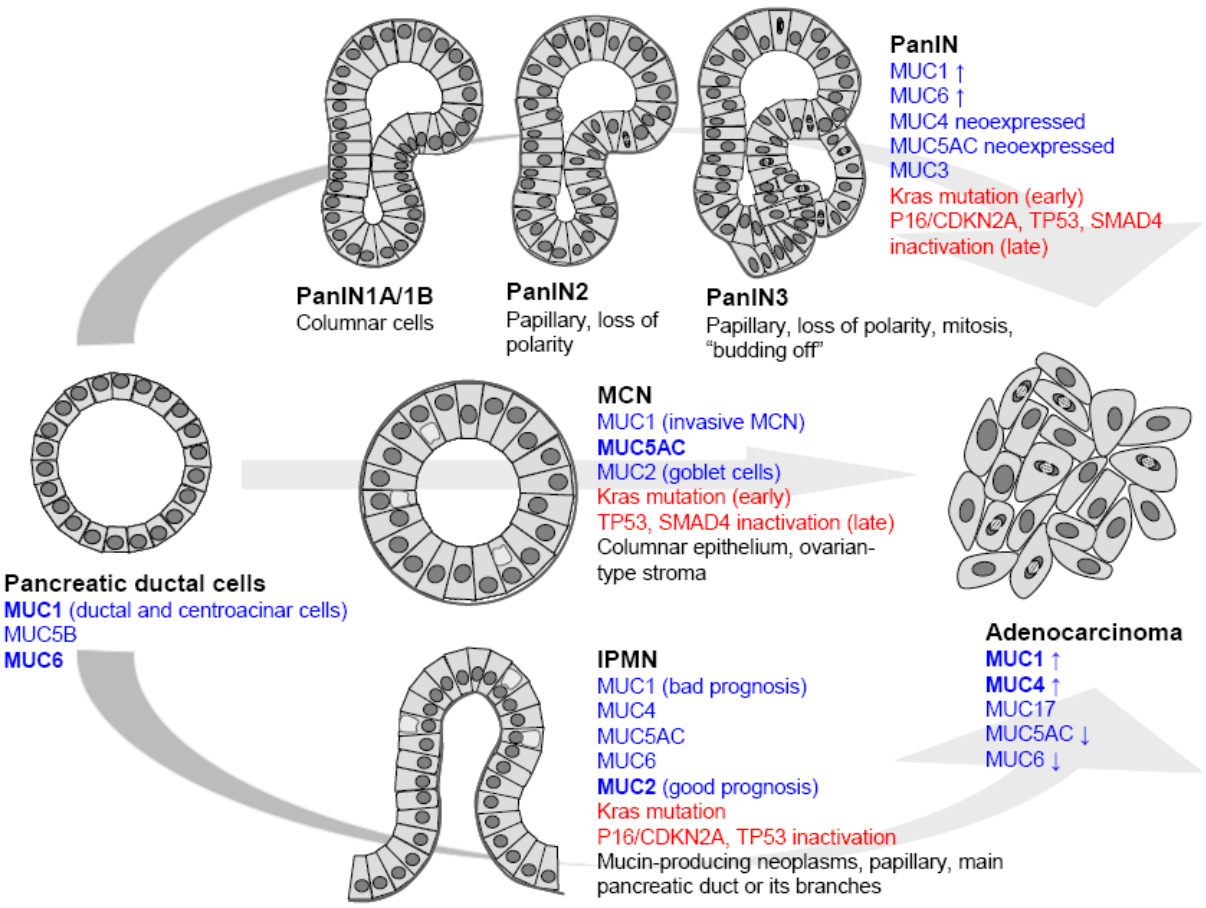


Figure 14: Mucin expression in precursor lesions leading to pancreatic ductal adenocarcinoma. Abbreviations: Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN). (Extracted from Jonckheere *et al.,* 2010 [20]).

Mucin expression, which means either protein backbone (apomucin) and/or glycosylated mucin, can be altered in disease status, including cancer [164, 166, 167]. This feature has converted mucins into an attractive target in terms of diagnosis and treatment of cancer and, as a consequence, many tumour markers now in clinical use are mucins [74]. Besides, mucins are often present in the sera of patients with PDAC. Thus, an in-depth investigation of these glycoproteins in serum of PDAC patients could lead to identifying promising PDAC biomarkers, an aspect that will be assessed in this thesis.

In the transformation process from normal ductal cells through different precursor lesions to pancreatic adenocarcinoma, it is important to highlight the overexpression of MUC1 and the neo-expression of MUC5AC (Figure 14) [20, 21].

3.1.2.1 MUC1

Mucin 1 (MUC1) is a single-pass type I transmembrane glycoprotein expressed in glandular or luminal epithelial cells of the mammary gland, oesophagus, stomach, duodenum, pancreas, endometrium, cervix, prostate, lungs [168] and, to a lesser extent, in hematopoietic cells [169, 170]. Despite the broad expression of MUC1, it is absent in the skin epithelium and in all kinds of mesenchymal cells [171]. In healthy tissues, the glycan chains of MUC1 oligomerise to form a mucinous gel that lubricates and protects the underlying epithelia from desiccation, changes in pH, pollutants and microbes. The cytoplasmic tail of MUC1 plays a role in signalling pathways involved in cell differentiation, proliferation, adhesion and motility [172].

The *MUC1* gene encodes a single polypeptide chain which is autoproteolytically cleaved into two subunits due to conformational stress. The resulting subunits, MUC1-N and MUC1-C, remain associated through stable hydrogen bonds formed in the extracellular domain (Figure 15) [170, 173]. In humans, there are several isoforms of MUC1 that result from alternative splicing, exon skipping and intron retention of the seven exons of the gene [174].

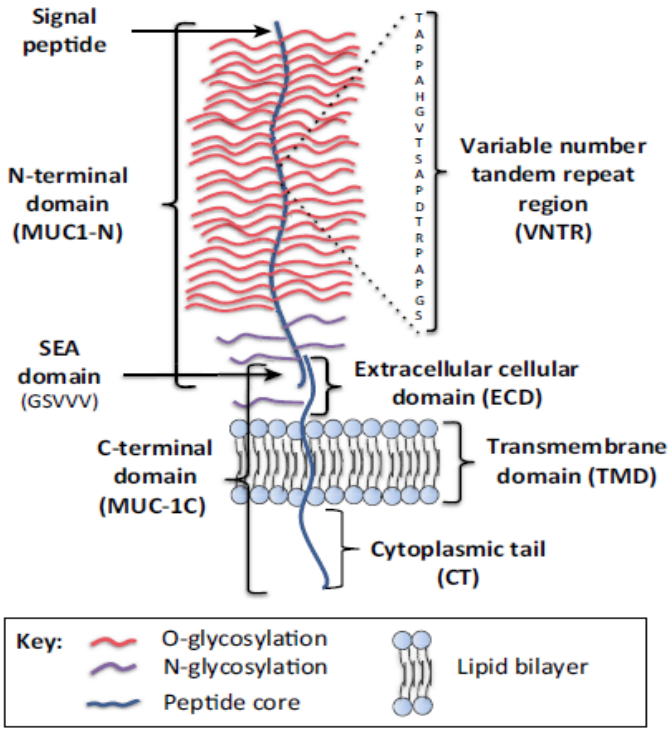


Figure 15: Schematic representation of the structure of MUC1. The N-terminal subunit (MUC1-N) and C-terminal (MUC1-C) of MUC1 associate around the SEA domain, forming a stable heterodimeric complex. MUC1-N contains the signal peptide, variable number tandem repeat (VNTR) region, and SEA domain. The VNTR region of MUC1-N is composed of 20 amino acids that are extensively O-glycosylated at the serine and threonine residues. MUC1-N and MUC1-C are sparingly N-glycosylated at asparagine residues. The MUC1-C consists of the extracellular domain (ECD), transmembrane domain (TMD) and cytoplasmic tail (CT). (Extracted from Nath and Mukherjee, 2014 [173]).

MUC1 is extensively *O*-glycosylated and moderately *N*-glycosylated to yield the mature and functional mucin [168]. Glycosylation contributes to 50-90% of the total weight of MUC1, which can vary depending on the number of tandem repeats and the degree of glycosylation. Around 40% of the amino acids in the variable number of tandem repeats (VNTR) are serine and threonine residues, which are abundantly *O*-glycosylated. *N*-glycosylation occurs at five sites, four of them in the MUC1-N and one in the extracellular domain of the MUC1-C [173]. The glycosylation pattern of MUC1 varies depending upon the tissue-specific expression of the glycosyltransferases [170]. *O*-glycosylation correlates with the biological properties of MUC1, whereas *N*-glycosylation is vital for protein folding, sorting, membrane trafficking, secretion and apical expression in polarized cells [175].

In tumours, several studies have indicated that MUC1 plays a critical role in the transcriptional regulation of genes associated with tumour invasion, metastasis, angiogenesis, proliferation, apoptosis, drug resistance, inflammation and immune regulation [166, 167, 173]. Overexpression of MUC1 is caused either by increased gene dosage and level of transcription induced by pro-inflammatory cytokines such as TNF-α and IFN-γ and/or epigenetic modification, and by a loss of post-transcriptional regulation caused by changes in microRNA expression. MUC1 expressed in cancer cells

differs from that expressed in normal cells, both in its biochemical features, including aberrant glycosylation, and its cellular distribution (MUC1 is redistributed over the cell surface and within the cytoplasm due to the loss of cell polarity) [166, 168, 173]. In PDAC, MUC1 does not seem to initiate malignant transformation but appears to play a critical role in creating the conditions necessary for cancer development, according to studies using murine models and pancreatic cancer cells [20, 173].

3.1.2.2 MUC5AC

MUC5AC is a secreted, gel-forming mucin expressed by epithelial cells lining the respiratory and gastrointestinal (especially stomach) tract and cervix, but it is rarely expressed in foetal or adult pancreases. It is a high-molecular-weight glycoprotein of approximately 641 kDa and, remarkably, up to 80% of the total weight is due to the large number of *O*-glycosylated chains [176].

On the surface of the normal respiratory epithelium, MUC5AC is one of the major contributors to the rheological properties of the mucus that plays a critical role in the defence against pathogenic and environmental challenges. In the gastric mucosa, MUC5AC and MUC6 are the main components of the protective layer over the surface, and act as a selective diffusion barrier to hydrochloric acid (HCl). According to descriptions, MUC5AC also protects the gastric epithelium from *Helicobacter pylori* [166, 176].

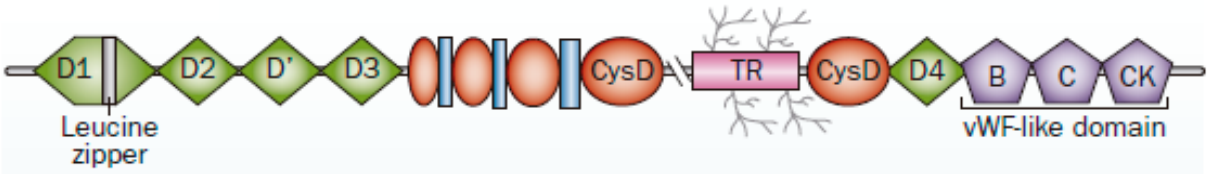


Figure 16: Depiction of MUC5AC domains. Abbreviations: TR, tandem repeat, vWD, von Willebrand factor domain. (Extracted from Kaur *et al.*, 2013 [177]).

MUC5AC is a polymeric mucin with three regions (Figure 16): the N-terminal, the central and the C-terminal region. At the N-terminal, D1, D2, D' and D3 cysteine-rich domains (Cys) are present, and are responsible for the disulphide-mediated polymerisation. In the central region, Cys domains are interspersed with domains rich in serine, threonine and proline. Also located in the central region is the tandem repeat (TR) domain, which

contains a high number of potential *O*-glycosylation sites. The C-terminal region has von Willebrand factor (vWF)-like domains involved in dimerization [176, 177].

Under pathological conditions, MUC5AC expression can be altered. Several factors, such as cytokines, inflammatory mediators, growth factors, some bacterial exoproducts and toxic agents like tobacco smoke and pollutants, can induce MUC5AC transcription, whereas glucocorticoids can down-regulate it [176]. It is noteworthy that the appearance of PDAC is accompanied by *de novo* expression of MUC5AC in PanIN lesions and their expression further increases with disease. MUC5AC is also expressed in the pancreas in mucinous cystic neoplasms and intraductal papillary mucinous neoplasms [21]. Therefore, understanding the role of MUC5AC in the malignant transformation of the pancreas is of great interest. Since this glycoprotein is *de novo* expressed in very early pancreatic precursor lesions, MUC5AC shows high potential as a biomarker. The monoclonal antibody PAM4 (in clinical trials known as clivatuzumab) targets MUC5AC and has been reported to be the only antibody able to differentiate PDAC (including PanINs and early PDAC stages) from benign, non-neoplastic tissues of the pancreas. Contrary to CA19-9, all inflamed tissues are negative and the labelled chronic pancreatitis specimens' reactivity is restricted to the presence of associated PanIN [136, 137, 178, 179].

Objectives

This work has focused on the study of alterations of glycan moieties of serum glycoproteins as potential candidates for PDAC biomarkers.

In particular, the glycan analysis of several acute-phase proteins and mucins has been assessed in pancreatic cancer compared to benign or healthy situations using different glycan analytical techniques and purification methods, to accomplish the following specific objectives:

1. Analyse the glycan pattern of serum alpha-1-acid glycoprotein in order to detect potential altered levels of fucosylation in pancreatic cancer patients.

2. Evaluate the levels of SLe^x antigen on serum ceruloplasmin from pancreatic cancer patients in comparison with control patients.

3. Identify the expression of sialylated epitopes SLe^x and STn on the MUC1 and MUC5AC mucins in pancreatic cancer specimens.

Results

The results of this PhD thesis have been published in scientific journals included in the Journal Citation Report of the Institute of Scientific Information. Therefore, this PhD thesis has been presented as a compendium of publications according to the current rules that regulate this format type at the Universitat de Girona, for the PhD Programme in Molecular Biology, Biomedicine and Health.

The results obtained in this Doctoral thesis are presented following an investigation regarding the identification of specific glycoforms to attempt to discover new pancreatic cancer biomarkers and they are divided into three scientific articles.

Chapter 1 corresponds to **Increased α1-3 fucosylation of α-1-acid glycoprotein (AGP) in pancreatic cancer** published in *Journal of Proteomics* in January 2016.

Chapter 2 corresponds to **Identification of potential pancreatic cancer serum markers: Increased sialyl-Lewis X on ceruloplasmin** published in *Clinica Chimca Acta* journal in March 2015.

Finally, **chapter 3** corresponds to the unpublished manuscript **Identification of potential pancreatic cancer biomarkers based on aberrant mucin glycoforms by in situ proximity ligation assay**, which will be submitted in 2016.

Results - Chapter 1

Increased α1-3 fucosylation of α-1-acid glycoprotein (AGP) in pancreatic cancer

This chapter has been published in the *Journal of Proteomics*.
Balmaña M, Giménez E, Puerta A, Llop E, Figueras J, Fort E, Sanz-Nebot V, de Bolós C, Rizzi A, Barrabés S, de Frutos M, Peracaula R.
J Proteomics. 2016 Jan 30;132:144-54. doi: 10.1016/j.jprot.2015.11.006
IF (2014): 3.888; Q1 in the Biochemical research methods category

Journal of Proteomics 132 (2016) 144–154

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Increased α1-3 fucosylation of α-1-acid glycoprotein (AGP) in pancreatic cancer

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ARTICLE INFO

Article history:
Received 20 July 2015
Received in revised form 29 September 2015
Accepted 6 November 2015
Available online 10 November 2015

Keywords:
α-1-acid glycoprotein
Capillary electrophoresis
Fucosylation
Aleuria aurantia lectin (AAL)
Mass spectrometry
Pancreatic cancer

ABSTRACT

Pancreatic cancer (PDAC) lacks reliable diagnostic biomarkers and the search for new biomarkers represents an important challenge. Previous results looking at a small cohort of patients showed an increase in α-1-acid glycoprotein (AGP) fucosylation in advanced PDAC using *N*-glycan sequencing. Here, we have analysed AGP glycoforms in a larger cohort using several analytical techniques including mass spectrometry (MS), capillary zone electrophoresis (CZE) and enzyme-linked lectin assays (ELLAs) for determining AGP glycoforms which could be PDAC associated. AGP from 31 serum samples, including healthy controls (HC), chronic pancreatitis (ChrP) and PDAC patients, was purified by immunoaffinity chromatography. Stable isotope labelling of AGP released *N*-glycans and their analysis by zwitterionic hydrophilic interaction capillary liquid chromatography electrospray MS (μZIC-HILIC–ESI-MS) showed an increase in AGP fucosylated glycoforms in PDAC compared to ChrP and HC. By CZE-UV analysis, relative concentrations of some of the AGP isoforms were found significantly different compared to those in PDAC and HC. Finally, ELLAs using *Aleuria aurantia* lectin displayed a significant increase in AGP fucosylation, before and after AGP neuraminidase treatment, in advanced PDAC compared to ChrP and HC, respectively. Altogether, these results indicate that α1-3 fucosylated glycoforms of AGP are increased in PDAC and could be potentially regarded as a PDAC biomarker.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths in Europe and the United States [1]. Its low survival rate (7% in 5 years) is due to the intrinsic aggressiveness of this tumour and also to a late diagnosis, usually when metastasis has occurred [1,2].

The only available biomarker for PDAC is CA19-9, but this marker is currently only used for monitoring disease progression because of its false positive rate [3]. Thus, it is important to search for new biomarkers able to differentiate PDAC from other benign diseases such as chronic pancreatitis (ChrP) which can also present high CA19-9 levels [4,5].

Glycosylation is a post-translational modification present in very many proteins, and alterations in protein glycosylation are a key feature of malignant transformation and tumour progression [6,7]. Those glycan alterations that are cancer associated usually affect the terminal structures of membrane-associated or secreted glycoproteins [8]. In this regard, glycomics and glycoproteomics have emerged as important tools in new biomarkers discovery [9,10]. Previously, our group performed *N*-glycan sequencing of major serum acute phase proteins, including AGP, in a small cohort of serum samples from healthy controls and PDAC patients [11]. α-1-Acid glycoprotein (AGP), or orosomucoid, is a 41–43 kDa glycoprotein with 5 complex-type *N*-linked glycans resulting in a carbohydrate content of 45% of its molecular mass [12]. *N*-glycan analysis showed different bi-, tri- and tetraantennary sialylated structures, including several sialofucosylated ones [11,13,14]. Interestingly, a minor biantennary, disialylated, core fucosylated glycan of AGP was found specifically increased in advanced PDAC [11].

Recently, we have also analysed the relative levels of AGP-released glycan species of eight pathological samples (two ChrP and six PDAC) vs. a control pool. This was done by stable isotope labelling of the

Abbreviations: AAL, *Aleuria aurantia* lectin; AGP, α-1-acid glycoprotein; ChrP, chronic pancreatitis; ELLA, enzyme-linked lectin assay; HC, healthy control; PDAC, pancreatic ductal adenocarcinoma; PhoSL, *Pholiota squarrosa* lectin; μZIC-HILIC–ESI-MS, zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry.

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http://dx.doi.org/10.1016/j.jprot.2015.11.006
1874-3919/

Table 1
Clinical and pathological characteristics of the patients.

Category	Cases	Stage (TNM system)	Gender	Age	Age average	±SD	Range
Healthy control (HC)	HC1[b]		Male	59	60.67	3.61	57–66
	HC2		Female	63			
	HC3[b]		Male	62			
	HC4		Male	57			
	HC5[b]		Male	66			
	HC6[b]		Female	57			
Chronic pancreatitis (ChrP)	ChrP1		Male	59	59.50	5.54	49–65
	ChrP2[a]		Female	62			
	ChrP3		Male	62			
	ChrP4		Female	49			
	ChrP5[a]		Male	65			
	ChrP6		Female	60			
Non-advanced PDAC	PDAC1	IB (T2N0M0)	Male	63	59.67	3.88	53–63
	PDAC2[a]	IIA (T3N0M0)	Female	62			
	PDAC3[a]	IIB (T3N1M0)	Male	57			
	PDAC4	IIA (T3N0M0)	Male	61			
	PDAC5	IIB (T3N1M0)	Male	62			
	PDAC6	IIB (T3N1M0)	Female	53			
Advanced PDAC	PDAC7	III (T4NxM0)	Male	58	60.46	8.01	45–73
	PDAC8	III (T4NxM0)	Female	59			
	PDAC9	III (T4NxM0)	Male	52			
	PDAC10	III (T4NxM0)	Female	69			
	PDAC11[a]	III (T4N1M0)	Male	67			
	PDAC12[a]	III (T4N0M0)	Male	55			
	PDAC13	III (T4N0M0)	Male	66			
	PDAC14	IV (TxNxM1)	Male	52			
	PDAC15	IV (T3N1M1)	Female	67			
	PDAC16	IV (TxNxM1)	Female	61			
	PDAC17	IV (T3N1M1)	Female	45			
	PDAC18[a]	IV (T3N1M1)	Male	73			
	PDAC19[a]	IV (T3N1M1)	Female	62			

HC, healthy control; ChrP, chronic pancreatitis; PDAC pancreatic ductal adenocarcinoma.
[a] Samples of the previous study [15].
[b] Samples that constitute the pool of HC for the *N*-glycan analysis by μZIC-HILIC–ESI-MS.

released glycans, separation of the various glycan species by zwitterionic hydrophilic interaction capillary liquid chromatography (μZIC-HILIC) and quantitation by electrospray mass spectrometry (ESI-MS). These previously obtained preliminary results showed an increase of several AGP fucosylated glycans in PDAC at advanced stages [15].

In the present work we have analysed the AGP glycosylation from serum samples in a larger cohort of individuals (N = 31) using different complementary analytical approaches to explore the potential value of AGP glycoforms as PDAC biomarker. This study is further aimed at establishing an enzyme linked lectin assay (ELLA) as a quantitative method that could be easily transferred into clinics.

Within this study, we have performed a relative quantification of the released AGP *N*-glycans using stable isotope labelling and subsequent μZIC-HILIC-MS. Further, AGP isoforms of the intact glycoprotein were analysed by capillary zone electrophoresis with UV detection (CZE-UV) and the different peaks, related to different carbohydrate moieties attached, were quantified and compared among groups. Finally, ELLAs using two different lectins specifically recognizing fucose were developed for determining differences in AGP glycoforms among groups of diseases. These methods showed specific differences in AGP glycosylation when comparing PDAC patients' serum with that of healthy controls and ChrP patients. In particular, significant increases in the α1-3 fucosylation levels were found in the AGP from PDAC patients.

2. Materials and methods

2.1. Serum samples

Serum samples were obtained from the Hospital Dr. Josep Trueta (Girona, Spain) following the standard operating procedures of its Ethics Committee. Samples corresponded to six healthy controls (two females and four males; age range 57–66), six chronic pancreatitis patients (three females and three males; age range 49–65), and nineteen pancreatic cancer patients (eight females and eleven males; age range 45–73; out of the cancer patients six were non-advanced stages, stage IB (T2N0M0), stage IIA (T3N0M0) and stage IIB (T1-2-3N1M0), and thirteen advanced stages, stage III (T4NxM0) and stage IV (T3NxM1) (Table 1). Patients were diagnosed by biopsy or image examination by the Digestive and Pathology Units.

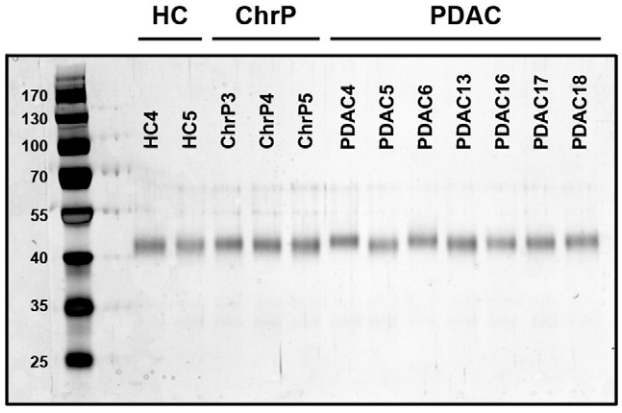

Fig. 1. Purity profiles of serum AGP isolated by immunoaffinity chromatography. Silver staining of 10% SDS-PAGE showing twelve representative samples.

2.2. Immunoaffinity column preparation

The immunoaffinity chromatography (IAC) column was made as previously described [16]. Briefly, Protein-Pak epoxy-silica of 40 μm particle size and 500 Å pore diameter (Millipore, Waters Chromatography Division, Milford, MA) was converted into diol-silica and oxidized to aldehyde-silica. The antibody goat anti-human AGP (Immune Systems Ltd., Paignton, Devon, UK) was transferred to coupling buffer (0.1 M sodium phosphate pH 5.7) and concentrated using centrifugal filter devices (Centricon 50, Amicon, Beverly, MA). The coupling was made by mixing the concentrated antibody and sodium cyanoborohydride with the supporting material for 65 h at 4 °C. The unbound aldehyde groups were reduced with sodium borohydride. The antibody-derivatized Protein-Pak material in phosphate buffered saline (PBS)

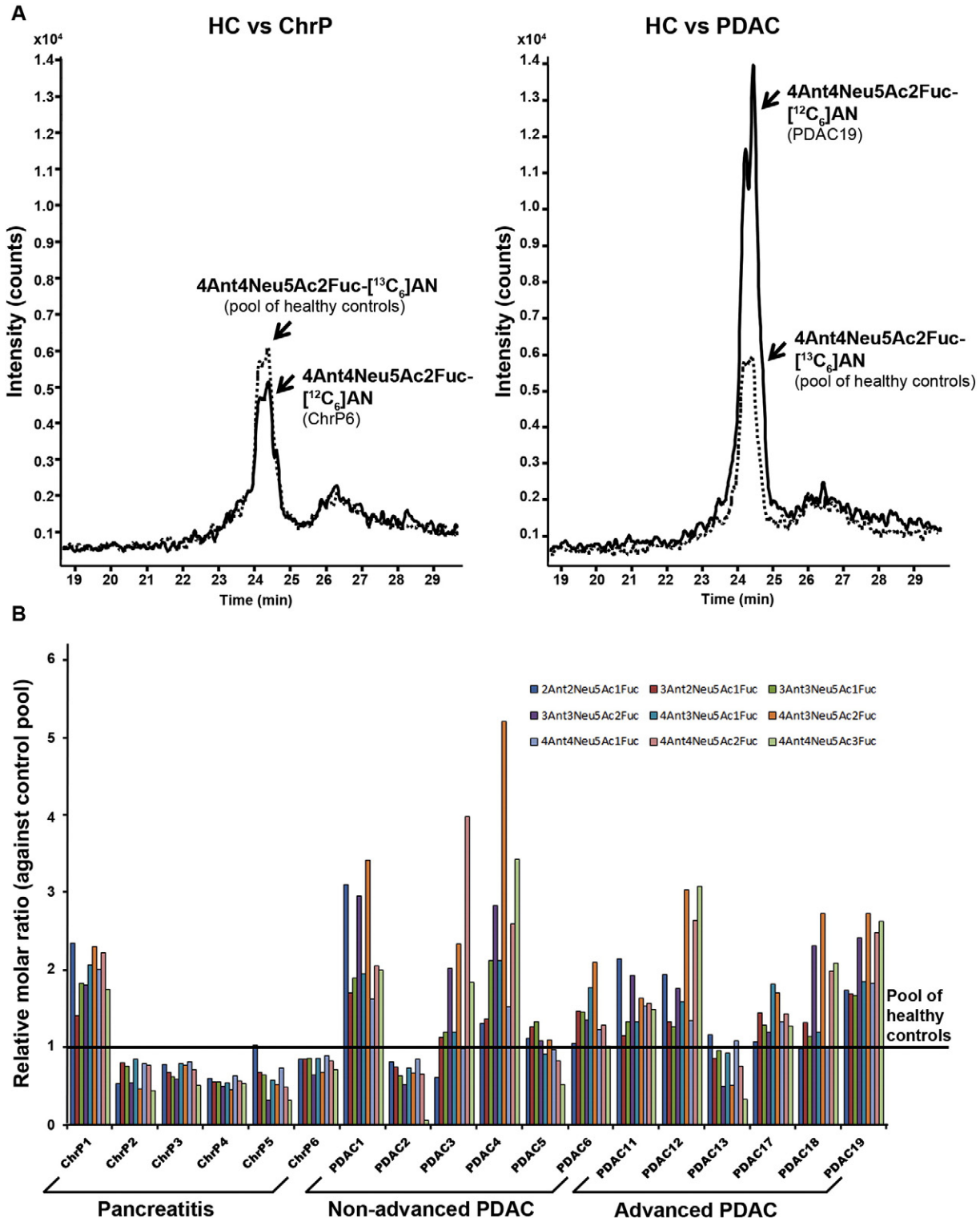

Fig. 2. μZIC-HILIC–ESI-MS analysis of aniline-labelled *N*-glycans released from AGP. (A) Extracted ion chromatograms of *N*-glycan species 4Ant4Neu5Ac2Fuc released from control AGP (dotted line) and PDAC or ChrP samples (solid line). (B) Experimental molar ratios determined for the sialofucosylated *N*-glycans of AGP. A molar ratio value of 1 is obtained when the glycan in the pathological sample has the same peak area as the glycan detected in the pool of healthy control samples.

was packed into the column by the slurry method. A 3 cm PEEK-lined stainless steel tubing of 4.6 mm i.d. provided with 1/4 to 1/16 reduction end fittings and with 2 μm pore diameter frits were used as the immunoaffinity column hardware (Grace Davison Discover Sciences, Deerfield, IL).

2.3. AGP immunopurification from serum samples

To purify AGP from sera, a previously developed method [16] with slight modifications was applied as follows. 100 μL of each serum sample was incubated for 30 min with 1% (*v*/*v*) of protease Inhibitor Cocktail (Sigma-Aldrich, Saint Louis, MO). Then 99 μL of water was added and the sera were pre-cleaned using a 0.22 μm Spin-X Centrifuge Tube Filter (Costar, Corning, NY) according to the manufacturer's protocol.

The immunopurification with the column described above was performed in an Äkta-FPLC system (GE Healthcare, Buckinghamshire, United Kingdom). PBS was used as mobile phase at 0.5 mL/min flow rate, and 0.1 M glycine/HCl pH 2.2 was used as desorption buffer. The sample (200 μL) was injected and elution with desorption buffer was carried out for 3 min at 0.5 mL/min (after this time, the absorbance signal returned to the baseline level). The eluted sample (about 1.4 mL) was collected and neutralized by the addition of 0.1 M Na_2HPO_4. This neutralized fraction was re-injected in the IAC column in order to properly purify AGP [17]. Three further desorption steps were performed but only the two first elution fractions were collected and neutralized. Afterwards, each sample was concentrated and desalted using a Microcon YM-3 Centrifugal Filter Device (Millipore, Billerica, MA) in order to avoid any undesired interference of salt ions in the following procedures. A blank cycle was performed prior to each serum sample immunopurification run.

2.4. Protein quantification and purity determination

Protein concentration in each sample was determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and using the extinction coefficient $E^{1\%} = 8.93$ at 278 nm.

AGP purity after isolation from serum was confirmed for each sample by loading an aliquot of 100 ng of protein under reducing conditions on a 10% SDS-PAGE with detection by silver-staining.

2.5. N-glycan analysis by zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry (μZIC-HILIC–ESI-MS)

Relative quantitation of AGP glycosylation variants by stable isotope coded labelling of AGP released *N*-glycans was carried out using $^{12}C_6/^{13}C_6$ aniline and μZIC-HILIC–ESI-MS as previously described [18]. Briefly, purified AGP was reduced and subjected to enzymatic digestion by PNGase F. The released *N*-glycans were purified by solid phase extraction using Hypercarb cartridges (Thermo Fisher Scientific, Waltham, MA). The dried glycans were differently labelled with 0.35 M aniline and precipitated. For their chromatographic separation, a 1200 series capillary liquid chromatography system (Agilent Technologies, Waldbronn, Germany) was employed, using a capillary column (150 mm × 0.33 mm) filled with 5 μm particles with surface-immobilized zwitterionic sulfobetaine moieties (SeQuant, Umeå, Sweden). This HPLC system was coupled to a 6220 oa-TOF LC/MS mass spectrometer (Agilent Technologies) [15].

2.6. Capillary zone electrophoresis

Capillary zone electrophoresis with UV–Vis detection (CZE-UV) was performed by an Agilent 7100 CE instrument (Waldbronn, Germany) using a detection wavelength of 214 nm. Agilent ChemStation software was used to control the instrument and to acquire and process data. The uncoated fused silica capillaries used (78.5 cm total length, 70 cm effective length, 50 μm i.d., 375 μm o.d.) were supplied by CM Scientific Limited (Silsden, United Kingdom). Separation was performed employing a method previously developed [16,19]. Shortly, purified AGP of each sample diluted all at the same concentration were injected at the anodic end of the capillary for 30 s at 35 mbar. The background electrolyte consisted of 10 mM tricine, 10 mM NaCl, 10 mM sodium acetate, 7 M urea and 3.9 mM putrescine, pH 4.5. Separation voltage and temperature were 25 kV and 35 °C, respectively. Peaks were aligned and assigned by using the effective electrophoretic mobility (μ_{eff}) as migration parameter for each peak and by comparison with the aligned AGP profile of the standard (G9885, Sigma-Aldrich) solution analysed on the same day. Each peak was quantified as the percentage of its corrected area (A_{corr}), which is calculated by multiplying the area counts by the migration velocity. The data is given as the percentage of corrected area (A_{corr} of a given peak in relation to the sum of A_{corr} of all peaks of the sample).

2.7. Enzymatic removal of sialic acid from purified AGP

One microgram of purified AGP at 0.2 mg/mL of each sample was digested overnight at 37 °C with 0.025 mg/mL of *Arthrobacter ureafaciens* neuraminidase (Roche Diagnostics, Mannheim, Germany) in 50 mM sodium phosphate pH 5.

2.8. Enzyme-linked lectin assay (ELLA) of purified AGP with Aleuria aurantia lectin (AAL) and Pholiota squarrosa lectin (PhoSL)

General fucosylation levels of purified AGP before and after treatment with neuraminidase were quantified by direct ELLA using biotinylated AAL (Vector Laboratories, Burlingame, CA). Core fucosylation levels were specifically determined using biotinylated PhoSL (kindly provided by Dr. Yuka Kobayashi from J-Oil mills, Inc., Yokohama, Japan). The procedure was optimized and performed as follows. All incubation steps, except the initial plate coating step with purified AGP, were performed at room temperature. Plate coating was performed with pure AGP samples diluted in coating buffer (0.05 M sodium carbonate, pH 9.6) and incubated for 1 h at 37 °C in 96-well plates (Thermo Fisher Scientific). The amounts of purified AGP used were established for each assay considering the LOQ, and the dynamic range (between 1.1 ng/μL and 9.4 ng/μL for AAL assay and between 0.6 ng/μL and 15 ng/μL for PhoSL assay). Thus, the fucosylation analyses prior to and after neuraminidase treatment were carried out with 2 ng/μL and 1 ng/μL AGP, respectively; and 8 ng/μL and 4 ng/μL AGP were used for core fucosylation determination. After washing the plate three times with washing buffer (0.9% NaCl solution, 0.05% Tween 20), wells were blocked for 1 h with 2% polyvinylpyrrolidone (PVP) (Sigma-Aldrich) in phosphate buffered saline (PBS). Wells were then washed and incubated for 2 h with either biotin labelled AAL at 4 μg/mL or biotin labelled PhoSL at 1 μg/mL in lectin buffer (150 mM NaCl, 0.1 M Tris–HCl, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). Plates were washed and incubated for 1 h with streptavidin-horseradish peroxidase-conjugated (GE Healthcare Bio-Sciences, Uppsala, Sweden) diluted 1/5000 in PBS, 0.1% Tween20, 1% BSA for the AAL assay and with the streptavidin-horseradish peroxidase conjugated from the Vectastain ABC kit (Vector Laboratories) diluted according to the manufacturer's instructions for the PhoSL assay. Plates were washed and colorimetric detection was performed using 100 μL/well of BM blue Peroxidase substrate soluble (Roche Diagnostics, Mannheim, Germany). Absorbance was read at 450 nm with a reference of 690 nm in an automated microplate reader (BIO-TEK, Winooski, VT) after stopping the reaction with 100 μL/well of 1 M H_2SO_4. In order to quantify the different glycan levels, a calibration curve of standard AGP was used. For each plate, duplicates of each sample were performed. The assays were conducted by triplicate, giving a total of 6 data points per sample. Only data with a $CV_{intraassay} < 10\%$ and a $CV_{interassay} < 20\%$ were considered for calculation of the mean values for each sample.

Table 2
Relative molar ratios of AGP *N*-glycan structures determined by μZIC-HILIC–ESI-MS for each individual patient and the mean and SD for each group of patients. The bold entries correspond to the mean and SD values of the samples of each group of patients for each glycan structure.

		Chronic pancreatitis							Pancreatic adenocarcinoma													
									Non-advanced							Advanced						
		ChrP1	ChrP2	ChrP3	ChrP4	ChrP5	ChrP6	Mean (±SD)	PDAC1	PDAC2	PDAC3	PDAC4	PDAC5	PDAC6	Mean (±SD)	PDAC11	PDAC 12	PDAC 13	PDAC 17	PDAC 18	PDAC 19	Mean (±SD)
Non-fucosylated structures	2Ant2Neu5Ac	1.34	0.95	0.66	0.79	0.73	0.68	**0.86 (±0.26)**	1.69	0.92	0.45	0.93	1.58	1.00	**1.10 (±0.46)**	1.24	0.76	1.38	1.09	0.52	0.68	**0.95 (±0.34)**
	3Ant2Neu5Ac	0.89	0.67	0.63	0.77	0.62	0.78	**0.73 (±0.11)**	0.87	0.92	0.36	0.67	1.37	1.12	**0.88 (±0.35)**	0.82	0.54	1.29	0.94	0.39	0.59	**0.76 (±0.32)**
	3Ant3Neu5Ac	1.01	0.73	0.63	0.72	0.69	0.90	**0.78 (±0.15)**	0.92	0.87	0.37	0.79	1.59	1.23	**0.96 (±0.41)**	0.93	0.62	1.33	1.02	0.52	0.91	**0.89 (±0.29)**
	4Ant3Neu5Ac	1.10	0.65	0.79	0.79	0.62	0.82	**0.79 (±0.17)**	0.70	1.09	0.34	0.51	1.06	1.25	**0.82 (±0.36)**	0.83	0.41	1.33	1.03	0.40	0.73	**0.79 (±0.36)**
	4Ant4Neu5Ac	1.16	0.57	0.72	0.75	0.68	0.77	**0.77 (±0.20)**	0.71	1.13	0.26	0.46	1.01	1.01	**0.76 (±0.35)**	0.92	0.49	1.35	1.10	0.38	0.66	**0.81 (±0.37)**
Fucosylated structures	2Ant2Neu5Ac1Fuc	2.33	0.53	0.78	0.60	1.03	0.85	**1.02 (±0.67)**	3.09	0.81	0.61	1.31	1.12	1.05	**1.33 (±0.89)**	2.14	1.93	1.16	1.07	0.98	1.74	**1.50 (±0.49)**
	3Ant2Neu5Ac1Fuc	1.40	0.80	0.68	0.55	0.68	0.85	**0.83 (±0.30)**	1.70	0.75	1.12	1.36	1.26	1.47	**1.28 (±0.32)**	1.15	1.33	0.85	1.44	1.32	1.69	**1.30 (±0.28)**
	3Ant3Neu5Ac1Fuc	1.83	0.75	0.62	0.56	0.64	0.86	**0.87 (±0.48)**	1.89	0.63	1.19	2.11	1.33	1.46	**1.43 (±0.53)**	1.33	1.26	0.96	1.28	1.14	1.66	**1.27 (±0.23)**
	3Ant3Neu5Ac2Fuc	1.80	0.54	0.59	0.50	0.31	0.64	**0.73 (±0.54)**	2.95	0.52	2.02	2.82	1.08	1.35	**1.79 (±0.98)**	1.92	1.76	0.49	1.20	2.31	2.41	**1.68 (±0.72)**
	4Ant3Neu5Ac1Fuc	2.06	0.85	0.79	0.55	0.58	0.86	**0.95 (±0.56)**	1.94	0.74	1.19	2.11	0.91	1.77	**1.44 (±0.57)**	1.33	1.59	0.92	1.81	1.20	1.84	**1.45 (±0.36)**
	4Ant3Neu5Ac2Fuc	2.30	0.46	0.77	0.45	0.52	0.67	**0.86 (±0.71)**	3.40	0.67	2.33	5.20	1.09	2.09	**2.46 (±1.65)**	1.64	3.03	0.51	1.70	2.72	2.72	**2.05 (±0.95)**
	4Ant4Neu5Ac1Fuc	2.00	0.79	0.81	0.63	0.73	0.88	**0.97 (±0.51)**	1.62	0.85	0.97	1.52	0.97	1.23	**1.19 (±0.32)**	1.53	1.34	1.08	1.33	0.99	1.82	**1.35 (±0.30)**
	4Ant4Neu5Ac2Fuc	2.22	0.77	0.71	0.56	0.48	0.82	**0.93 (±0.65)**	2.05	0.66	3.97	2.59	0.82	1.29	**1.90 (±1.25)**	1.56	2.64	0.75	1.43	1.98	2.47	**1.81 (±0.70)**
	4Ant4Neu5Ac3Fuc	1.74	0.44	0.51	0.53	0.32	0.71	**0.71 (±0.52)**	1.99	0.05	1.83	3.42	0.52	1.02	**1.47 (±1.21)**	1.48	3.06	0.33	1.27	2.08	2.62	**1.81 (±0.99)**

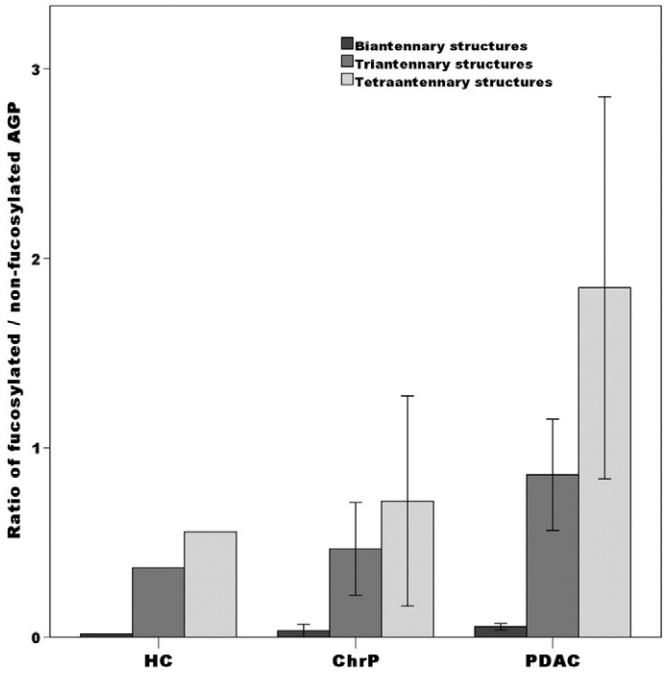

Fig. 3. Ratio of fucosylated/non-fucosylated AGP glycan structures. This was determined by dividing the molar ratios obtained by *N*-glycan μZIC-HILIC–ESI-MS analysis of fucosylated structures by the corresponding values of non-fucosylated structures. *N*-glycans with the same number of antennae are considered together and the averages of each group of samples are depicted as histograms with ± standard error (SE). HC does not present SE since it corresponds to a pool of samples and is analysed by μZIC-HILIC–ESI-MS as a single one.

2.9. Statistical analysis

Mean or median comparisons of the variables were performed using the SPSS statistical software for Windows (version 19; SPSS Inc., Chicago, IL). The variables of each group (HC, ChrP and PDAC) were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene's tests. When these criteria were accomplished (capillary zone electrophoresis data) intergroup differences were analysed by means of ANOVA with Tukey post-test. On the other hand, for non-normally distributed groups (data from lectin assays) Kruskal–Wallis test was performed. For all the analyses, $p < 0.05$ was considered statistically significant.

3. Results

3.1. AGP immunopurification

AGP from serum samples was purified using an immunoaffinity column [16,20] in order to assure that the glycans analysed by the following approaches belonged only to AGP and could not come from other serum proteins. Silver staining of a SDS-PAGE of the purified AGP fractions collected from the immunoaffinity column confirmed AGP purity (Fig. 1).

3.2. N-glycan analysis by μZIC-HILIC–ESI-MS

Glycan analysis by mass spectrometry is a technique widely used to determine glycan structures. In this study, we applied our described methodology involving isotope-coded labelling for improved quantification. Technically, this approach ionizes two differently labelled samples at the same time and compares intensity differences in the obtained profile. In this way we could monitor changes in the glycosylation pattern of AGP in control vs. patient samples by direct comparison.

The levels of *N*-glycan structures released from AGP isolated from 6 ChrP and 12 PDAC samples, comprising 6 non-advanced and 6 advanced PDAC, were determined in comparison to a pool of control (Fig. 2A). The pool of control was prepared by mixing the same amount of AGP purified from four healthy control (HC; n = 4, HC1, HC3; HC5 and HC6, Table 1) sera, followed by the subsequent release of the *N*-glycans with PNGase F and labelling. HILIC-HPLC combined with ESI-MS provided information regarding the monosaccharide composition of each glycan species considered. Several bi-, tri- and tetraantennary sialylated and sialofucosylated *N*-glycans were identified; their occurrence in AGP had been previously described by other authors [12,15,21–23]. Bi-, tri- and tetraantennary glycans with one sialic acid, however, were not detected using this approach. Among the structures identified (Table 2), the following sialofucosylated structures were found: a biantennary, disialylated structure with one fucose (2Ant2Neu5Ac1Fuc), a triantennary, disialylated structure with one fucose (3Ant2Neu5Ac1Fuc), a triantennary, trisialylated structure with one fucose (3Ant3Neu5Ac1Fuc) and two fucoses (3Ant3Neu5Ac2Fuc), a tetraantennary trisialylated structure with one fucose (4Ant3Neu5Ac1Fuc) and two fucoses (4Ant3Neu5Ac2Fuc) and a tetraantennary tetrasialylated structure with one fucose (4Ant4Neu5Ac1Fuc), two fucoses (4Ant4Neu5Ac2Fuc) and three fucoses (4Ant4Neu5Ac3Fuc). The absolute levels of the biantennary structure (2Ant2Neu5Ac1Fuc) were found being very low, accounting for less than the 0.7% of all *N*-glycan structures. This is of interest, as this structure is considered – as will be discussed later – to be the only one with core α1-6-fucosylation according to the literature [11]. The rest of sialofucosylated structures, which is considered to contain exclusively external (i.e. antenna-linked) α1-3-fucoses, because only type II structures (Galβ1-4GlcNAc) has been described in AGP [11], accounted for 19–68% of *N*-glycan structures, with different percentages depending on the samples. Di- and tri-fucosylated structures on AGP had not been described before by this technology. However, they had been previously reported by other authors using different methodologies [11,21]. They accounted for 2–31% of *N*-glycan structures also depending on the samples.

Using the μZIC-HILIC–ESI-MS approach, differences between PDAC and ChrP samples were found in the relative levels of the nine sialofucosylated glycan structures (Fig. 2B). ChrP samples showed lower levels of all sialofucosylated structures when compared to healthy control pool (with the exception of one sample, ChrP1). In contrast, PDAC samples contained higher levels of the sialofucosylated glycans than the control pool, with the exception of one non-advanced PDAC (PDAC2) (Fig. 2B). The profile of the nine sialofucosylated glycans was different among the PDAC samples, and these structures were more highly abundant in most of the advanced cancers than in the control pool. Four and five of the sialofucosylated structures of PDAC5 and PDAC13, respectively, were equally or less abundant than in the control pool while the rest of the structures showed higher levels than in the control pool. For each sample the ratio sum of fucosylated/sum of non-fucosylated structures was calculated and an increasing trend in this ratio was observed in PDAC samples (Fig. 3).

In contrast, no differences among groups could be detected when comparing the content of sialic acids and the extent of branching of *N*-glycan structures (data not shown).

3.3. Capillary electrophoresis analysis of AGP

Capillary zone electrophoresis with UV detection was able to separate purified AGP from each of the 31 serum samples (6 HC, 6 ChrP, 19 PDAC) into 12 peaks corresponding to isoforms. This is shown in Fig. 4A for a typical sample of PDAC. Migration in CZE is related to the charge to size ratio of the molecules and, therefore, differences in AGP glycosylation between AGP isoforms which contribute to changes in size and/or charge can modify the AGP electropherogram [17]. From the twelve peaks obtained, peak 1 was omitted in the analysis

because of its weak signal which made it undetectable in some of the samples. The corrected area percentage values of the eleven analysed peaks were calculated for each of the serum samples and statistically different profiles between HC and PDAC sera were found (Table 3 and Fig. 4B). In particular, a decrease in the peak areas of isoforms with lower charge/size ratio (the earlier migrating peaks 4, 5 and 6) concomitant to an increase of the areas for AGP isoforms with higher charge/size ratio (peaks 8, 9 and 10) were observed when comparing the PDAC samples with HC.

ChrP samples showed an intermediate percentage of peak areas, non-significantly different from PDAC or HC peak areas. Interestingly, the peak area distribution of ChrP1 sample was more similar to some PDAC samples than to any ChrP sample (data not shown), a similar behaviour already detected when the *N*-glycans from ChrP1 were analysed by μZIC-HILIC–ESI-MS.

3.4. Fucosylation levels of AGP using AAL and PhoSL lectins

Fucosylation levels of AGP were analysed with an enzyme-linked lectin assay (ELLA) using *A. aurantia* lectin (AAL) which recognizes fucoses α1-2, α1-3 or α1-6-linked [7] and with *P. squarrosa* lectin (PhoSL) which recognizes only α1-6-linked fucose [24]. Both assays were optimized with standard AGP previously purified by immunoaffinity chromatography, and the amount of AGP/well for each assay

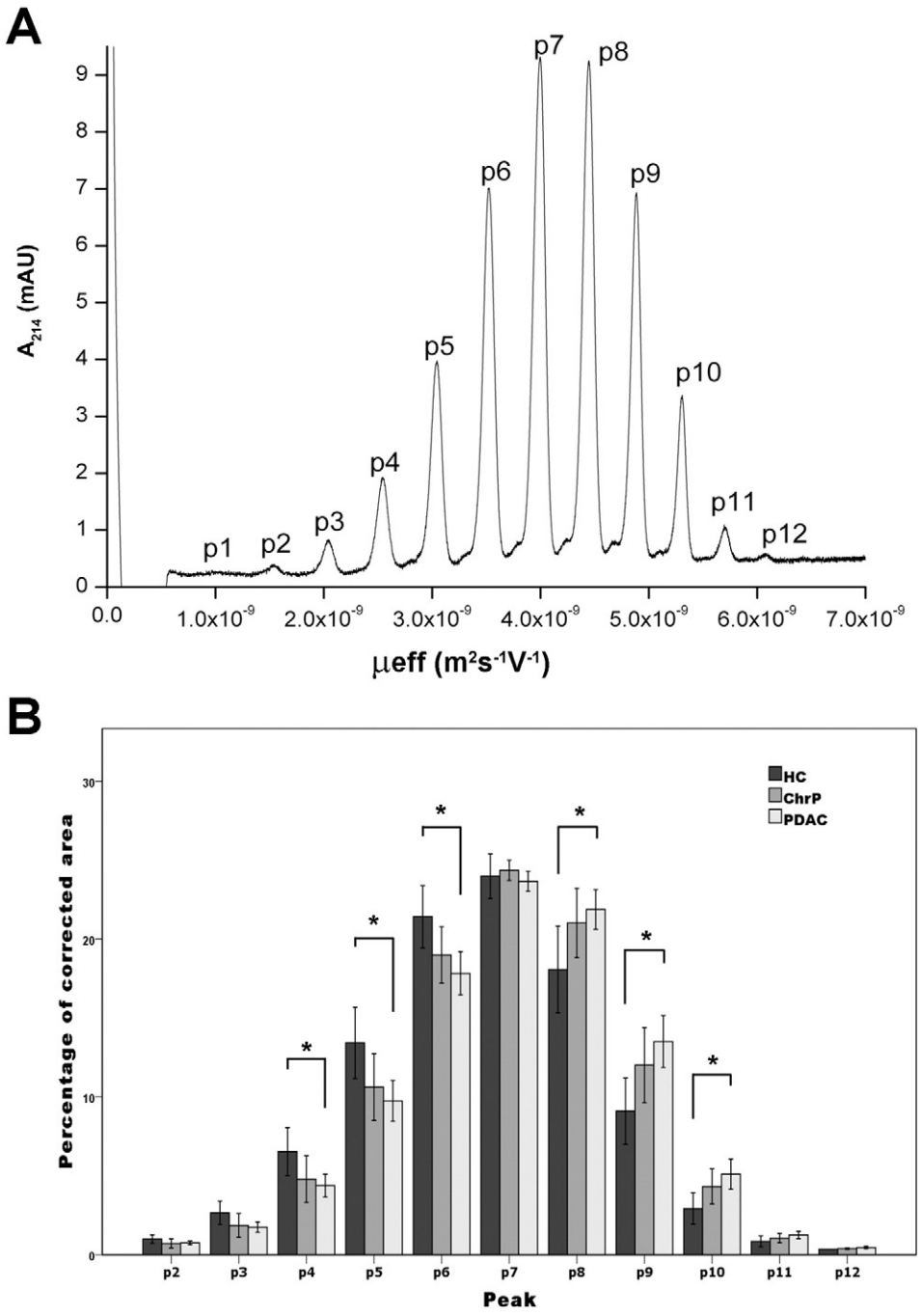

Fig. 4. AGP analysis by capillary electrophoresis. (A) Example of capillary electrophoresis profile of purified serum AGP. Each identified peak is labelled according to its migration time from p1 (the first to migrate) to p12 (the last one to migrate). (B) Statistical comparison of percentage of corrected area of each peak of serum AGP from different samples grouped in HC, ChrP and PDAC analysed by CE-UV. ANOVA test with Tukey post-test was used ($p < 0.05$ was considered statistically significant, labelled as *).

Table 3
Capillary zone electrophoresis. Mean values of the percentage of the peak corrected area for each group of patients. The bold entries correspond to the p values that show statistically significant differences between the groups of study.

Peak	Mean (±SD)			p-Value (ANOVA)		
	HC	ChrP	PDAC	HC vs ChrP	HC vs PDAC	ChrP vs PDAC
p2	0.99 (±0.33)	0.72 (±0.35)	0.75 (±0.23)	0.229	0.194	0.966
p3	2.66 (±0.92)	1.85 (±0.93)	1.75 (±0.72)	0.208	0.056	0.959
p4	6.54 (±1.86)	4.79 (±1.81)	4.38 (±1.57)	0.186	**0.027***	0.857
p5	13.42 (±2.77)	10.62 (±2.59)	9.75 (±2.81)	0.204	**0.022***	0.781
p6	21.42 (±2.41)	19.00 (±2.18)	17.83 (±3.00)	0.301	**0.026***	0.644
p7	23.98 (±1.74)	24.35 (±0.79)	23.66 (±1.38)	0.885	0.868	0.528
p8	18.07 (±3.36)	21.02 (±2.70)	21.88 (±2.72)	0.189	**0.021***	0.798
p9	9.1 (±2.57)	12.01 (±2.92)	13.51 (±3.59)	0.296	**0.022***	0.606
p10	2.93 (±1.20)	4.33 (±1.37)	5.11 (±2.09)	0.401	**0.045***	0.643
p11	0.84 (±0.43)	1.05 (±0.35)	1.25 (±0.51)	0.727	0.166	0.628
p12	0.36 (−)	0.38 (±0.04)	0.45 (±0.13)	–	–	–

* Statistically significant ($p < 0.05$).

was established according to the sensitivity (based on LOQ and LOD) and dynamic range of the corresponding AAL and PhoSL ELLAs.

General fucosylation levels of the different AGP samples were determined using AAL. In this case, 2 ng/μL of purified AGP/well from the cohort of 6 HC, 6 ChrP and 19 PDAC patients' sera were used. The ratio of fucosylated AGP was calculated dividing the value of fucosylated AGP – obtained from the ELLA – by the amount of AGP loaded in the ELLA (Fig. 5A and Table 4). As shown in Fig. 5A, the group of advanced PDAC showed significantly higher AGP fucosylated ratio than the ChrP patients. The lower reactivity of the ChrP group to this lectin may indicate either a lower AGP fucosylation level or a higher masking effect on the fucose by the presence of other monosaccharides (such as sialic acid attached nearby an external fucose). To better elucidate this issue, we investigated whether or not the removal of sialic acid affects AAL recognition. For that, all AGP samples were treated with neuraminidase, and ELLA with AAL was performed using 1 ng/μL of digested AGP/well. Doing so, higher fucosylation levels were obtained with neuraminidase treated AGP samples (calculated according to a calibration curve with non-treated standard AGP). Statistical analysis of fucosylation levels measured with AAL after neuraminidase digestion showed again a fucosylation increase in PDAC samples compared with HC and ChrP samples. This increase was more pronounced with AGP from advanced PDAC patients leading to fucosylation levels significantly different from those observed in HC (Fig. 5B and Table 4). Thus, sialic acid hinders recognition by AAL, being the ratio of fucosylated AGP detected with AAL after sialic acid digestion higher than before the removal of the sialic acid.

Interestingly, the samples that showed higher fucosylation ratios in the ELLA with intact AGP were the same that showed higher fucosylation ratios in the ELLA with AGP after neuraminidase digestion, and vice versa. This could indicate homogeneous sialylation levels among the samples, which agrees with our previous results obtained by μZIC-HILIC–ESI-MS that did not show significant differences in the sialic acid content of the AGP *N*-glycan structures among groups. Interestingly, the extreme value in the ChrP group of the AGP neuraminidase digested samples corresponded to ChrP1, the same sample that showed higher fucosylation levels by μZIC-HILIC–ESI-MS and a more different profile among the ChrP samples when analysed by CZE-UV. Also, the samples PDAC2 and PDAC13, which showed low levels of fucosylated structure by μZIC-HILIC–ESI-MS, were the PDAC samples with a lower AAL signal after sialic acid digestion.

The measure of core fucosylation (α1-6-linked fucose) levels of AGP *N*-glycans that are contained in the minor biantennary disialylated structure was addressed by an ELLA with PhoSL. For that, 4 ng/μL and 8 ng/μL of purified AGP/well were used. No differences in AGP core fucosylation levels among the three different groups were detected (data not shown). Thus, the increased fucosylation levels of AGP associated to PDAC is attributed to α1-3-linked fucoses.

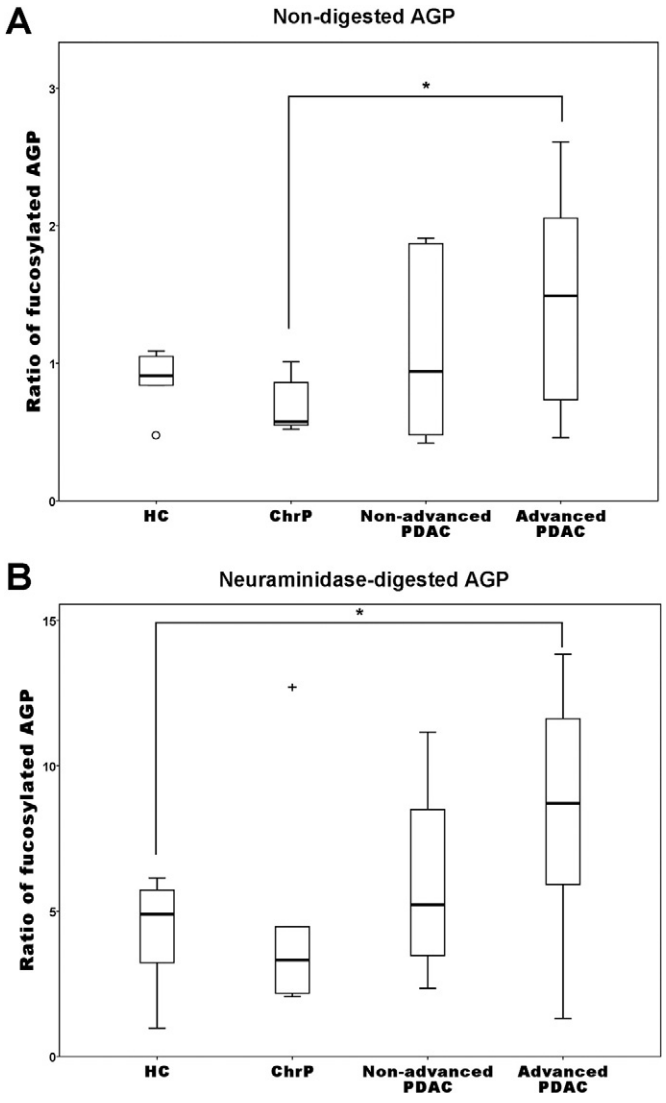

Fig. 5. ELLA analysis of purified AGP using *Aleuria aurantia* lectin (AAL). Boxplots describe the median and the sample distribution within a group. (A) Ratio of fucosylated AGP/total AGP analysed by ELLA. (B) Ratio of fucosylated AGP/total AGP by direct ELLA after neuraminidase treatment for removal of sialic acids. Non-parametric Kruskal–Wallis test was used for statistical analysis ($p < 0.05$ was considered statistically significant, labelled as *). Outlier data are indicated as o and extreme values as +.

Table 4
Aleuria aurantia lectin assays. Mean values of the ratios of fucosylated AGP/total AGP before and after neuraminidase digestion. The bold entries correspond to the p values that show statistically significant differences between the groups of study.

	Mean (±SD)				p-Value (Kruskal–Wallis)					
	HC	ChrP	Non-advanced PDAC	Advanced PDAC	HC vs ChrP	HC vs non-advanced PDAC	HC vs advanced PDAC	ChrP vs non-advanced PDAC	ChrP vs advanced PDAC	Non-advanced PDAC vs advanced PDAC
AGP	0.87 (±0.25)	0.68 (±0.20)	1.09 (±0.65)	1.45 (±0.74)	0.273	0.715	0.154	0.522	**0.025***	0.303
Neuraminidase-digested AGP	4.31 (±1.92)	4.68 (±4.05)	5.99 (±3.74)	8.31 (±3.84)	0.423	0.670	**0.028***	0.286	0.096	0.213

* Statistically significant ($p < 0.05$).

4. Discussion

Fucosylation is one of the most important oligosaccharide modifications involved in cancer and inflammation [25]. Different studies link the presence of fucosylated epitopes on specific glycoproteins with cancer. In particular, Breborowicz and collaborators [26] demonstrated that fucosylated alpha-fetoprotein is more specific as a hepatocellular carcinoma (HCC) biomarker than alpha-fetoprotein (AFP) and nowadays fucosylated AFP (AFP-L3) is used for HCC risk assessment [27]. Likewise, other authors reported that high levels of fucosylated haptoglobin were produced in the advanced stages of several types of cancer such as ovarian, lung, breast and also pancreatic cancer [28,29]. In PDAC, Shimomura and co-workers described recently a lectin assay using PhoSL to analyse α1-6-fucosylated haptoglobin in serum. They concluded that the increase in the fucosylation levels of haptoglobin in PDAC was more related to fucose linked α1-3 than α1-6 [30]. Remarkably, these results are in agreement with the ones we describe here for AGP, suggesting an increase in α1-3 fucosylated acute phase proteins in PDAC.

AGP is a highly glycosylated protein with 5 *N*-glycosylation sites. It is known to contain bi-, tri-, and tetraantennary structures. Biantennary structures can contain one or two sialic acids and the disialylated biantennary structure can contain a fucose. Regarding the fucosylated structure 2Ant2Neu5Ac1Fuc, our group previously showed that the fucose of this minor structure was α1-6-linked (core fucose), while the rest of tri- and tetraantennary fucosylated structures contained only external fucoses α1-3-linked [11]. Both *N*-glycan sequencing [11] and mass spectrometry [15] showed that this fucosylated biantennary structure represents a low proportion among all AGP glycan structures. AGP triantennary structures can be di- or trisialylated and one or two external fucose linked α1-3 can be present. For tetraantennary glycans, AGP show many different structures with 2, 3 or 4 sialic acids. The trisialylated tetraantennary structures can present one or two external fucoses and the tetrasialylated forms can present 1, 2 or 3 external fucoses linked α1-3 [11]. Our previous studies using a smaller cohort of patients indicated that an increase in monofucosylated glycans could be associated to PDAC [11,15]. Here, using a larger cohort of patients and extending the analysis to di- and tri-fucosylated glycans, we have shown an increase in most of the fucosylated structures of AGP released *N*-glycans in PDAC compared to ChrP and HC using μZIC-HILIC–ESI-MS. These results have been confirmed using an AAL lectin assay that recognizes fucosylated structures including α1-3 and α1-6 linked ones. Advanced PDAC showed a significant increase in AGP fucosylation compared with HC detected by AAL after sialic acid removal. The same outcome was found for PDAC vs. ChrP patients when no removal of sialic acids was performed. In addition, it must be pointed out that, although not being statistically significant, AGP from non-advanced PDAC sera also showed an increased tendency for fucosylation compared to non-malignant situations. No differences between PDAC, ChrP and HC could be detected using the specific core fucose recognizing lectin PhoSL. Both methods, MS and ELLA, have provided complementary information on AGP glycans. While MS identified and quantified the fucosylated glycans of AGP by the type of branching and the content of sialic acid, ELLAs with different binding specificity quantified the AGP fucosylated glycans distinguishing between fucose linkage positions. Altogether both methods have converged in that structures containing α1-3 linked fucose were found increased in PDAC. Thus, a quantitative ELLA based on AAL is a potential tool to detect fucosylated AGP. This methodology could be used to validate the usefulness of fucosylated AGP as a PDAC biomarker in a larger cohort.

CZE-UV analysis of AGP isoforms showed a general increase of peaks with larger charge/size ratio for cancer samples compared to chronic pancreatitis and controls. The same trend had been found in the CZE-UV analysis of AGP samples from the serum of bladder cancer patients compared to that of controls [31]. The CZE-MS analysis of glycans and of the intact AGP for the same samples showed an increased expression of AGP isoforms with fucosylated tri- and tetraantennary glycans, and a decrease on tri- and tetraantennary non-fucosylated AGP isoforms in bladder cancer patients when compared to healthy controls [32]. Though it is not clear whether different fucosylation of the AGP isoforms or rather different degrees of sialylation are the cause of the shift observed in the quantitative isoform pattern by CZE-UV (Fig. 4B), this shift can be seen as an additional diagnostic tool distinguishing the PDAC group from ChrP and HC groups.

AGP, as well as alpha-fetoprotein and haptoglobin mentioned above, are acute phase proteins. Aberrantly glycosylated acute phase proteins may modulate macrophage activity in tumour microenvironment by interacting with its receptors and contribute to tumour cell survival, growth and metastasis [33]. Several studies propose AGP glycoforms for diagnosis and management of cancer. Hashimoto and collaborators [34] showed by cross-affinity immunoelectrophoresis (CAIE) using AAL and concanavalin A that cancer patients who had fucosylated tri- and tetraantennary AGP for long periods after surgery had poor prognosis. Also, Mackiewicz and Mackiewicz [35] described a decrease in concanavalin-A reactivity of serum AGP from cancers originating in the liver, pancreas or bile duct. By contrast, these changes were not detected in metastatic liver tumours. AGP has also been analysed as a biomarker for breast cancer but the studies reveal that, although increased levels of AGP can be detected in plasma of patients with breast cancer, the concentration is unrelated to disease progression. However, monosaccharide composition and oligosaccharide profiles of the AGP glycosylation were found to differ between normal, non-invasive and invasive groups of breast cancer patients [36]. Likewise, Croce and collaborators reported a correlation between AGP and sLe^x expression in colorectal cancer tissues [37]. Also, specific changes in both expression and glycosylation of genetic variants of AGP in human malignant mesothelioma have been found [38]. Most of the methods reported in the aforementioned studies allow only a semi-quantification of the different AGP glycoforms. Here, we have used quantitative methods to analyse the N-glycans of purified AGP and have described fucosylation changes therein by mass spectrometry and ELLA. In order to avoid potential unspecific binding of other major serum fucosylated proteins that could produce unspecific signal in sandwich assays, here we have used immunoaffinity purified AGP for the accurate quantification of fucosylated AGP in the ELLA with AAL.

The mechanisms of post-translational modifications of AGP during diseases are still unknown. During the last phase of acute inflammation, high levels of AGP are found in plasma but its glycan branching degree

corresponded to that observed in non-inflamed status. Hence, different pathways regulating the expression of the protein on the one hand and the modification of the oligosaccharide structures attached on the other hand may probably exist [21]. Fucosylation involves the action of fucosyltransferases, GDP-fucose synthetic enzymes, and GDP-fucose transporter [39]. The reason for the increased fucosylation of AGP in the serum of patients with pancreatic cancer could be explained by different mechanisms, which are not exclusive one from another. Although AGP is produced mainly by the liver, there are studies reporting AGP expression in other tissues as well [21,37]. Also, there is the possibility of PDAC cells secreting factors that could induce the production of fucosylated AGP by the liver. This hypothesis has already been described by Okuyama and collaborators for another acute-phase protein, haptoglobin. They detected haptoglobin mRNA in a pancreatic adenocarcinoma cell line (PSN-1) and also described an increased production of fucosylated haptoglobin by hepatic cells (Hep3B) after stimulation with PSN-1 conditioned medium [40]. Moreover, inflammatory cytokines, such as interleukin-1β, have been reported to increase the secretion levels of AGP by HuH-7 hepatocyte cell line; and also to alter AGP sugar chain expression resulting in higher binding affinities to AAL, *Sambucus sieboldiana* agglutinin and concanavalin-A lectins [41]. In addition, the stimulation of HuH-7 cells with interleukin-1β induces an enhanced expression of the fucosyltransferase gene 6 (*FUT6*), which could explain the increase in α1-3 fucosylated structures [42]. In this regard, we have also described higher expression levels of *FUT3* and *FUT6* in PDAC tissues compared to normal tissues, which may account for the increase of α1-3 fucosylated structures in PDAC [43]. Discovering the mechanisms that produce these changes in AGP fucosylation in PDAC could help to develop novel targeted cancer therapies.

5. Conclusions

We have identified increased fucosylation levels of serum AGP in pancreatic cancer compared to healthy controls and chronic pancreatitis patients. *N*-glycan analysis showed an increase of the AGP sialofucosylated glycan structures in PDAC samples. We would be able to distinguish between HC and PDAC patient groups by capillary zone electrophoresis of AGP isoforms or by ELLA with AAL of neuraminidase digested AGP. We could also differentiate ChrP and PDAC patients by ELLA with AAL of purified AGP. These results suggest that AGP fucosylation levels could be useful as a PDAC marker, what should be confirmed in a larger cohort study in combination with the measure of other potential biomarkers. The different approaches used in this work show the suitability and complementarity of these techniques for the discovery of new potential biomarkers based on altered glycosylation of serum proteins.

Transaparency document

The Transparency document associated with this article can be found, online version.

Acknowledgements

M.B. acknowledges the University of Girona for a pre-doctoral fellowship. This work was supported by the Government of Catalonia (grant 2014 SGR 229), the Spanish Ministry of Science and Innovation (grant BIO 2010-16922, awarded to R. P) and the Spanish Ministry of Economy and Competitiveness (grant CTQ2013-43236, awarded to M.F. and grant CTQ2011-27130, awarded to V. S-N).

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Results - Chapter 2

Identification of potential pancreatic cancer serum markers: Increased sialyl-Lewis X on ceruloplasmin

This chapter has been published in the journal *Clinica Chimica Acta*.
Balmaña M, Sarrats A, Llop E, Barrabés S, Saldova R, Ferri MJ, Figueras J, Fort E, de Llorens R, Rudd PM, Peracaula R.
Clin Chim Acta. 2015 Jan 14; doi: 10.1016/j.cca.2015.01.007
IF (2014): 2.824; Q1 in the Medical Laboratory category

Clinica Chimica Acta 442 (2015) 56–62

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Identification of potential pancreatic cancer serum markers: Increased sialyl-Lewis X on ceruloplasmin

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ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form 19 December 2014
Accepted 11 January 2015
Available online 14 January 2015

Keywords:
Acute-phase proteins
Biomarker
Ceruloplasmin
Pancreatic cancer
Pancreatitis
Sialyl-Lewis X

ABSTRACT

Pancreatic adenocarcinoma (PDAC) usually shows an enhanced expression of sialyl-Lewis X (sLe^x) and related epitopes. PDAC may secrete some of the proteins carrying such increased sLe^x determinant into serum, so they could be used as PDAC markers. Previously, we identified acute-phase proteins with increased sLe^x in both PDAC and in chronic pancreatitis patients. In this study, depleted sera from the main acute-phase proteins has been analysed for the search of proteins with increased sLe^x levels in PDAC. Sera from healthy controls, chronic pancreatitis and PDAC patients were depleted, electrophoresed and subjected to sLe^x immunodetection. Proteins that differentially expressed sLe^x in PDAC were trypsin digested and identified by LC-ESI-QTOF mass spectrometry. Five protein bands that differentially expressed sLe^x in PDAC were identified and corresponded to seven different acute-phase proteins. Among them, ceruloplasmin (CP) was selected for further analysis. *N*-glycan sequencing of CP confirmed the increase of sLe^x levels in CP in PDAC patients. Healthy controls, chronic pancreatitis and PDAC patients' sera were immunoprecipitated with anti-CP antibodies, and their sLe^x and CP levels were analysed by western blot. The sLe^x/CP ratio tended to be higher for the PDAC group, which altogether suggests that the sLe^x/CP ratio could be a useful biomarker for PDAC.

1. Introduction

Pancreatic cancer (PDAC) has the lowest 5-year survival rate (about 5%) of all cancer types. Although only representing around 3% of all cancer cases, it was the fourth leading cause of cancer death in Europe and the United States [1]. This poor survival may be attributed to its late diagnosis, usually performed after metastases have occurred. Early detection of pancreatic cancer would improve 5-year survival rate to 20% [1,2].

CA19-9 serum detection is currently used to monitor PDAC patients. However, its use in diagnosis is restricted by its false positive results, as it is also increased in patients with benign pancreaticobiliary disorders such as chronic pancreatitis (ChrP) [3,4]. Thus, the availability of adequate biomarkers for PDAC detection is of major interest.

Glycosylation changes are a universal feature of malignant transformation and tumour progression. These changes can be found either in tumour cell surface or in secreted glycoconjugates. Glycan changes in malignant cells take a variety of forms, usually affecting terminal glycan structures [5]. In particular, sialyl-Lewis X (sLe^x) and related Lewis antigens have been found to be overexpressed in PDAC cell lines [6,7] and tissues [8–10]. An increase of sialylated Lewis antigens and both fucosylation and sialylation of certain glycoproteins have been detected in the sera of PDAC patients compared to healthy individuals and ChrP patients [11–13]. These data suggest that pancreatic tumour may shed into the blood glycoproteins carrying sLe^x, which could be used as PDAC tumour markers.

In a previous work, we identified serum glycoproteins carrying increased sLe^x in both advanced PDAC and chronic pancreatitis patients [14]. However, these proteins corresponded to major acute-phase proteins (APP); alpha-1-acid-glycoprotein, haptoglobin and transferrin, which are produced mainly by the liver. Other APPs were also found to bear increased sLe^x levels only in chronic pancreatitis patients (alpha-1-antitrypsin and fetuin). Although sLe^x on these APPs may be used as cancer prognostic factors, these modifications are not specific enough to be used as PDAC markers.

Abbreviations: A2M, alpha-2-macroglobulin; APP, acute-phase proteins; CP, ceruloplasmin; ChrP, chronic pancreatitis; ITIH4, inter-alpha-trypsin inhibitor heavy chain H4; PDAC, pancreatic cancer; sLe^x, sialyl-Lewis X.

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http://dx.doi.org/10.1016/j.cca.2015.01.007
0009-8981/

In the present work, a glycoproteomic strategy to identify potential pancreatic cancer biomarkers based on changes in sLe^x glycan antigen in serum proteins was performed. For this purpose, the most abundant serum proteins were depleted in order to identify other glycoproteins with enhanced sLe^x from PDAC patients and have found ceruloplasmin (CP) as an interesting candidate for further analysis.

CP is an acute-phase protein produced by the liver and secreted in plasma. Its function is related to copper transport in serum and it is suggested to have a role in cancer since it is involved in angiogenesis and neovascularisation [15,16]. CP has 4 described N-glycosylation sites with complex type, bi, tri and tetrantennary structures both sialylated and fucosylated, containing sLe^x epitope mainly in triantennary structures, but also bi- and tetra-antennary [17,18]. In this study, the sLe^x levels on CP from sera of PDAC, ChrP and healthy controls were analysed and tended to be increased in the PDAC group.

2. Materials and methods

2.1. Serum samples

Serum samples were obtained from 13 healthy controls (HC) (7 females and 6 males; age range 44–69 years), 20 PDAC patients (11 females and 9 males; age range 45–70 years, 3 stage IIA, 7 stage IIB, 4 stage III and 6 stage IV) and 14 ChrP patients (6 females and 8 males; age range 46–79 years) from the Hospital Josep Trueta (Girona, Spain) following the standard operating procedures of its Ethics Committee. Patients were diagnosed by biopsy or image examination by the Pathology and Digestive Units.

2.2. Serum depletion

Serum samples (20 μL of each) were depleted using the ProteomeLab IgY-12 high-capacity spin column (Proteome Partitioning Kit, Beckman Coulter, Fullerton, CA), following centrifugation using a 0.22 μm Spin-X Centrifuge Tube Filter (Costar, Corning, NY) for 10 min at 2000 rpm according to manufacturer's protocols. This column facilitates the removal of albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α1-acid-glycoprotein, α2-macroglobin, apolipoprotein A-I, apolipoprotein A-II and fibrinogen in a single step. The final volume of each serum sample following immunodepletion was concentrated up to 50–100 μL using Microcon YM-3 Centrifugal Filter Device (Millipore, Billerica, MA).

2.3. Protein quantification

Protein concentration was determined by the Bradford protein assay using bovine serum albumin as standard (Quick Start Bradford Protein Assay, BioRad, Hercules, CA).

2.4. SLe^x immunodetection

After immunodepletion and concentration of serum samples, 25 μg of total protein was electrophoresed under reducing conditions on polyacrylamide gels, which were either Coomassie stained or transferred onto a PVDF membrane (Millipore, Billerica, MA). Transferred proteins were Ponceau stained (Ponceau S solution, DIG Glycan Differentiation Kit, Roche Diagnostics, Mannheim, Germany), and after that, sLe^x was immunodetected as previously described [14]. Chemiluminescence was visualised using the imaging system Fluorochem SP (AlphaInnotech, San Leandro, CA) under non-saturating conditions.

2.5. MS analysis

Proteins contained in the bands with specific sLe^x immunodetection for the PDAC patients group were in-gel digested with trypsin, extracted and analysed in an LC-ESI-QTOF mass spectrometer as described by Sarrats et al. [14]. Data were generated in PKL file format and submitted for database searching in the MASCOT server against SwissProt 2010_04 database. The search parameters were human taxonomy, 1 missed cleavage allowed, carbamidomethyl of cysteine as a fixed modification and oxidation of methionine as a variable modification. The peptide tolerance was 200 ppm and 0.25 Da, respectively for MS and MS/MS spectra. The significance threshold was set at $p < 0.05$. In the peptide report, only proteins with at least 2 peptides identified were accepted as positive hits.

2.6. N-glycan analysis

N-glycans were extracted from the gel pieces of CP bands according to the procedure described by Royle et al. [19]. Briefly, the gel pieces were washed and treated with PNGase F to release the N-linked glycans. Afterwards, *N*-glycans were fluorescently labelled with 2-aminobenzamide (2AB) by reductive amination using a Ludger Tag 2-AB labelling kit [20]. The excess of 2AB reagent was removed by ascending chromatography on Whatman 3MM paper in acetonitrile.

The 2AB-labelled glycans were digested in 10 μl of 50 mM sodium acetate buffer, pH 5.5, for 18 h at 37 °C, using arrays of the following enzymes (all purchased from Prozyme, San Leandro, CA) at the indicated concentrations: ABS—*Arthrobacter ureafaciens* sialidase (EC 3.2.1.18), 0.5 U/ml; NAN1—*Streptococcus pneumonia* sialidase (EC 3.2.1.23), 1.7 U/ml; BTG—Bovine testes β-galactosidase (EC 3.2.1.23), 1 U/ml; BKF—bovine kidney alpha-fucosidase (EC 3.2.1.51), 1 U/ml. After incubation, enzymes were removed by filtration through a protein binding EZ filters (Millipore, Billerica, MA), and *N*-glycans were then analysed by HILIC.

2-AB derivatised *N*-glycans were separated by ultra-performance liquid chromatography with fluorescence detection on a Waters Acquity UPLC instrument consisting of a binary solvent manager, sample manager and fluorescence detector under the control of Empower 2 chromatography workstation software (Waters, Milford, MA). Separations were performed using BEH glycan column 2.1 × 150 mm, 1.7 μm BEH particles. Solvent A was 50 mM formic acid adjusted to pH 4.4 with ammonia solution. Solvent B was acetonitrile. The column temperature was set to 30 °C. A 30 min method was used with a linear gradient 70–53% acetonitrile at 0.56 ml/min. An injection volume of 20 μl sample prepared in 60% v/v acetonitrile was used throughout. The fluorescence detection excitation/emission wavelengths were $\lambda_{ex} = 330$ nm and $\lambda_{em} = 420$ nm, respectively. Retention times were converted into glucose unit (GU) values by time-based standardisation against a dextran hydrolysed ladder.

2.7. Ceruloplasmin immunoprecipitation

CP from sera was purified by affinity immunoprecipitation. For each sample, 2.2 μg of streptavidin magnetic beads (Roche Diagnostics, Mannheim, Germany) were washed with buffer A (50 mM Tris pH 7.5, 150 mM NaCl, 10 μL/mL Triton X-100) and incubated for 1 h with 8 μg of biotin-conjugated polyclonal rabbit antibody anti-ceruloplasmin (Abcam, Cambridge, UK) dissolved in buffer B (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20, 1%BSA). Beads were afterwards washed three times with buffer A. Then 50 μL of serum was incubated with the streptavidin magnetic beads conjugated to the antibody anti-ceruloplasmin for 1 h in buffer B. After three washes with buffer A, CP was detached with 100 μL of gentle elution buffer (Pierce Biotechnology, Rockford, IL). All steps were performed at room temperature with shaking. As previously described in Section 2.2, the final volume of CP immunopurified from each serum was concentrated up to 40 μL using Microcon YM-3 Centrifugal Filter Device (Millipore, Billerica, MA).

The protein profile of 25 μL of immunoprecipitated serum was analysed by SDS–PAGE and silver staining. Resolving gel was prepared at 8% of polyacrylamide. Standard CP (0.5 μg), two immunoprecipitated serum samples and a control were reduced and loaded on the gel.

2.8. Immunodetection of sLe^x on ceruloplasmin

Immunoprecipitated CP samples were electrophoresed and sLe^x immunodetection was performed as described in Section 2.4, except that purified mouse anti-human CD15s (BD Biosicences, San Jose, CA) diluted 1/50 was used as a primary antibody and Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) was used as a developer. In order to verify the amount of CP loaded on the gel, the membrane was treated with a stripping solution (TBS, 2% SDS, 100 mM β-mercaptoethanol), previously to CP immunodetection. CP was detected using a polyclonal sheep horseradish-conjugated anti-human ceruloplasmin (AbD Serotec, Kidlington, UK) diluted 1/2000 in incubation buffer for 2 h at RT. SLe^x and CP bands were quantified with *AlphaEaseFC 4.0* software (AlphaInnotech, San Leandro, CA).

2.9. Cell lines and ceruloplasmin analysis from conditioned medium

The human pancreatic adenocarcinoma cell line Capan-1 and the human melanoma SK-MEL-28 cell line were obtained from American Tissue Culture Collection (ATCC, Rockville, MD). The human fibroblast CCD-18Co cell line was obtained from EucellBank (University of Barcelona, Barcelona, Spain). The human skin fibroblast cell line 1BR3G was obtained from European Collection of Cell Cultures (ECACC, Porton, UK). Cells were maintained in DMEM supplemented with 10% FBS and 100U/mL penicillin-streptomycin (GIBCO BRL, Grand Island, NY) at 37 °C under a humidified atmosphere containing 5% CO_2 until 90% confluence was achieved.

Conditioned media from CCD-18Co, Capan-1, 1BR3G and SK-MEL-28 were collected from flasks left 60 h with DMEM without FBS. The medium was concentrated using Amicon Ultra-15 and Amicon Ultra-0.5 (Millipore, Cork, Ireland) up to 50 μL. Protein concentration was determined by Bradford assay as previously described. Twenty-five micrograms of medium from each cell line and standard CP were subjected to sLe^x and CP immunodetection as described in Section 2.8.

3. Results

3.1. Glycoproteins with increased sLe^x in PDAC

Depletion of serum samples with ProteomeLab IgY-12 high-capacity spin column reduced total protein amount by about 90% (range 83–95%). Depleted serum samples from three HC, seven PDAC patients and five ChrP patients were electrophoresed in a 12% polyacrylamide gel, transferred onto a PVDF membrane and subjected to sLe^x immunodetection. Different immunoreactive bands were observed in all the samples. Most of the PDAC samples showed stronger sLe^x signal in the molecular weight region higher than 64 kDa. Some of these bands were neither observed in the HC patients nor in the ChrP patients and were analysed for the identification of the serum glycoproteins bearing this PDAC specific increase of sLe^x. For that, representative samples of each group of patients that showed higher signal in the region above 64 kDa were selected and electrophoresed in an 8% polyacrylamide gel to improve band separation in the high molecular weight range (Fig. 1). The same samples were run in duplicate gels. After electrophoresis, one gel was Coomassie stained, and the other was subjected to western blot for sLe^x immunodetection. Five bands showed stronger sLe^x immunoreactivity in the PDAC patients than in the chronic pancreatitis or HC (Fig. 1a). These bands were cut from the Coomassie-stained gel and subjected to protein identification (Table 1).

Band 1 was found to contain alpha-2-macroglobulin (A2M). A2M corresponds to one of the proteins depleted by the ProteomeLab IgY-12 column. Thus, this protein was not 100% removed from the sera after the depletion protocol. Depletion process could have removed A2M at different yields in the different samples. However, similar intensities of the bands were observed in the Coomassie-stained gel, except for PDAC2 and HC1 sera, which were in less amount. Healthy patients analysed in the present study showed no sLe^x immunoreaction in band 1 (corresponding to A2M). However, sLe^x was positive in band 1 on most of the PDAC patients, suggesting an interesting specific glycan alteration in PDAC.

Band 2 was found to contain ceruloplasmin (CP). A2M was also found in this band but at a much lower score and number of identified

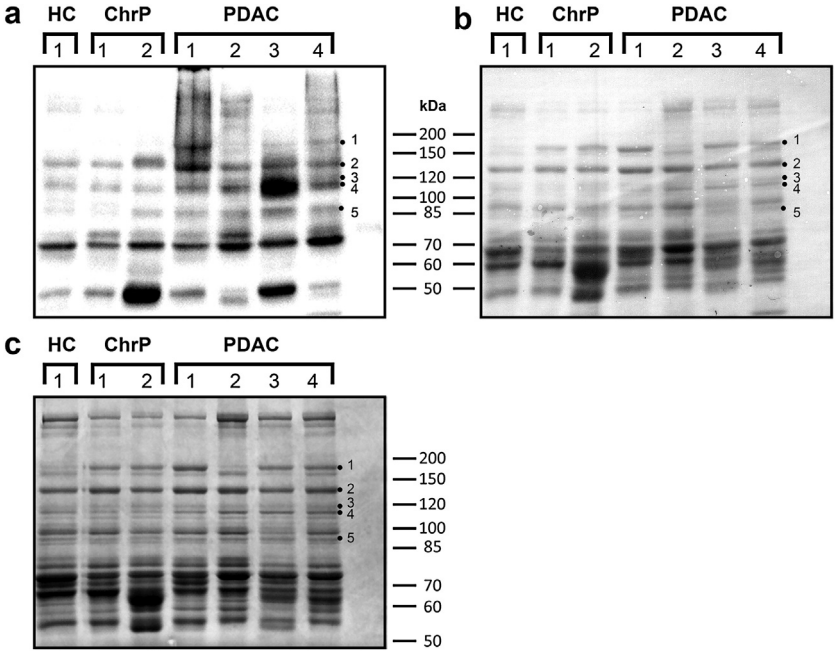


Fig. 1. SLe^x immunodetection of immunodepleted serum samples. Panels a and b correspond to western blot and Ponceau stain, respectively, of depleted serum samples electrophoresed in an 8% polyacrylamide gel. Panel c corresponds to a Coomassie-stained gel of the same depleted serum samples. Bands analysed by MS are indicated with dots and labelled 1 to 5 according to Table 1.

Table 1
Identification by MS analysis of the proteins in sLe^x positive bands.

Band	Band mass (Da)	Identification[1]	Mass (Da)	Accession number	Protein score	Sequence coverage	Peptides matched
1	~180000	Alpha-2-macroglobulin	164614	P01023	451	10%	12
2	~140000	Ceruloplasmin	122983	P00450	612	24%	21
2	~140000	Alpha-2-macroglobulin	164614	P01023	98	2%	2
3	~120000	Inter-alpha-trypsin inhibitor heavy chain H4	103521	Q14624	469	12%	8
4	~110000	Inter-alpha-trypsin inhibitor heavy chain H4	103521	Q14624	316	20%	9
4	~110000	Complement C3	188569	P01024	315	9%	10
4	~110000	Complement component C6	108367	P13671	166	6%	4
5	~90000	Complement C4-A	194247	P0C0L4	346	8%	8
5	~90000	Complement C4-B	194212	P0C0L5	346	8%	8

[1] Only glycoproteins identified are listed (unglycosylated proteins are not listed).

peptides (612 vs 98 and 21 vs 2, respectively). Although the contribution of A2M to the increased sLe^x observed in this band cannot be ruled out, we assumed that CP was the main protein present in this band. Band 2 intensities in the Coomassie-stained gel were similar in all the patients, which suggests that the enhanced sLe^x detection may be due preferentially to an increase of this epitope on the CP protein rather than an augment in CP serum levels.

Band 3 was found to contain inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4). Although the theoretical molecular weight of this protein is 103 kDa, it shows an experimental molecular weight of 120 kDa [21].

Band 4 was found to contain also ITIH4 and Complement C3 at similar scores and number of peptides. Complement component C6 was also identified in this band at a lower score/number of peptides. Thus, the increased sLe^x of this band may be attributed to all these proteins.

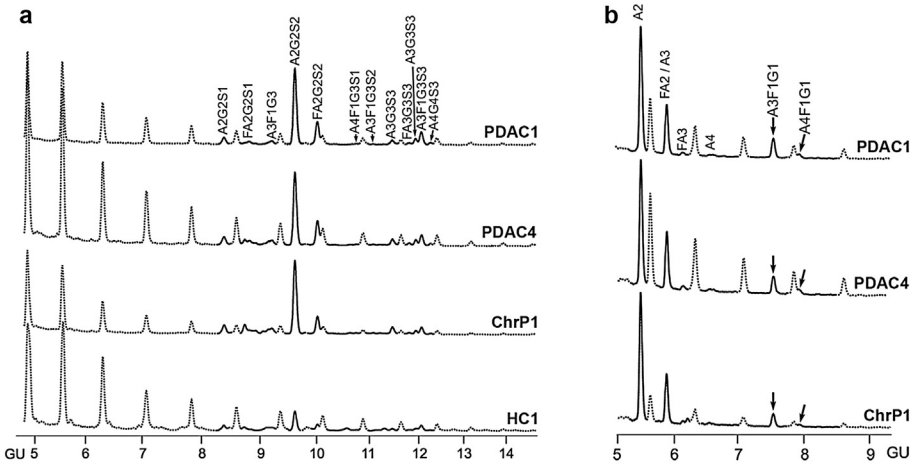
Peptides identified in band 5 were matched to complement C4-A and complement C4-B, which are serological isotypes of C4. Although sharing >99% sequence identities, they have different biological activities [22]. In fact, we cannot know unambiguously which of the isotypes were present in band 5 as both C4-A and C4-B were matched to the same set of peptides. Increased sLe^x in band 5 in all PDAC patients and in one ChrP patient may be due solely to glycosylation as similar levels of proteins were observed in all the samples (Fig. 1c).

3.2. SLe^x expression on CP in PDAC samples analysed by N-glycan sequencing

The analysis of the sLe^x epitope was further investigated on CP because of the higher protein score and sequence coverage of CP compared with the other proteins identified and the fact that the increased sLe^x immunostaining on CP in the PDAC samples was not due to an increase of the CP expression level.

First, *N*-glycans of each selected CP band from the Coomassie-stained gel were released by PNGase F digestion in order to perform glycan analysis. CP glycans from samples HC1, ChrP1, PDAC1 and PDAC4 (Figs. 1c, band 2) were fluorescently labelled with 2AB and analysed by NP-HPLC in combination with exoglycosidase digestions (Fig. 2). Glycan structures were assigned on the basis of their elution time according to a dextran ladder calibrator and measured in Glucose Units (GU).

The glycan content of CP from the healthy control HC1 was very low and generated a very weak signal what precluded further analysis. Thus, only glycan structures of CP from PDAC patients and ChrP patients' sera were analysed by exoglycosidase digestions. First, sialidase NAN1 removed all sialic acid bound α2,3 to a non-reducing terminal galactose in order to identify the structures likely to contain sLe^x, which were A4F1G3S1, A3F1G3S2 and A3F1G3S3 (depicted in Fig. 2a). Digestion with sialidase ABS combined with galactosidase BTG was also performed. In this case, all sialic acids were removed regardless of the



c

ABS+BTG digestion	A3F1G1	A4F1G1	Total
ChrP1	6.8%	1.3%	8.1%
PDAC1	10.2%	2.2%	12.4%
PDAC4	9.7%	2.4%	12.1%

Fig. 2. Ceruloplasmin (CP) *N*-glycan analysis. (a) Glycan profile of total *N*-glycans of CP. Profiles containing a polymer contamination are represented in dotted lines but peaks corresponding to *N*-glycans structures are in solid lines. (b) Sialidase (ABS) and galactosidase (BTG) digestion profiles of CP *N*-glycans with structures originally containing sLe^x highlighted. Profiles are standardised against a dextran hydrolysate (GU). *N*-glycans structures are abbreviated as follows: all *N*-glycans have two core N-acetylglucosamines (GlcNAc) and a trimannosyl core; F at the start of the abbreviation indicates a core fucose; A represents the number a of antenna; F after A represents fucose linked α1–3 to antenna GlcNAc; G represents the galactoses linked β1–4 on antenna; S represents the sialic acids linked to the galactose. (c) Percentage of sLe^x containing structures and the total percentage of sLe^x.

type of linkage. Also, galactoses were removed with galactosidase only when a fucose on the same arm was not present. Thus, those structures with a Lewis antigen can be spotted. This was the case for A3F1G1 and A4F1G1, which are the structures that contain the fucose linked to galactose (Fig. 2b). The information obtained from these digestions allowed us to quantify the proportion of structures containing sLe^x epitope (Fig. 2c), which were higher in PDAC patients (12.4 and 12.1%) compared to ChrP patients (8.1%), confirming our previous findings.

3.3. SLe^x levels on immunoprecipitated CP from serum samples

In order to assess the level of sLe^x on CP in a large cohort of sera, a new methodology was developed. It consisted of an immunoprecipitation method based on streptaviding coated magnetic beads and a biotinylated anti-CP antibody to capture the serum CP. Then the resulted immunoprecipitated ceruloplasmin was analysed, as previously described, by WB with antibodies against sLe^x and reblotted with antibodies anti-ceruloplasmin to quantify the ceruloplasmin bands. Silver staining of SDS–PAGE of an immunoprecipitated serum showed that immunoprecipitated samples were concentrated in ceruloplasmin and contained minor amounts of other serum proteins that are in the low molecular weight area of the gel, and thus they do not interfere with the CP band (Fig. 3). Prior to sample analysis, a dilution series of standard CP were performed to evaluate that stripping did not affect the CP quantification of the samples. A standard curve was obtained for CP immunodetection after the stripping step. Linear chemiluminescent signal between 0.075 and 0.6 μg of standard CP indicated that this treatment affected equally to all samples (Fig. 4a). The amounts of immunoprecipitated CP from 50 μL of serum using 8 μg of capture antibody yielded a band of about 0.1 μg of CP (Fig. 4b), which lies in the linearity range of the standard curves for sLe^x and CP quantification.

The analysis of the sLe^x levels of the immunoprecipitated CP of 12 HC, 12 ChrP and 16 PDAC was performed by western blot, followed by CP immunodetection of the stripped membranes (Fig. 5a). Considering that CP signal of samples was similar between membranes (coefficient of variation less than 15% both interassay and intraassay), the sLe^x/CP ratio of the 40 samples analysed was calculated. The interassay variation of sLe^x/CP ratio was determined using 0.1 μg of standard CP loaded in all membranes, and also of one sample analysed in two of them. The analysis gave coefficients of variation less than 20% and 10%, respectively.

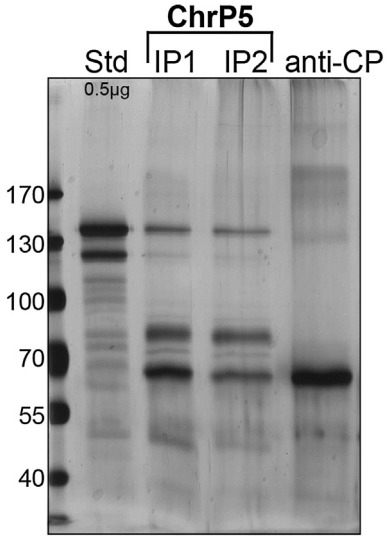


Fig. 3. Immunoprecipitation of ceruloplasmin (CP) from serum. Silver staining of an 8% SDS–PAGE gel. IP1 and IP2 correspond to the CP immunoprecipitated from 25 μL of serum using 10 and 5 μg of capture antibody respectively. Antibody lane is the control of the immunoprecipitation without sample.

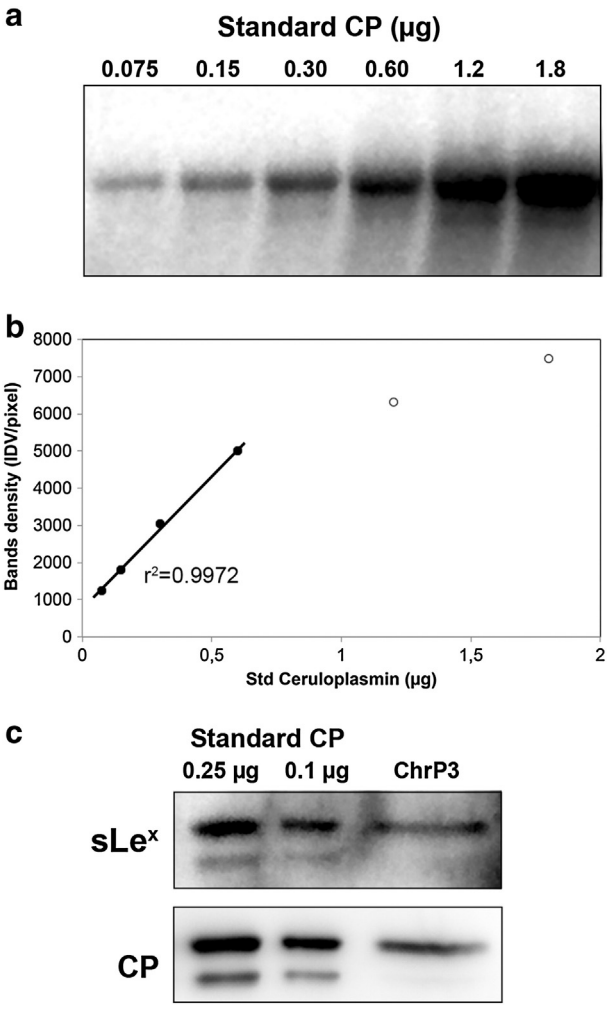


Fig. 4. Ceruloplasmin (CP) immunodetection analysis. (a) CP immunodetection of a dilution series of standard CP of a reblotted membrane after sLe^x detection. (b) Calibration curve obtained by densitometry analysis of CP bands. The linearity is indicated with a solid line. (c) SLe^x immunodetection and its corresponding CP immunodetection of 0.1 and 0.25 μg of standard CP and CP immunoprecipitated from 50 μL of serum using 8 μg of capture antibody.

The sLe^x/CP ratio of the PDAC group tended to be increased compared to both HC and ChrP groups, corroborating our previous findings. As shown in Fig. 5b, only 15% of HC and 16% of ChrP had a sLe^x/CP ratio higher than 0.3 in contrast to 56% of PDAC samples. No different behaviour between non-advanced and advanced PDAC could be detected.

3.4. CP expression in the human pancreatic adenocarcinoma Capan-1 cell line

In non-malignant conditions, CP is synthesised by hepatic cells. We have examined the ability of human pancreatic adenocarcinoma cells to produce CP with sLe^x. For this purpose, the conditioned medium of the pancreatic cell line Capan-1 was analysed by western blot to detect CP production. The conditioned media of CCD-18Co and 1BR3G were used as negative controls due to their non-epithelial origin. We also tested other tumour cell line, the human melanoma SK-MEL-28. CP was only detected in the conditioned medium from Capan-1, which was also positive for sLe^x in the CP band (Fig. 6).

4. Discussion

Early and effective detection of PDAC is required to reduce its high mortality rate. Since aberrant glycosylation in tumour cells such as the

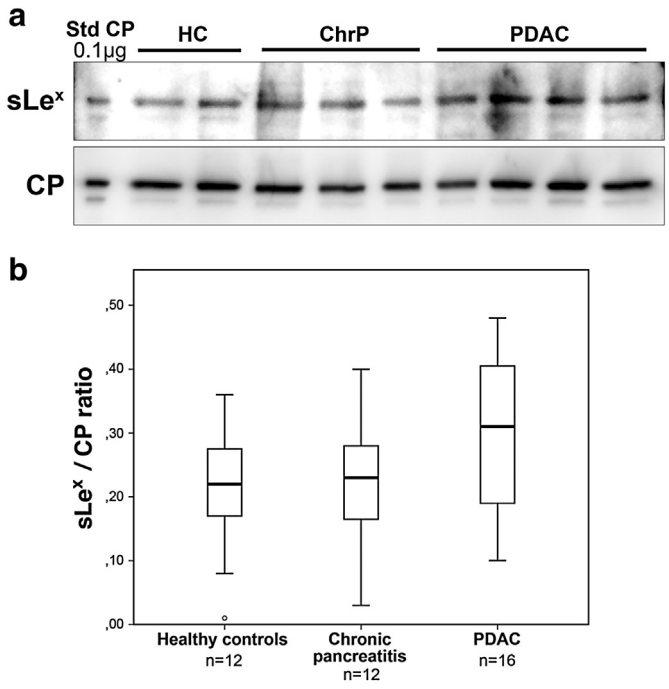


Fig. 5. Analysis of sLe^x on ceruloplasmin (CP). (a) Representative example of sLe^x immunodetection and its corresponding CP immunodetection of 50 µL of immunoprecipitated sera from healthy controls (HC), chronic pancreatitis (ChrP) and PDAC patients. (b) SLe^x/CP ratio of HC, ChrP and PDAC patient sera quantified by western blot.

overexpression of sLe^x is linked to tumour progression and malignancy, we have focused our search for biomarkers on the expression pattern of sLe^x epitope on different serum proteins in PDAC samples.

We have thus performed a glycoproteomic strategy to identify potential pancreatic cancer biomarkers based on changes in sLe^x glycan antigen in serum proteins, and we have identified the aforementioned serum glycoproteins with increased sLe^x in PDAC patients. These proteins were different from the high abundant APPs that we had previously described [14]. Although these proteins turned out to be again APPs, they may be used in the differentiation of PDAC and ChrP. In agreement with our results, an increase in both fucosylation and sialylation of serum glycoproteins, such as serum amyloid P-component, beta-2-glycoprotein 1 and alpha-1-β glycoprotein (also mainly liver-derived), has been described using glycoprotein microarrays with multi-lectin detection techniques, for PDAC patients compared to healthy controls and pancreatitis patients [11,13].

First, we have depleted the main serum proteins, including several acute-phase proteins and have performed immunoblotting of the depleted serum samples with anti-sLe^x antibody. Thus, we have found 5 immunoreactive bands to sLe^x epitope in PDAC depleted serum samples that were identified by mass spectrometry. They corresponded to 7 proteins that belong to acute-phase reactants.

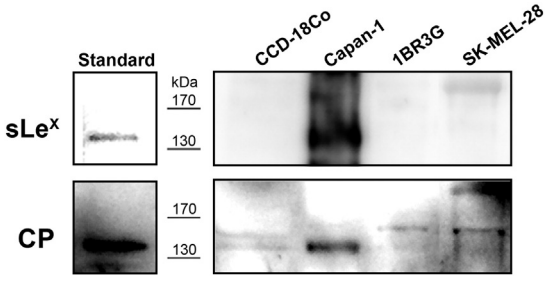


Fig. 6. Ceruloplasmin (CP) expressed by pancreatic tumour cells. SLe^x immunodetection and its corresponding CP immunodetection of conditioned media of different cell lines.

From all the proteins identified, we discarded A2M for further analysis because this protein corresponds to one of those that should be depleted by the ProteomeLab IgY-12 column, so it cannot be ruled out that the remaining A2M glycoforms are representative of A2M after this purification step. Likewise, we considered proteins in bands 4 and 5 (ITIH4, Complement C3, Complement component C6, Complement C4-A and C4-B) poor candidates because its contribution to sLe^x pattern is not clear since each band contains more than one glycoprotein. Thus, we selected CP to address the significance of the increased sLe^x in PDAC samples because it was the one with higher score in MS identification and the increased sLe^x immunostaining on CP was not associated to an increase in CP protein levels.

Next, we focused on the characterisation of the sLe^x content of CP in PDAC patients vs. ChrP and HC. For that, glycan sequencing of purified CP of PDAC, ChrP and HC samples was analysed. An increase of A3F1G1 and A4F1G1 was shown in the CP of two PDAC samples (PDAC1 and 4) vs a ChrP patient (ChrP1) after a combined sialidase and galactosidase digestion. Since all CP glycan structures are sialylated and α2,3-sialic acid has been found on tri- and tetra-antennary structures, this combined digestion is indicating that sLe^x is found in tri- and tetra-antennary CP *N*-glycan structures of the PDAC in higher proportion than in ChrP samples.

In order to analyse the sLe^x content of CP of a cohort of serum samples, we set up an immunoprecipitation methodology that allowed the CP purification from sera. The analysis of a set of 40 samples (16 PDAC, 12 ChrP and 12 HC) by immunoblotting with anti-sLe^x and further immunodetection of CP showed a higher ratio of sLe^x/CP in the PDAC samples. These results were in agreement with the data of the *N*-glycan sequencing.

Serum levels of CP have been proposed as markers of different solid cancers, as they were increased in different cancer patients compared to healthy controls. Abd-el-Fattah et al. [23] described five acute-phase proteins, including CP, that have a biphasic profile in tumour process, which consists on a little rise during the initial cancer stage, and a huge increase in a second phase. Taking into account that these changes can be attributed to the growing of the tumour mass, CP levels in serum were proposed as a marker for cancer monitoring and also, to evaluate the response to treatment [24,25]. Higher fucosylation has been detected in hepatocellular carcinoma [26] and esophagus adenocarcinoma [27]. Other studies showed different CP heterogeneity in immunoelectrophoresis attributed to changes in its charge/size ratio in patients with lung cancer compared with patients with other benign lung diseases. However, CP glycan microheterogeneity analysed by crossed affinoimmunoelectrophoresis with WGA (wheat germ aglutinnin, a lectin that recognises mainly *N*-acetyl-glucosamine and that has also found to have affinity to sialic acid) was not useful as an indicator of malignancy [28]. This lectin study could not give information about the detailed glycan structures of CP.

To better elucidate this issue, glycan characterisation of this protein should be performed in a larger study with more PDAC and ChrP patients, including also other types of tumours, especially the ones with signs and symptoms similar to PDAC patients.

The increased sLe^x epitope on CP in PDAC is consistent with previous findings of acute-phase proteins being good carriers of pathological information of cancers from a non-liver origin [29–31]. Glycan-altered acute-phase proteins have been described to modulate macrophage activity in tumour microenvironment by interacting with its receptors and contributing to tumour cell survival, growth and metastasis [32]. Furthermore, we have previously described that the overexpression of the α2,3-sialyltransferases involved in sLe^x synthesis promotes several steps of tumour progression such as adhesion, migration and metastasis formation [33].

CP could also be produced by tumour cells as indicated in previous reports that show CP mRNA expression by colon (WiDr) and breast (MCF-7) human cancer cell lines [15]. In agreement with this, here we have shown that the human pancreatic adenocarcinoma Capan-1 cells

produce CP, which contains sLe^x. In this line, we could hypothesise that PDAC cells could contribute to the synthesis of the increased sLe^x on CP detected in PDAC patients. A more sialylated CP would evade the clearance by the liver (via the asialoglycoprotein receptor), and thus it would remain in blood for longer enabling it to perform its role in angiogenesis and neovascularisation.

5. Conclusions

Increased levels of sLe^x epitope on CP are present in most sera from PDAC patients. Although being an acute-phase protein produced mainly by the liver, we have shown that pancreatic cancer cells can also produce CP. These results show a trend that should be studied in wider cohorts in order to validate the usefulness of sLe^x/CP ratio in the detection, monitoring or prognosis of PDAC patients.

Acknowledgements

M. B. and A. S. acknowledge University of Girona for pre-doctoral fellowships. This work was supported by the Spanish Ministry of Science and Innovation, grants BIO 2007-61323 and BIO 2010-16922, awarded to R. P., and by the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 260600 ("GlycoHIT"), awarded to R. S.

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Results - Chapter 3

Identification of potential pancreatic cancer biomarkers based on aberrant mucin glycoforms by *in situ* proximity ligation assay

Identification of potential pancreatic cancer biomarkers based on aberrant mucin glycoforms by *in situ* proximity ligation assay

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Abbreviations: PDAC, pancreatic cancer; PLA, *in situ* proximity ligation assay; SLe^x, sialyl-Lewis x; STn, sialyl-Tn

Keywords: biomarker; immunohistochemistry; mucin; glycosylation; pancreatic cancer; PLA

Abstract

Pancreatic adenocarcinoma (PDAC) is a major problem in public health, due to the lack of biomarkers and the ineffectiveness of currently available treatments. Mucins are glycoproteins expressed by epithelial cells and carry aberrant glycosylation moieties in malignancy. Our objective was to identify specific cancer-related glycan epitopes on MUC1 and MUC5AC that could help to find specific biomarkers for PDAC. In particular we have focused on the tumour-associated carbohydrate antigens sialyl-Lewis x (SLe^x) and sialyl-Tn (STn) expressed in PDAC.

We have selected 21 PDAC tissues that expressed SLe^x and/or STn by immunohistochemistry and 3 normal pancreas specimens as controls. All selected PDAC tissues showed positive staining for SLe^x, and 76% of the cases showed STn expression. The evaluation of the expression of MUC1 and MUC5AC was performed using specific monoclonal antibodies. Both mucins were detected in 90% of PDAC tissues. Next, we performed *in situ* proximity ligation assay (PLA) combining antibodies against mucins and the glycan epitopes SLe^x and STn to identify specific mucin glycoforms in PDAC tumours.

The aberrant glycoforms MUC1-SLe^x and MUC5AC-SLe^x were found in 68% and 84% of the mucin expressing PDAC tissues respectively, while STn hardly colocalized with any of the studied mucins. The SLe^x glycoforms on MUC1 and MUC5AC could thus be regarded as potential PDAC markers.

General discussion

Surgical resection is the only potentially curative treatment for pancreatic cancer (PDAC). However, only 10-20% of diagnosed patients can undergo surgical resection due to advanced stage presentation at the time of diagnosis [5, 7]. Hence, the identification of new diagnostic biomarkers is of great importance for future PDAC management, and a priority for the scientific community. Important efforts to improve PDAC diagnosis are aimed at discovering new molecules produced by tumour cells at early stages of carcinogenesis or by the host cells in response to the tumour. Despite all the efforts made in recent decades, still no biomarker has shown enough potential for PDAC diagnosis [180, 181]. Ideally, a clinical biomarker for early detection of cancer should be based on the identification and quantification of substances released into a biological fluid [182]. Blood, urine, stool and saliva are considered the most interesting sources for biomarkers due to the minimally or non-invasive procedures for obtaining them.

Urine can be considered as an ultrafiltrate of plasma cleared by the kidneys that may contain valuable biomarkers [183]. Two different studies based on proteomic analysis show that PDAC is associated with different urine-based proteins, some of which were previously described as overexpressed in PDAC tissues [184, 185]. Regarding stools, which have been considered in the study of pathologies of the gastrointestinal tract such as Chron's disease [186, 187], various studies show some aberrantly methylated genes in PDAC stools [13, 188]. Little research has been performed using saliva because of the unapparent presence of potential biomarkers of pancreatic disease in the oral cavity; although some studies have propose some noncoding RNAs and mRNAs of the saliva as PDAC biomarkers [189-191]. On the other hand, pancreatic juice, despite being obtained through an invasive procedure, must be regarded as a source for specific PDAC biomarkers. Traditional serum biomarkers, such as CEA and CA19-9, can be found in pancreatic juice but they have little or no diagnostic advantage over serum for PDAC patients [181]. Nevertheless, studies of KRAS mutations and telomerase activity in pancreatic juice have demonstrated their potential as early diagnostic tools [192-194].

Biomarkers generally used in clinics, such as carbohydrate antigens CA125 and CA19-9, and PSA, were discovered using tumour cell lines or tumour extracts [195]. The physical as well as the physiological status of a patient plays an important role in tumour biology, thus, cell culture-based systems have been thought to diminish heterogeneity among

individuals. Furthermore, contrary to the limited availability of patient tissue specimens, cell cultures allow cells to be easily propagated for an unlimited source of samples. The cell secretome could indirectly represent the proteins that can be found in a patient's body fluids. The inability of 2D culture systems to mimic tumour microenvironments can be overcome by novel 3D culture techniques. However, once a potential biomarker is established, candidates must eventually be confirmed using patient samples. Different biomarkers have been identified for several cancer types using tissue culture-based approaches [196, 197]. Regarding PDAC, the proteomes of the conditioned media of six pancreatic cancer cell lines, and a normal human pancreatic ductal epithelial cell line were characterised and 5 biomarker candidates were identified [198]. However, a further validation of these candidates showed slight improvement in performance when analysed in combination, but individually CA19-9 continued to be the one with the largest area under the ROC curve (AUC) [198, 199].

In addition to the original sources of biomarkers, a wide range of molecules can also be used. With the emergence of high-throughput proteomics technology, proteins were regarded as an endless source of information; however, other biomarker discovery research has detected circulating tumour cells in peripheral blood, as well as aberrant circulating free DNA, microRNAs and exosomes [200-203].

Despite the steadily increasing number of publications reporting potentially new and useful biomarkers, the number of those approved by the FDA has continued to decrease [118, 204]. This demonstrates the complexity of discovering new molecules with both high sensitivity and specificity for the diagnosis of pathologies. Current strategies for discovering cancer biomarkers might include emerging technologies, such as tissue or serum gene-expression profiling, tissue proteomic profiling, or peptidomics (which attempts to find novel biomarkers in the low-molecular-weight range in plasma or serum proteome). Other possibilities include more rational strategies based on analysing the role of related members of a protein that is already an established biomarker. Additional approaches to cancer biomarker discovery include the use of animal models involving human tumour xenograft experiments, mass-spectrometry imaging of tissues and autoantibodies analyses. [182, 205].

The phases of biomarker development consist of (i) preclinical exploratory studies, (ii) assay development and validation, (iii) retrospective longitudinal clinical repository studies, (iv) prospective screening studies and (v) randomized control trials [206]. It should be noted that cancer biomarker candidates also have to be approved by the FDA, meet the cost-benefit ratio, and gain acceptance and recognition among clinicians [118]. The work performed in this doctoral thesis is focused on the preclinical phase, where tumour and non-tumour samples are compared to generate potential biomarker candidates for detecting PDAC.

Briefly, protein biomarker discovery is commonly based on finding a particular protein that is differently expressed in a selected sample source comparing healthy and pathological processes. The methods employed for protein-based biomarkers can vary but usually include a final mass spectrometry (MS) step to identify the target. Then, this putative biomarker is verified in a different cohort of samples and a method is developed to directly target the protein [207, 208].

Proteomic analysis uses large-scale and high-throughput methods to characterise the total-protein expression in a given cell type or biological fluid at a given time point and to search for differentially expressed proteins. The proteins are found using several techniques, including two-dimensional electrophoresis, multi-dimensional liquid chromatography and protein chip technology, all of which are frequently combined with mass spectrometry (MS), as previously mentioned [42]. For example, the workflow performed by Guo and collaborators consisted of applying serum samples from PDAC patients and healthy individuals to strong anion exchange chromatography (SAX) protein chips for protein profiling using surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). Then, the multiple protein peaks were statistically analysed. They obtained a sensitivity of 83.3% and a specificity of 100% by distinguishing PDAC from healthy controls and benign pancreatic diseases. Then they performed ProteinChip immunoassay to determine putative proteins [209]. A similar strategy was followed by Qian and co-workers. They used weak cation exchange magnetic beads combined with SELDI-TOF-MS of serum proteins in a cohort of healthy individuals and patients with PDAC and acute and chronic pancreatitis. The results allowed the authors to construct a software model based on three biomarkers (7762, 8560, 11654 Da), which achieved 93.3% sensitivity with 95.6% specificity in

distinguishing PDAC from non-cancer samples [210]. Although the results are promising, none of these findings have been applied clinically.

Although proteomics, and genomics to some extent, can provide information about changes in protein expression, the altered protein profile of tissues or cells found in different circumstances, such as cancer, is frequently the result of protein modifications rather than altered gene expression. Therefore, proteomic studies should take post-translational modifications into consideration [211]. Glycosylation, considered the most complex post-translational modification in mammals, plays a key role in malignant transformation and tumour progression [57, 59, 98]. Therefore, in recent years, altered glycan moieties of serum glycoproteins have been studied to find new biomarkers [57, 59, 74]. The analysis of a specific glycoform rather than the protein alone is expected to provide more specificity to the biomarker. This is the case of the AFP-L3, the fucosylated glycoform of alpha-fetoprotein which was approved by the FDA for hepatocellular carcinoma (HCC) prognosis and risk assessment [212, 213].

As described in detail in the introduction of this thesis, high-throughput technologies have also been developed for glycan analyses [58, 98]. However, these tools can only provide potential biomarker candidates that must still be verified by targeted quantitative techniques, such as ELISA or protein chips [208, 214]. Different approaches and complementary techniques are used throughout this thesis to identify and verify new biomarkers based on the altered glycosylation of proteins in PDAC. The first two strategies employed in this work are focused on identifying serum glycoproteins as PDAC biomarkers in cohorts of PDAC and control patients.

Glycoforms of serum α-1-acid glycoprotein and ceruloplasmin associated with PDAC

Previous results from a small cohort of patients (N=9; 3 healthy controls (HC), 2 chronic pancreatitis (ChrP), and 4 PDAC) had shown that α-1-acid glycoprotein (AGP) displayed an increase in core fucosylation in PDAC patients when analysed by *N*-glycan sequencing. The identification of fucosylated AGP as a potential biomarker candidate

motivated more thorough analysis of the fucosylation of the AGP in a larger cohort to verify its possible use as a PDAC biomarker (Figure 17).

Fucosylated AGP was analysed as a putative PDAC biomarker by first purifying AGP from serum samples using a home-made immunoaffinity column [215] in a larger cohort of serum samples (N=31; 6 HC, 6 ChrP, and 19 PDAC including 6 non-advanced and 13 advanced stages). Purified AGP was then analysed using three different techniques: capillary zone electrophoresis (CZE-UV) to determine the different glycoform patterns of this glycoprotein, MS to analyse the different *N*-glycan structures and enzyme-linked lectin assays to quantify AGP fucose levels.

Stable-isotope labelling for relative quantitation and zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry (ZIC-HILIC-MS) were used to distinguish the linkage/branching of *N*-glycan isomers. This strategy reliably compares the glycosylation pattern of a glycoprotein from different patients (e.g., disease vs. control), as this relative quantitation is independent of the ionization yields and the charge-state distributions for different glycans. Moreover, separating isomeric glycans using ZIC-HILIC stationary phases distinguishes between samples exhibiting various proportions of isobaric isomers differing in branching and linkage positions [156, 216]. The *N*-glycan spectra obtained for all pairwise, control and pathological samples (either ChrP or PDAC) were thoroughly analysed for branching and for sialic acid and fucose content. Several fucosylated structures (2Ant2Neu5Ac1Fuc, 3Ant2Neu5Ac1Fuc, 3Ant3Neu5Ac1Fuc, 3Ant3Neu5Ac2Fuc, 4Ant3Neu5Ac1Fuc, 4Ant3Neu5Ac2Fuc, 4Ant4Neu5Ac1Fuc, 4Ant4Neu5Ac2Fuc, 4Ant4Neu5Ac3Fuc) increased more in PDAC than in ChrP patients [217].

CZE-MS had previously been used to analyse whole glycoprotein isoforms of AGP and the content of the different peaks based mainly on the number of sialic acid residues but also on the fucose amount and the branching differences [218, 219]. In this work, CZE-UV analysis showed different retention times of the AGP from PDAC patients' sera compared to HC and ChrP, meaning differences in the size, charge or shape of the protein [220]. Considering the CZE-MS results and the results previously reported by ZIC-HILIC-MS, we suggested that the profile obtained using CZE-UV could be influenced by different degrees of fucosylation among samples [217]. The different protein isoform

profiles obtained using CZE have previously been reported as useful biomarkers for different diseases [215, 221, 222].

Both ZIC-HILIC-MS and CZE-UV have shown different glycosylated AGP in PDAC than in ChrP. Next, different enzyme-linked lectin assays (ELLAs) were developed to analyse fucosylated-AGP glycoforms. Like ELISA, the ELLA methodology allows a sensitive and quantitative analysis of several samples in a relatively short time. ELISA and its variants are one of the most commonly used tests in clinical diagnostics. ELLAs were performed using *Sambucus nigra* agglutinin (SNA) to detect α2,6-sialylation, *Aleuria aurantia* lectin (AAL) to detect fucosylation (with high specificity for α1-6 fucose, followed by α1-3 fucose and α1-2-fucose), and *Pholiota squarrosa* lectin (PhoSL) to specifically detect core fucosylation (α1-6-linked fucose) on purified AGP. The ELLA assays using the aforementioned lectins were performed to quantify glycosylation differences. A cut-off value could be established to separate samples in non-tumour and tumour patient groups and to stage the cancer group. In particular, significant differences in AAL ELLA between advanced PDAC and ChrP were found before neuraminidase digestion and between advanced PDAC and HC after neuraminidase digestion [217]. These results were in agreement with the MS analysis data.

The three methods – CZE-UV, ZIC-HILIC-MS and ELLAs – showed that fucosylated-AGP is associated with advanced PDAC. However, this marker's potential to discriminate between groups (PDAC and ChrP) was lower than that of CA19-9.

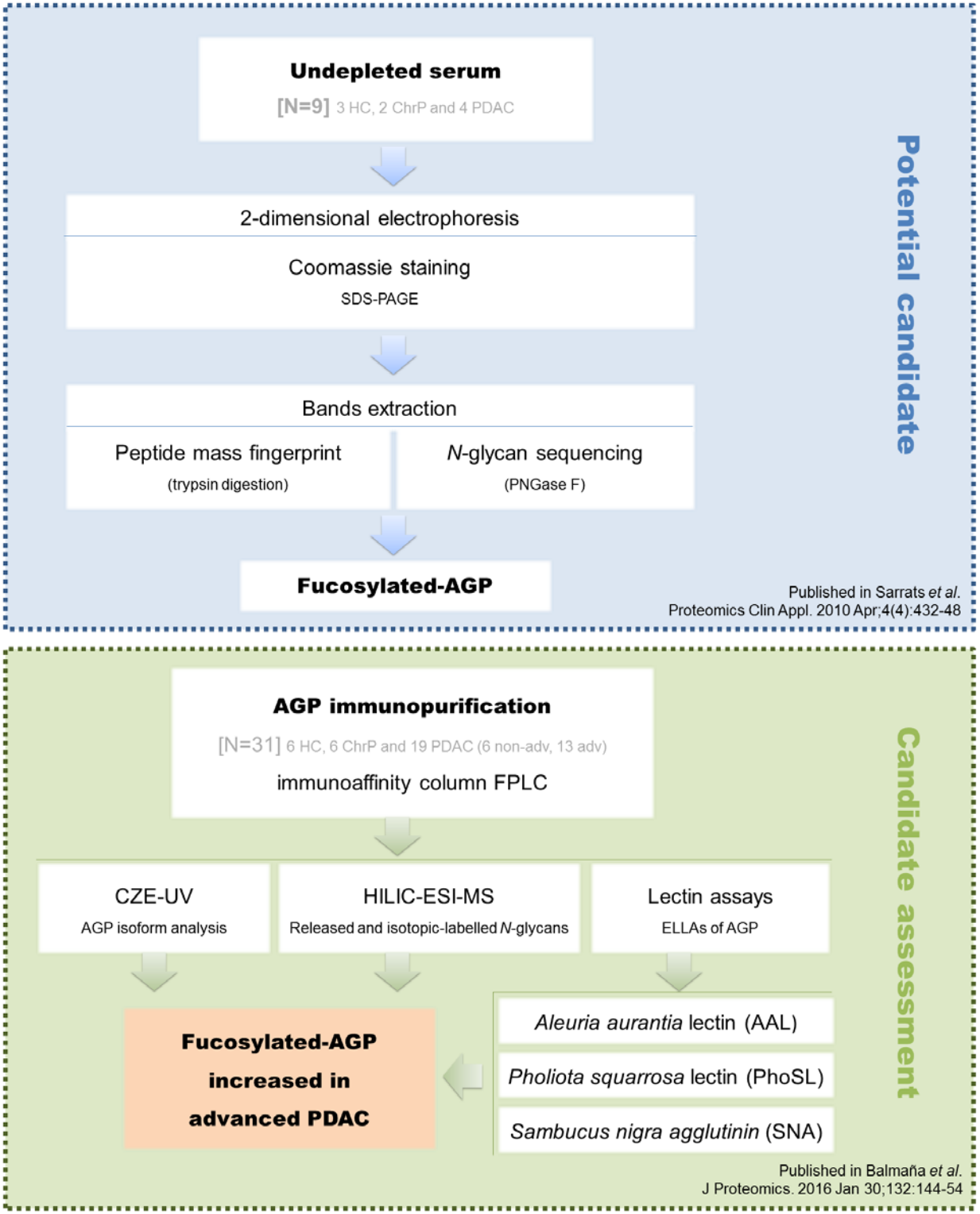

Figure 17: Increased AGP fucosylation in cancer. 17A. Differential fucosylated pattern was determined in α-1-acid glycoprotein (AGP) in serum of healthy individuals, pancreatic cancer patients and chronic pancreatitis patients. 17B. AGP fucosylation was analysed using different methods in a larger cohort of patients.

CZE: capillary zone electrophoresis; ELLA: enzyme-linked lectin assay; FPLC: fast protein liquid chromatography; HILIC-ESI-MS: hydrophilic liquid chromatography electrospray ionization mass spectrometry.

Fucosylation is considered to be one of the most important glycan modifications involved in cancer and inflammation [223]. Its role in PDAC has been studied in depth and noteworthy is the fucosylated-haptoglobin case [224-226]. Besides, fucose is involved in the structure of Lewis antigens and their sialylated versions. CA19-9, the biomarker used for PDAC monitoring, is based on increases in SLe^a levels in serum. The relationship between SLe^x and PDAC is well established [89-92, 227] and especially interesting because, contrary to SLe^a, SLe^x is absent in healthy pancreatic tissue and faintly expressed in a few chronic pancreatitis tissues [92]. Thus, SLe^x, which is expressed in more than 80% of PDAC tissues, presents high PDAC specificity [91, 92, 228].

Our first approach to the PDAC biomarker search led us to identify a non-pancreatic protein (an acute-phase protein) with altered glycosylation in advanced PDAC patients. In a subsequent strategy, we sought SLe^x glycoepitope overexpressing proteins. To achieve this (Figure 18), we depleted the most abundant proteins, including major acute-phase proteins, from the serum to detect the minor glycoproteins that could carry the information from the cancer cells, in particular the SLe^x carbohydrate antigen, and identified them as potential PDAC biomarkers [229]. The cohort selected (N=15) consisted of 3 HC, 5 ChrP and 7 PDAC sera.

Serum contains 60-80 mg of protein/mL in addition to various small molecules including salts, lipids, amino acids and sugars [230]. Over the years, attempts have been made to reveal the total number of serum proteins. Although the exact number remains elusive, it is well established that human serum contains at least 880 proteins [231]. The presence of higher abundant proteins interferes with the identification and quantification of lower abundant ones. Given that the concentration difference between the most abundant protein (albumin at 35-50mg/mL) and one of the least abundant protein (e.g. interleukin 6 at 0-0.005ng/mL) represents a protein dynamic range wider than 10^{10}, the proteome profiling methods must be able to handle this substantially large variability. In addition, only 22 high abundance proteins comprise about 99% of the plasma protein content, and the remaining 1% is made up of low abundance proteins, which would correspond to the sub-proteome where cancer biomarkers are expected to be found [205, 232].

Methods currently used to deplete more abundant proteins that could prevent the recognition of more interesting proteins include precipitation-based methods (salting out with inorganic salts, precipitations with organic solvents, rivanol or polyethylene glycol) and chromatographic methods (ion exchange, affinity ligands, size exclusion chromatography, etc.) [205, 233]. The selection of each method (or a combination of them) would depend on several factors, including the purity required for the final purpose and the amount of plasma/serum to treat.

To deplete serum from the major proteins, we used the ProteomeLab IgY-12 spin column, which is an affinity-based depletion system that allows the removal of the 12 major serum proteins (albumin, transferrin, haptoglobin, fibrinogen, α2-macroglobulin, α1-antitrypsin, AGP, apolipoprotein A-I, apolipoprotein A-II and the immunoglobulins IgG, IgA and IgM). One of the most important advantages of using IgY antibodies is the low cross-reactivity they present with human proteins due to their avian nature [234]. In this part of the work, a differential SLe^x expression pattern was found and liquid chromatography-electrospray ionization-quadrupole-time of flight-MS (LC-ESI-Q-TOF-MS) was performed to identify the protein candidates bearing SLe^x. This technique identifies proteins based on the unique peptide masses generated by trypsin digestion. MS analyses revealed several candidate proteins: alpha-2-macroglobulin, ceruloplasmin (CP), complement C3, complement C4-A and C4-B, complement component C6 and inter-alpha-trypsin inhibitor heavy chain H4. Considering the works that associate CP with cancer [235-240], this serum glycoprotein was selected for further analysis: specifically, to analyse whether the SLe^x-CP glycoform increased more in PDAC samples than in ChrP and HC ones.

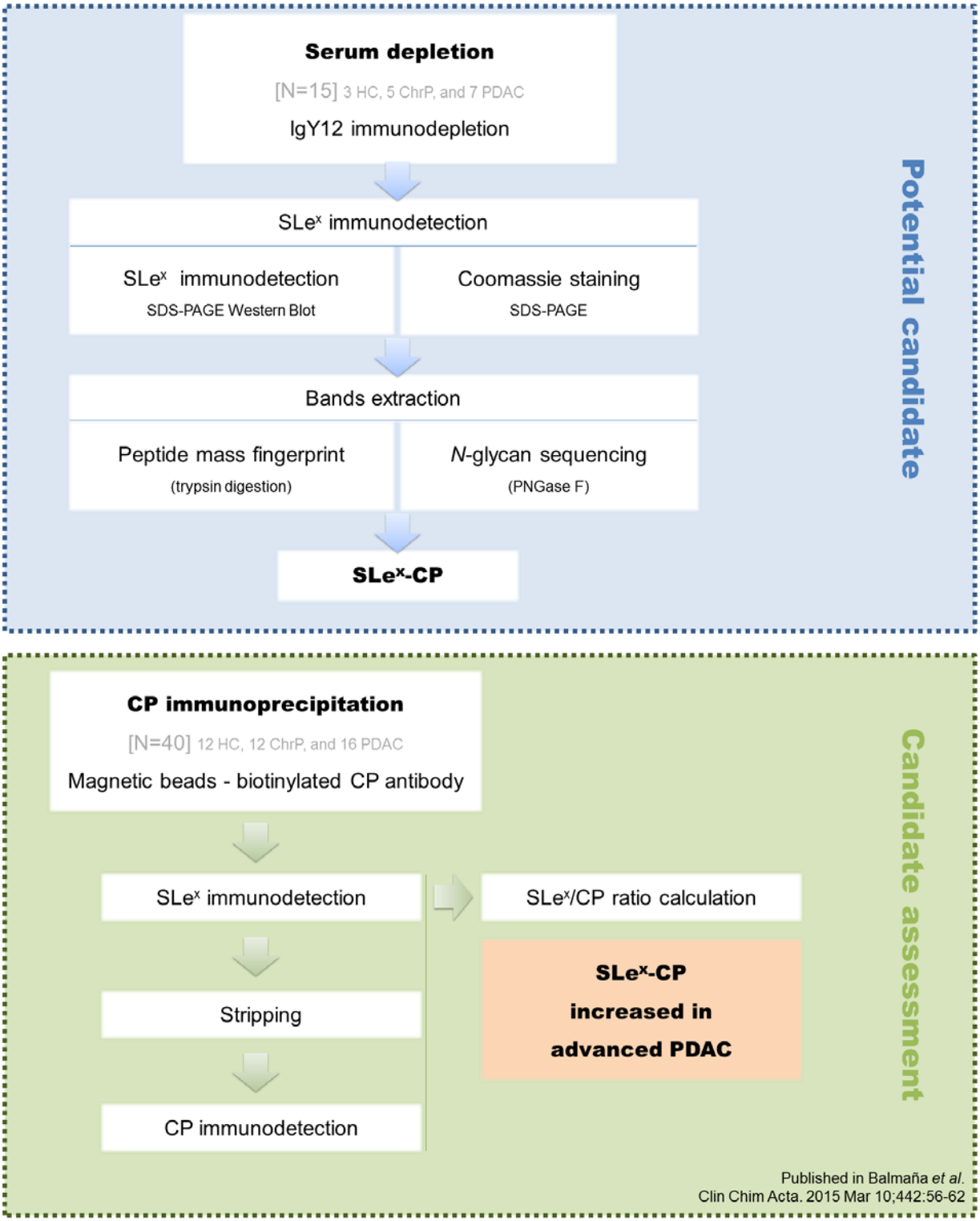

Figure 18: Identification of SLe^x glycoproteins in cancer: ceruloplasmin. 18A. After depletion of the twelve most abundant proteins of serum of healthy individuals, pancreatic cancer patients and chronic pancreatitis patients, a differential SLe^x pattern was observed in ceruloplasmin (CP). 18B. SLe^x/CP ratio was determined in a larger cohort of patients.

N-glycan sequencing allowed us to confirm the presence of the SLe^x epitope on the CP in some selected samples (N=4). Next, we developed an approach to verify the SLe^x on CP in a larger cohort (N=40; 12 HC, 12 ChrP, and 16 PDAC). After that, we performed CP immunoprecipitation from the sera. Then, a western blot of SLe^x and subsequent CP immunodetection allowed the SLe^x/CP ratio to be calculated for each sample. Finally, statistical analysis of the ratios for each group of samples (PDAC, ChrP and HC) showed that the SLe^x epitope on the CP tended to increase in PDAC patients [229].

The two candidates analysed so far, AGP and CP, are both acute phase proteins (APPs) and are in the range of abundant serum proteins. Consequently, none of the approaches performed so far allow us to reach the serum sub-proteome of the low abundant proteins. PDAC is a tumour with a high inflammatory component [241, 242] to which the liver responds by producing APPs. Most of the APPs are glycoproteins and thus, they may show altered glycan moieties as a result of the acute-phase response both for acute injuries or chronic conditions, such as cancer [138]. The liver responds by secreting both major positive APPs and moderate or minor positive APPs. While major positive APPs often increase markedly during the first 48h after disturbances and also rapidly decrease because of their short life, moderate and minor positive APPs, which comprise AGP and CP, may increase more slowly and be more prolonged in duration, depending on the status of the triggering event. Furthermore, these APPs can be observed during chronic inflammatory processes [142], which could be considered a lack of specificity. However, they could still be integrated into a complementary biomarker panel. The molecular heterogeneity of tumours from patient to patient and the epidemiological differences require panels of biomarker analytes to gain enough sensitivity and specificity, especially for the pre-symptomatic detection of cancer, rather than the idealized single, cancer-specific biomarker [243]. Therefore, the two presented glycoforms, SLe^x-CP and fucosylated-AGP, could complement diagnostic screening with novel and/or current biomarkers.

Mostly in advanced stages, both AGP and CP showed an aberrant glycosylation pattern, which means they might be a consequence of a tumour and/or sustained inflammation [140]. ChrP samples included in both studies frequently had lower levels of these glycoforms than the PDAC samples. Hence, these alterations seem to be cancer specific. In this regard, Kontro and collaborators used SNA affinity chromatography to enrich

with sialylated glycopeptides the depleted sera of PDAC, acute pancreatitis and HC samples and compared their relative abundance using ultra performance LC-MS. The identification analyses conducted on seventeen high-abundant serum proteins, mainly immunoglobulins and APPs, including AGP and CP, agreed with our results [244]. Although their diagnostic value is questionable, both AGP and CP might be regarded as potential biomarkers for other purposes such as disease monitoring or recurrence detection. On the other hand, it is important to analyse whether these glycosylation changes described on AGP and CP are a general cancer feature or only PDAC-related. Since APPs are proteins secreted by hepatocytes, AGP and CP glycosylation might be altered in other malignancies, particularly in hepatocellular carcinomas [245, 246]. Moreover, other APPs have also been proposed to carry aberrant glycosylation in PDAC [244, 247]. In particular, the aforementioned fucosylated-haptoglobin has been widely studied as a PDAC biomarker. In agreement with our results, fucosylated-haptoglobin is increased in sera from advanced-stage PDAC patients and its clinical usefulness depends on combining fucosylated-haptoglobin with CA19-9 to improve the performance of CA19-9 alone [224-226].

The research conducted in this thesis, and also by other authors, employing serum as the primary source for finding PDAC biomarkers has repeatedly resulted in APP identification [138], even after serum depletion steps were performed [229, 244]. A new strategy to overcome this weakness was then proposed.

Mucin glycoforms in PDAC tissues

Tumour tissue, where the putative candidates are present in major concentrations, is the most direct source for biomarker identification. Another approach for biomarker discovery performed in this thesis aimed to analyse potential PDAC-specific glycoforms in tissues. The glycoforms chosen were SLe^x and STn on the mucins MUC1 and MUC5AC (Figure 19A). SLe^x and STn are expressed by more than 80% of human carcinomas and are associated with adverse outcome and decreased overall survival of patients [80, 248]. Many studies have linked these epitopes with cancer [57, 59, 80, 249], including PDAC [89, 92, 250]. Both SLe^x and STn are absent in a healthy pancreas, and faintly expressed in ChrP tissues; therefore, their expression in PDAC is an interesting goal to

reach cancer specificity. On the other hand, mucins are heavily glycosylated proteins that could easily carry altered glycan moieties when the glycosylation machinery is altered. MUC1, a widely studied transmembrane protein, is overexpressed in PDAC [165, 166]; therefore it is considered a potential carrier of pathological information. MUC5AC is a secreted gel-forming mucin which is rarely expressed in a normal pancreas but is present in around 80% of PDAC tissues [20]. This peculiarity makes MUC5AC a very interesting candidate for PDAC specificity. However, this protein is also expressed in other tissues such as the respiratory and gastrointestinal epitheliums, the endocervix and the gallbladder [164, 176]. Our working hypothesis is that a particular glycoform could be both cancer- and tissue-specific.

The novel *in situ* proximity ligation assay (PLA) was chosen to probe for specific glycoforms (SLe^x-MUC1, SLe^x-MUC5AC, STn-MUC1, and STn-MUC5AC) in a cohort of 21 PDAC and 3 normal pancreas tissues. This method (Figure 19B) consists of detecting two targets located in close molecular proximity through the recognition of two specific oligonucleotide-conjugated antibodies (PLA probes). When the antibodies bind in close proximity, a bridging sequence links the two oligonucleotide sequences and the closed nucleotide circle can be amplified by a DNA polymerase to generate repeated copies of the circular DNA strands.

Fluorescently labelled oligonucleotides hybridize with the amplified product to produce easily visible fluorescent spots [98]. Contrary to our expectations, STn was poorly expressed on MUC1 and MUC5AC. However, the SLe^x-MUC1 and SLe^x-MUC5AC glycoforms were found in 68% and 84%, respectively, of the mucin expressing PDAC tissues. The decoration of the MUC1 and MUC5AC mucins with SLe^x has been described for other tissues [251-256] but until now not detected in serum. SLe^x-MUC5AC must be regarded as an interesting target for further research both for the neo-expression of this mucin in malignant pancreas and the wide colocalization found in PDAC tissues. This glycoform could reach the peripheral bloodstream when the correct tissue architecture is disrupted during cancer dysplasia. Therefore, a further step will attempt to detect SLe^x-MUC5AC in serum.

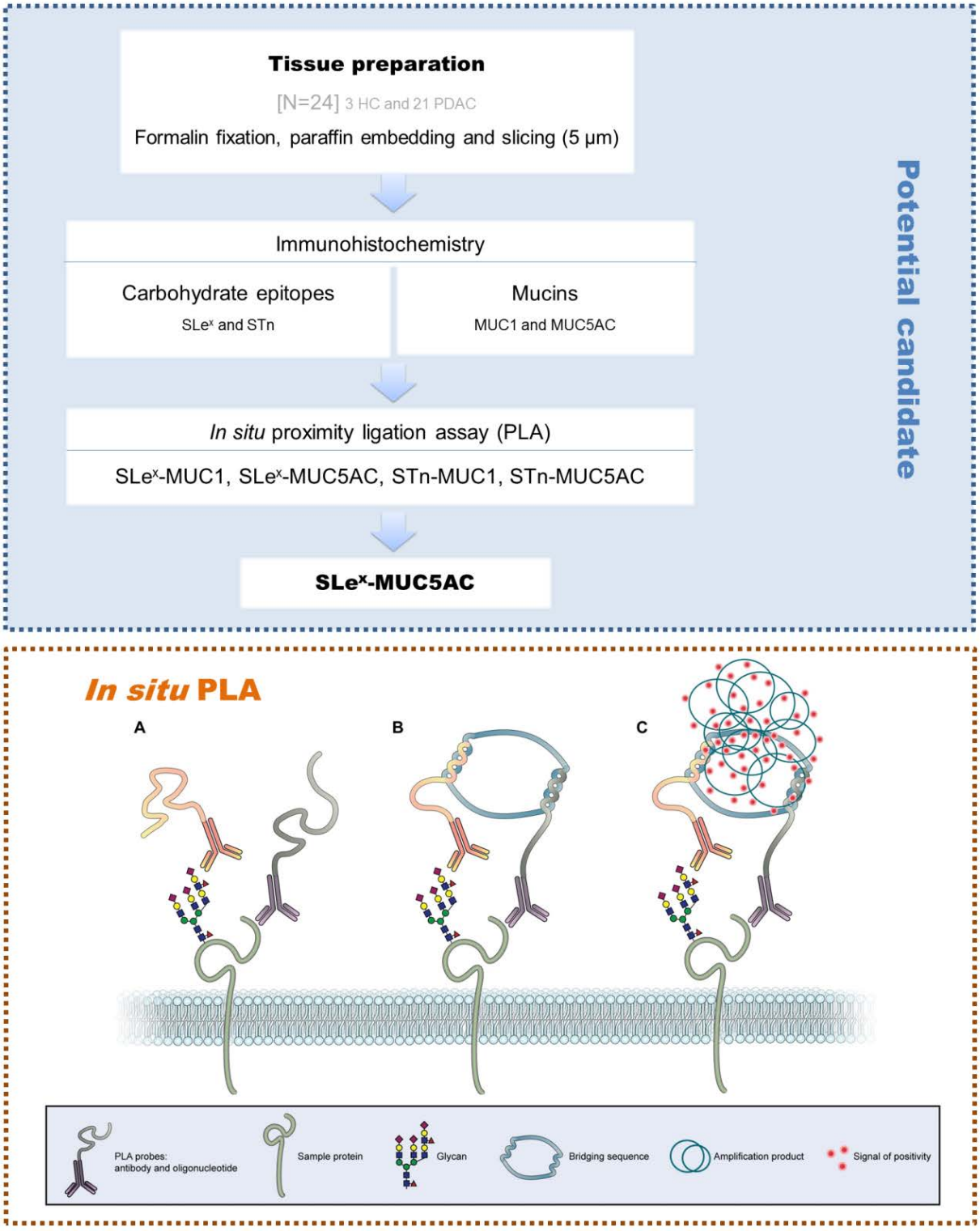

Figure 19: Biomarker candidate identification from tissue. 19A. Tissue immunohistochemistry of different epitopes (SLe^x, STn, MUC1 and MUC5AC) and subsequent PLA for colocalization analyses were performed to identify a biomarker. 19B. The main steps of the PLA technique: (A) Two antibodies, one specific for the protein backbone and the other specific for a glycan epitope, are conjugated with two different oligonucleotide chains (PLA probes). (B) If the antibodies bind to molecules in close proximity, a bridging sequence links the two oligonucleotide sequences. (C) A subsequent polymerase-induced amplification in combination with labelled nucleotides leads to the formation of a fluorescent or chromogenic signal at the co-expression site of the protein and glycan, allowing the in situ detection of the PLA signal. (Extracted from Mereiter *et al*., 2016 [98]).

Concluding remarks

General functions of glycans have been described [56, 57, 59], although the role of a particular glycan on specific glycoconjugate remains unknown. In fact, and as described throughout this dissertation, the structural characterization of glycoproteins is extremely important for better diagnosis of pathologies such as cancer. Moreover, understanding the role of glycans is important in improving our knowledge of biological processes and in maintaining quality control over procedures such as pharmaceutical product manufacturing [257].

Despite the efforts made in biomarker research, not a single new biomarker for PDAC management has shown better performance than CA19-9, mainly due to the incapacity of novel candidates to fulfil initial expectations after pre-clinical and/or clinical tests to improve the specificity and the sensitivity.

Since the research for new biomarkers is a priority for the scientific community, the experience and expertise developed in this work may not only be applied to PDAC diseases but might also be useful for other diseases. This work shows that changes in protein glycosylation can give rise to a more accurate diagnosis than the protein concentration in serum, in agreement with the aforementioned AFP-L3 for HCC. However, the two studied glycoforms (fucosylated-AGP and SLe^x-CP) are APPs, which are usually more indicative of advanced stages of malignancy. This leads us to consider these glycoforms as putative complementary biomarkers for monitoring disease or detecting recurrence.

On the other hand, employing tissues as a source for biomarkers makes it possible to discover specific biomarkers that must then be screened in biological fluids like serum to verify their usefulness. Using this approach, we could identify candidates from the sub-proteome of low abundant molecules that might carry the specific-tissue pathological information. This differs from the prior strategy that started the workflow directly with serum samples, and led to APP detection. Once the putative candidate is established, its detection in the serum sub-proteome cannot be regarded as a drawback since current methodologies directly target serum proteins in the concentration order of 0.005 ng/mL (e.g., PSA test measurable ranges are 0.002 – 100 ng/mL [258]). Thus, the challenge for the scientific community is to decipher the usefulness of the vast amount of

glycoproteins contained in the sub-proteome through the development of novel strategies and methodologies.

Conclusions

1. An increase in fucosylation of AGP in advanced PDAC has been shown using different techniques. Zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry (μZIC-HILIC-ESI-MS) showed differences in the relative abundance of fucosylated glycans of purified AGP between ChrP and PDAC patients. AGP isoforms analysed by CZE displayed significantly different profiles between PDAC and HC. The ELLA assay with *Aleuria aurantia* lectin showed significantly increased fucosylation levels of AGP and sialic acid-digested AGP in PDAC patients compared to ChrP and HC, respectively.

All these techniques have proven useful for finding novel glycoprotein biomarkers in cancer research.

2. Serum depletion of major serum proteins led to the identification of several proteins with increased SLe^x levels in PDAC, all of them being acute-phase proteins. Among them, the analysis of the SLe^x epitope on ceruloplasmin (CP) showed an increased tendency in the SLe^x-CP/CP ratio in sera from PDAC compared to HC and ChrP patients.

3. Serum levels of acute phase proteins hinder the identification of minor proteins with altered glycosylation from a pancreatic tumour origin, even after the depletion of the most abundant serum proteins.

4. The analysis of specific mucin glycoforms (STn-MUC1/MUC5AC and SLe^x-MUC1/MUC5AC) on PDAC tissues using *in situ* proximity ligation assay (PLA) has shown poor expression of STn-MUC1/MUC5AC and high expression of SLe^x-MUC1/MUC5AC in PDAC tissues. Considering that MUC5AC is a secreted mucin neo-expressed in PDAC, the wide expression of SLe^x-MUC5AC glycoform in PDAC tissues suggests that it could reach the bloodstream and become a potential PDAC biomarker.

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Annex 1

Quantitative analysis of N-glycans from human alfa-acid-glycoprotein using stable isotope labeling and zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry as tool for pancreatic disease diagnosis

This article has been published in the journal *Analytica Chimica Acta*.
Giménez E, **Balmaña M**, Figueras J, Fort E, de Bolós C Sanz-Nebot V, Peracaula R, Rizzi A.
Anal Chim Acta. 2015 Mar 25;866:59-68; doi: 10.1016/j.aca.2015.02.008
IF (2014): 4.667; Q1 in the Analytical Chemistry category

Analytica Chimica Acta 866 (2015) 59–68

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Quantitative analysis of *N*-glycans from human alfa-acid-glycoprotein using stable isotope labeling and zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry as tool for pancreatic disease diagnosis

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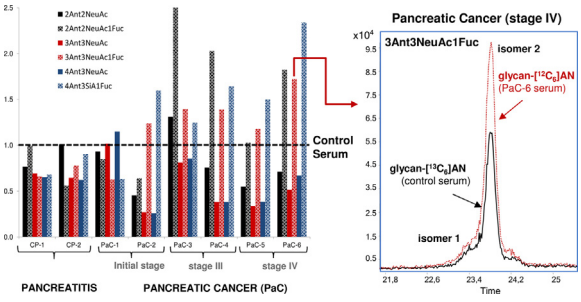
HIGHLIGHTS

- The method enables relative quantitation of hAGP glycans from pathological samples
- Pancreatic cancer samples clearly showed an increase of hAGP fucosylated glycans.
- Fucosylated glycans could be potential biomarkers for diagnosing pancreatic cancer.
- The established method could be extremely useful to find novel glycoprotein biomarkers

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 November 2014
Received in revised form 30 January 2015
Accepted 5 February 2015
Available online 7 February 2015

Keywords:
Glycan analysis
Human α_1-acid-glycoprotein
Pancreatitis
Pancreatic cancer
Zwitterionic hydrophilic interaction liquid

ABSTRACT

In this work we demonstrate the potential of glycan reductive isotope labeling (GRIL) using [^{12}C]- and [^{13}C]-coded aniline and zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry (μZIC-HILIC-ESI-MS) for relative quantitation of glycosylation variants in selected glycoproteins present in samples from cancer patients. Human α_1-acid-glycoprotein (hAGP) is an acute phase serum glycoprotein whose glycosylation has been described to be altered in cancer and chronic inflammation. However, it is not clear yet whether some particular glycans in hAGP can be used as biomarker for differentiating between these two pathologies. In this work, hAGP was isolated by immunoaffinity chromatography (IAC) from serum samples of healthy individuals and from those suffering chronic pancreatitis and different stages of pancreatic cancer, respectively. After de-*N*-glycosylation, relative quantitation of the hAGP glycans was carried out using stable isotope labeling and μZIC-HILIC-ESI-MS analysis. First, protein denaturing conditions prior to PNGase F digestion were optimized to achieve quantitative digestion yields, and the reproducibility of the established

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http://dx.doi.org/10.1016/j.aca.2015.02.008

chromatography
Mass spectrometry

methodology was evaluated with standard hAGP. Then, the proposed method was applied to the analysis of the clinical samples (control *vs*. pathological). Pancreatic cancer samples clearly showed an increase in the abundance of fucosylated glycans as the stage of the disease increases and this was unlike to samples from chronic pancreatitis. The results gained here indicate the mentioned glycan in hAGP as a candidate structure worth to be corroborated by an extended study including more clinical cases; especially those with chronic pancreatitis and initial stages of pancreatic cancer. Importantly, the results demonstrate that the presented methodology combining an enrichment of a target protein by IAC with isotope coded relative quantitation of *N*-glycans can be successfully used for targeted glycomics studies. The methodology is assumed being suitable as well for other such studies aimed at finding novel cancer associated glycoprotein biomarkers.

1. Introduction

Glycosylation is one of the most common co- and post-translational modifications present in proteins, and has a variety of structural and functional roles in membrane-associated and secreted proteins [1]. *O*- and *N*-glycan structures of glycoproteins may be altered in many diseases such as congenital disorders of glycosylation, chronic inflammation or cancer [1]. Hence, searching for specific glycan-structures serving as adequate biomarkers became a widely accepted target in these fields [2,3]. Fucosylation, sialylation or extend and type of branching have been reported as alterations in glycan structures associated with cancer and some inflammatory diseases [2,4]. Several glycoproteins have been described as tumor markers, as for instance prostate-specific antigen for prostate cancer, CA 19-9 for pancreatic and colorectal cancer, TAG-72 for gastric and pancreatic cancer and CA-125 for ovarian cancer. However, some of them are not cancer-specific enough as they can be over-expressed in other diseases as well. In this context, the glyco-pattern of these proteins is discussed as a means for increasing this specificity. For this purpose, a reliable quantitative glycan characterization of potential tumor markers is necessary to determine even minor differences in the glycosylation of these proteins in presence and absence of cancer, and, particularly, to distinguish cancer from other pathologies in which these markers can be altered in abundance as well.

To determine the glycosylation variants present in a protein, glycans are usually released either by enzymatic digestion or by chemical release, derivatized e.g., by permethylation or by reductive amination (attaching for instance a fluorescence label) and subsequently analyzed by liquid chromatography (HPLC) coupled to fluorescence or mass spectrometric detection, or analyzed *via* glycan microarrays [5]. In a previous work, glycan reductive isotope labeling (GRIL) using $[^{12}C_6]/[^{13}C_6]$ coded aniline followed by zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry (μZIC-HILIC-ESI-MS) analysis was established for relative quantitation of *N*-glycans [6]. Unlike other methods reported for glycan analysis, this strategy permits to reliably compare the glycosylation pattern of a glycoprotein (e.g., disease *vs*. healthy control) as this relative quantitation is independent on the ionization yields and the charge-state distributions for different glycans, and is not influenced by ion suppression effects and the tuning of the mass spectrometer. Moreover, the separation of isomeric glycans by ZIC-HILIC stationary phases enables one to distinguish between samples exhibiting different proportions of isobaric isomers differing in branching and linkage positions [6].

In this work, the *N*-glycans of human α_1-acid-glycoprotein (hAGP) have been analyzed using stable-isotope coding for relative quantitation and ZIC-HILIC-MS for distinguishing of linkage/branching-isomers with the particular aim of applying the proposed methodology to the analysis of relevant samples in cancer research. hAGP is selected as an acute phase serum glycoprotein of approximately 40 kDa containing five complex type *N*-glycans attached to the polypeptide backbone (at Asn15, Asn38, Asn54, Asn75 and Asn85) [7,8]. Certain variability in the microheterogeneity profile of these five AGP oligosaccharides has been described, depending on type and severity of diseases [8–10]. There is wide agreement that changes in hAGP glycosylation are associated with cancer and in chronic inflammation and concern mainly an increased glycan branching and the expression of the sialyl-Lewis epitopes (sLe^x). However, it is not clear yet if there are some other (quantitative) differences in glycan-structures suited to differentiate between both pathologies [11,12] and serving in this respect as potential differential-biomarkers. In this work, *N*-glycans of hAGP from pathological sera, *i.e.*, with chronic pancreatitis at the one hand and different stages of pancreatic cancer on the other hand, were compared with those released from hAGP of healthy volunteers. Immunoaffinity chromatographic (IAC) enrichment of a target glycoprotein, in this instance hAGP, followed by isotope coded labeling of the released glycans and relative quantitation of isobaric linkage variants by means of μZIC-HILIC-ESI-MS is presented here as a powerful strategy in targeted patho-glycomics and cancer research.

2. Materials and methods

2.1. Chemicals

All chemicals used for the preparation of buffers and solutions were analytical reagent grade. Acetic acid (HAc, glacial), formic acid (HFor 98–100%), dimethylsulphoxide (DMSO), ethanol (EtOH), trifluoroacetic acid (TFA), sodium hydrogen phosphate (Na_2HPO_4) and acetone were supplied by Merck (Darmstadt, Germany). DL-Dithiothreitol (DTT, ≥99%), iodoacetamide (IAA), ammonium hydrogencarbonate (NH_4HCO_3), sodium cyanoborohydride ($NaBH_3CN$), $[^{12}C_6]$-aniline ($[^{12}C_6]$AN), $[^{13}C_6]$-aniline ($[^{13}C_6]$AN), 2-mercaptoethanol (β-ME) and sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Hydrogen chloride (HCl, 37%) was supplied by Panreac (Barcelona, Spain). "NP-40 alternative" was provided by Calbiochem (Darmstadt, Germany) and RapiGest® by Waters (Milford, Massachusetts, USA). Acetonitrile (ACN), ammonium acetate (NH_4Ac) and water LC–MS quality grade provided by Merck and Fluka (Madrid, Spain), respectively, were used for μZIC-HILIC-ESI-TOF-MS analysis. A standard sample of human α_1-acid-glycoprotein (hAGP, 99%) was purchased from Sigma–Aldrich. Peptide *N*-glycosidase F (PNGase F) and glycine were purchased from Roche Diagnostics (Basel, Switzerland). Polyclonal goat anti-human AGP antibody (affinity purified) was purchased from Immune Systems Ltd. (Devon, UK). ESI low concentration (ESI-L) tuning mix was supplied by Agilent Technologies (Waldbronn, Germany) for tuning and calibrating the TOF mass spectrometer.

2.2. hAGP purification from serum samples

Control and pathological human serum samples were provided by Hospital Universitari Dr. Josep Trueta, Girona, Spain, following the standard procedures of its Ethics Committee. Four serum samples of healthy volunteers were used as controls and eight pathological serum samples were analyzed: two serum samples from individuals with pancreatitis (CP-1 and CP-2) and six serum samples from individuals suffering from pancreatic cancer (two with an initial stage (PaC-1 and PaC-2); two with stage III (PaC-3 and PaC-4); and two samples with stage IV (PaC-5 and PaC-6). The study was approved by the Ethics Committee (CEIC) of the Hospital. Control and pathological serum samples (0.1 mL) were incubated with 1% (v/v) protease inhibitor cocktail for 30 min at −20 °C and diluted to 0.2 mL with Milli-Q water before immunoaffinity chromatography (IAC) purification [13]. The IAC-column was prepared as reported in [13]. Briefly, the polyclonal anti-hAGP antibody was bonded to an epoxy-silica chromatographic support from Waters (Protein-Pak epoxy-activated affinity products) after different activation steps, the IAC-support was packed into a PEEK-lined stainless steel tubing

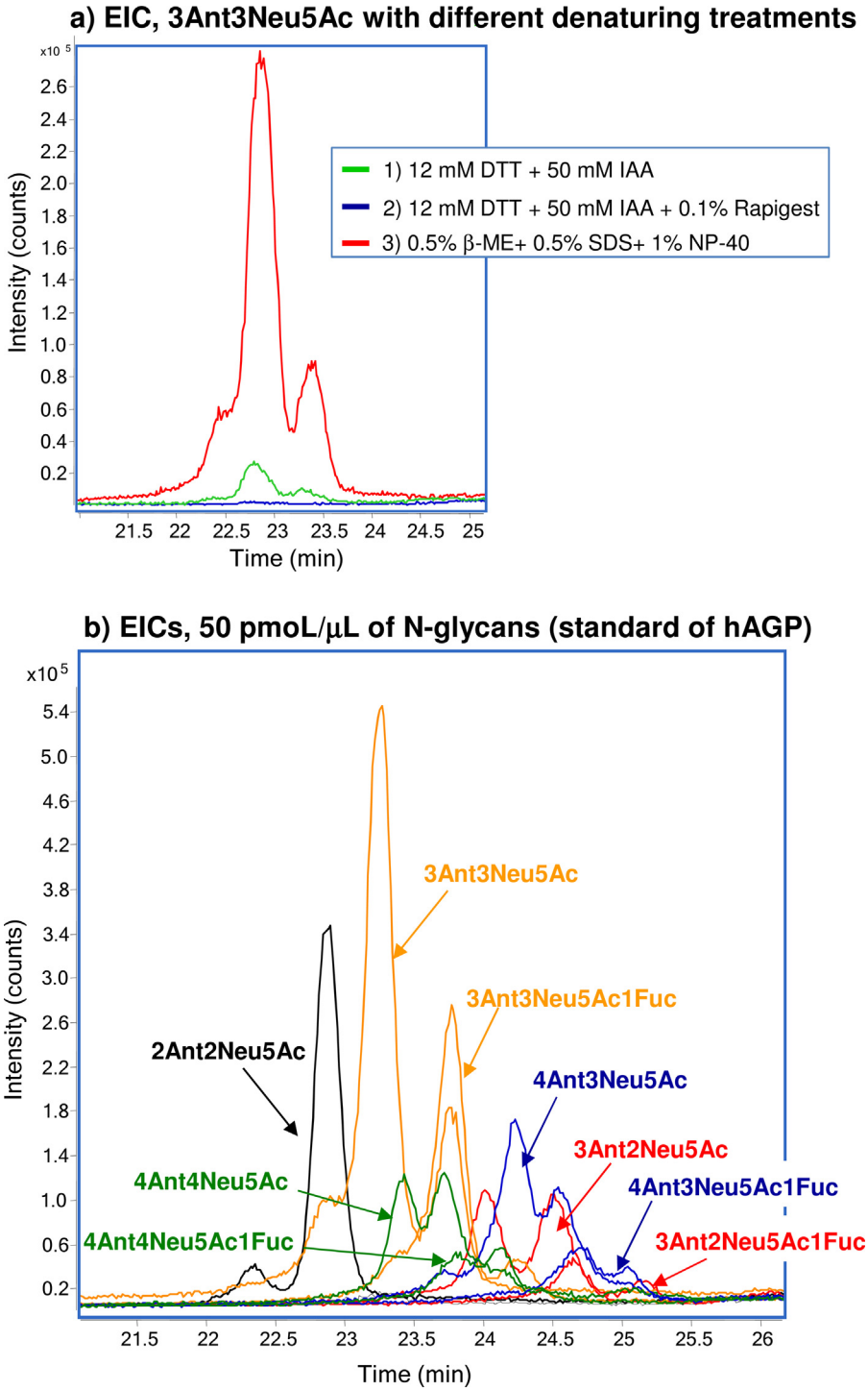

Fig. 1. (a) Extracted ion chromatogram (EIC) of 3Ant3Neu5Ac glycan labeled with [$^{12}C_6$]AN by μZIC-HILIC-ESI-MS after three different denaturing methods: (1) 12 mM DTT + 50 mM IAA, (2) 12 mM DTT + 50 mM IAA + 0.1% Rapigest, (3) 0.5% β-ME + 0.5% SDS +1% NP-40. (b) EICs of the detected hAGP derived aniline-labeled *N*-glycans analyzed by μZIC-HILIC-ESI-MS (standard hAGP at 50 pmol μL^{-1} of *N*-glycan).

(30 × 4.6 mm I.D) using 2 μm pore diameter frits [13]. Immunopurification of serum samples was performed by use of an Äkta-FPLC instrument (GE Healthcare, Waukesha, WI, USA) equipped with a UV–vis detector following a two-step procedure. First, the column was conditioned with phosphate buffered saline (PBS) (10 mM sodium phosphate, 138 mM NaCl and 2.7 mM KCl, pH 6.85) pumping at a flow rate of 0.5 mL min^{-1} over 10 min. Then, it was "cleaned" (for removing any memory from previous runs), by injecting three times 1.4 mL of the "elution buffer", *i.e.*, 0.1 M glycine-HCl buffer at pH 2.2. When baseline was stabilized, 0.2 mL pretreated serum sample was injected. When the absorbance signal was returned to the baseline level (after about 10 min), the buffer was changed to the "elution buffer" effecting the elution of the compounds specifically retained in the IAC column. The eluted compounds were collected and neutralized by addition of 0.1 M Na_2HPO_4. This eluted and neutralized fraction was called F0. As a second purification step, the neutralized fraction F0 was re-injected into the IAC column (as described in detail in [13]), and 1.4 mL of "elution buffer" was injected three times, *i.e.*, at 10 min, 25 min and 40 min after injection, respectively. The corresponding eluted fractions (named F1, F2 and F3) were collected. The purity of the eluted fractions was evaluated by analyzing these fractions by SDS-PAGE (10% acrylamide gels) and silver staining. F3 fraction was discarded, and F1 and F2 were mixed, neutralized by addition of 0.1 M Na_2HPO_4, desalted, and concentrated by using centrifuge filter devices with 3 kDa cut off membrane (Amicon Ultra 0.5 mL 3 K).

After IAC, the eluted hAGP (from F1 and F2 fractions) was quantified using a Nanodrop spectrophotometer (Thermo Scientific) by measuring absorbance at 280 nm and using the parameter E1% = 8.93 following the recommendations of the manufacturer for hAGP standard. Finally, a pool of hAGP control was prepared by mixing similar amounts of hAGP purified from the four healthy serum controls. hAGP samples were evaporated to dryness by Speed Vac and stored at −20 °C.

2.3. Release, purification and labeling of N-glycans

hAGP standards as well as serum samples purified by immunoaffinity chromatography were denatured following three different procedures. Method 1: samples were reduced with 12 mM DTT in 50 mM NH_4HCO_3 (pH 7.9). The mixture was incubated in a water bath at 56 °C for 1 h and then alkylated with 50 mM IAA for 30 min at room temperature in the dark. Excess of reagents were removed by ultracentrifugation with Microcon YM-10 (M_W cut-off 10 kDa; Millipore, Bedford, MA, USA). The final residue was reconstituted in 50 μL of 50 mM NH_4HCO_3 (pH 7.9). Method 2: samples were reduced and alkylated with DTT and IAA as in method 1 but also 0.1% (w/v) of Rapigest® was added in the reaction mixture. Excess of reagents were also removed with a Microcon YM-10 filter and the final residue was reconstituted in 50 μL of 50 mM NH_4HCO_3 (pH 7.9) with 0.1% Rapigest®. Method 3: samples were reduced with 0.5% of β-ME in the presence of 0.5% of SDS in 50 mM NH_4HCO_3 (pH 7.9) and boiled in a thermo-block for 30 min. A volume of 50 mM NH_4HCO_3 (pH 7.9) with 1% (v/v) of NP-40 alternative was added to achieve a final concentration of 0.1% of SDS and β-ME in the samples. With all three methods, 0.5 μL of PNGase F (0.5 U) solution were added to the mixture which was carefully vortexed and incubated at 37 °C for 18 h. To ensure total deglycosylation of hAGP samples, 0.5 μL of PNGase F solution were added after 18 h and incubation was continued for additional 18 h. Digestion was stopped by adding ~1 μL of HFor (pH must be adjusted to approximately 3). In case of method 2, at the end of digestion RapiGest® was hydrolyzed to avoid interferences with MS analysis. For this purpose, HFor was added to a concentration of 5% (v/v) and the mixture was incubated at 37 °C for 45 min. Then, the solution was centrifuged for 10 min at 12,000 rpm to separate precipitated by-products from RapiGest®. The supernatant was carefully collected and stored at −20 °C until analysis.

Released *N*-glycans were purified by solid phase extraction (SPE) using Hypercarb cartridges (25 mg, 1 mL volume, Thermo Fisher Scientific). SPE cartridges were first conditioned with 1 mL of 60% ACN, 0.1% HFor and equilibrated with 2 mL of water. After dissolving in ~500 μL of water, the digested hAGP sample was loaded and the SPE cartridge was rinsed with 1 mL of water. Retained *N*-glycans were eluted with 500 μL of 60% ACN, 0.1% HFor and the eluate was evaporated to dryness by Speed Vac. Dried *N*-glycans were stored at −20 °C until used.

Dried hAGP glycans were mixed with 10 μL of the reaction mixture and incubated at 70 °C for 2 h. The reaction mixture consisted of 0.35 M aniline and 1 M $NaCNBH_3$ in DMSO with 30% HAc [6,14]. Samples were cooled to room temperature and the labeled glycans were precipitated from the DMSO solution with acetone as described in [6]. Finally, the excess of acetone was removed by Speed Vac and the dried glycans were stored at −20 °C. Centrifugations were carried out in a Mikro 220R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).

2.4. μZIC-HILIC-ESI-MS

The μHPLC-ESI-TOF-MS experiments were performed using a 1200 series capillary liquid chromatography system (Agilent Technologies) coupled to a 6220 oa-TOF LC/MS mass spectrometer (Agilent Technologies). For the chromatographic separation a ZIC-HILIC column packed with 3.5 μm particles, 150 × 0.3 mm L_T × ID (SeQuant, Umeå, Sweden) was used; the stationary phase consisting of a surface with immobilized zwitterionic sulfobetaine moieties. Experiments were performed at room temperature with gradient elution at a flow rate of 4 μL min^{-1} and injecting 0.15 μL of glycan sample labeled with aniline (glycan concentration: ~50–25 pmol μL^{-1}). Eluting solvents were A: 5 or 10 mM NH_4Ac solution (for maltohexaose or released *N*-glycans, respectively) and B: acetonitrile. Gradient conditions were used: solvent B from 90 to 50% within 40 min as linear gradient, followed by cleaning and equilibration steps of B: 50 → 0% (within 5 min), 0% (over 10 min), 0 → 90% (within 5 min) and 90% (over 10 min) [6].

The mass spectrometer was equipped with a dual-nebulizer ESI source whereof the orthogonal nebulizer was used for the μLC-TOF-MS experiments; the other one, which is generally used to introduce the internal reference mass standard solution in conventional LC–MS experiments, was disabled to avoid any interference with the μLC-TOF-MS experiments. Tuning and calibration of the mass spectrometer followed the manufacturer's instructions. Later, the measurement parameters were fine-tuned by direct infusion of maltohexaose labeled with [$^{12}C_6$]-aniline maximizing the signal for the singly charged molecular ion [6]. The best ("optimum") operational conditions in negative mode were: capillary voltage −3500 V, drying gas (N_2) temperature 200 °C, drying gas flow rate 4 L min^{-1}, nebulizer gas (N_2) 15 psi, fragmentor voltage 190 V, skimmer voltage 70 V and OCT 1 RF Vpp voltage 300 V. Data were collected in profile (continuum) at 1 spectrum s^{-1} (approx. 10,000 transients/spectrum) between *m/z* 100 and 3000 working in the highest resolution mode (4 GHz). μLC-TOF-MS control, data acquisition and analysis were performed using the MassHunter workstation software (Agilent Technologies).

3. Results and discussion

3.1. Glycan-profile and efficiency of glycan release

In a previous work, glycan reductive isotope labeling (GRIL) using [^{12}C]- and [^{13}C]-coded aniline was optimized and used for relative quantitation of *N*-glycans of different model glycoproteins

such as ovalbumin, bovine α_1-acid-glycoprotein and fetuin by μZIC-HILIC-ESI-MS. The established methodology provided a reproducible relative quantitation and good separation of isobaric glycans [6]. Though relative quantitation of glycan variants enzymatically released from selected target glycoproteins is assumed not to be very sensitive to the (protein-site specific) degree of glycan release, (as it is based on the comparison between the same protein/glycan analytes in corresponding samples), the over-all sensitivity of glycan determination is affected by the degree of release. In order to achieve a widely complete de-*N*-glycosylation of intact glycoproteins, different protein denaturing conditions prior to PNGase F digestion were tested here for human α_1-acid-glycoprotein. Method 1 uses DTT and IAA to reduce and alkylate the disulfide bonds in the glycoprotein. This method was previously used in [6] and is widely used in proteomics prior to protein digestion [15,16]. Method 2 contains in addition to DTT and IAA also Rapigest, an acid-labile anionic surfactant which was described to increase the de-glycosylation yield of some glycoproteins *via* glycoprotein denaturation, making glycans more accessible to enzymatic cleavage with PNGase F [17,18]; and method 3 uses β-ME (0.1%) for reduction, SDS (0.1%) for denaturation, and NP-40 alternative (1%) to preserve PNGase F activity. Fig. 1a shows, as an example, the extracted ion chromatogram (EIC) obtained for the 3Ant3Neu5Ac glycan (triantennary complex type glycan with three *N*-acetyl-neuraminic acid units) labeled with [$^{12}C_6$]-aniline released from 50 μg of standard hAGP, using the three denaturing methods. The release efficiency of such a triantennary glycan is assumed being dependent on protein folding and can thus be seen as an indicator for sufficient glycoprotein denaturation. In our studies, the highest signal for this glycan was obtained by using β-ME and SDS (method 3). The denaturing treatment previously used in [6] (method 1) also leads to the detection of this triantennary glycan but to a lower extent. Surprisingly, method 2 gave poorer sensitivity in comparison with both other methods. Rapigest is hydrolyzed after protein digestion under acidic conditions giving place to a sulfonate salt that probably is not completely removed during glycan purification by the hypercarb cartridges. It is further not yet clear whether Rapigest or its remaining parts after decomposition can interfere with the glycan labeling or impedes ionization process in μZIC-HILIC-ESI-MS. As we were able to detect even minor abundant "bulky" glycans of hAGP when using method 3, this denaturation procedure was used for our following study.

3.2. Analysis of isotopically labeled N-glycans released from standard hAGP

Table 1 shows the [$^{12}C_6$]AN-labeled *N*-glycans of hAGP detected with the established methodology (*N*-glycans released from 50 μg of standard hAGP), together with the peak areas measured from their EICs, the RSD of the peak areas calculated from three experiments as well as the mass accuracy. In this targeted glycomics study, we evaluated the abundances of bi-, tri-, and tetra-antennary species with different content of sialic acids (from 0 to 4) and fucose units (from 0 to 2), but also the presence of other types of *N*-glycans. No signals corresponding to high-mannose- or hybrid-type *N*-glycan species were detected. All complex *N*-glycans previously reported by other authors were detected with the exception of the biantennary, triantennary and tetraantennary glycans with one Neu5Ac. Further, we did not observe in the hAGP standard sample the 3Ant4Neu5Ac and 4Ant4Neu5Ac glycans with one additional unit of *N*-acetyllactosamine (HexHexNAc or LacNAc) reported by Nakano et al. [19], and the 4Ant4Neu5Ac2Fuc described by Sarrats et al. [12]. As previously described for bovine AGP [6], the ZIC-HILIC methodology was able to separate isobaric glycans. It is assumed, but still has to be confirmed, that these isomers differ with respect to the 2,3- and 2,6-linkage of the terminal sialic acids, respectively. We detected at least two isomers for all hAGP released glycans carrying sialic acids, except for 2Ant2Neu5Ac1Fuc that only showed one (see Table 1). Fig. 1b shows the EICs for the detected glycans. The most abundant glycans in standard hAGP were the triantennary ones with three Neu5Ac (3Ant3Neu5Ac and 3Ant3Neu5Ac1Fuc) followed by the biantennary glycan with 2 NeuAc (2Ant2NeuAc). This is in agreement with the data of other authors [19,20]. 2Ant2Neu5Ac1Fuc was detected in very low concentration. For this minor glycan the detection limit was ~25 pmol μL^{-1} of glycan

Table 1
N-glycans released from 50 μg of hAGP labeled with [$^{12}C_6$]AN and analyzed by μZIC-HILIC-ESI-MS in negative ion mode. RSD calculated from three parallel experiments ($n = 3$). *N*-glycan concentration: 50 pmol μL^{-1}.

hAGP glycan-[$^{12}C_6$]AN	M_{theo}[a]	M_{exp}[a]	Error (ppm)	Peak area	$RSD_{peakarea}$
2Ant2Neu5Ac isomer 1	2299.8232	2299.8111	5.3	603737	25.6
2Ant2Neu5Ac isomer 2	2299.8232	2300.1498	−8.6	4857503	22.6
2Ant2Neu5Ac1Fuc	2445.8810	2445.8718	3.9	281795	21.2
3Ant2Neu5Ac isomer 1	2664.9554	2664.9396	5.9	213899	5.0
3Ant2Neu5Ac isomer 2	2664.9554	2664.9419	5.1	1784623	18.7
3Ant2Neu5Ac isomer 3	2664.9554	2664.9399	5.8	1743872	17.7
3Ant2Neu5Ac1Fuc isomer 1	2811.0132	2810.9982	5.4	868149	21.6
3Ant2Neu5Ac1Fuc isomer 2	2811.0132	2810.9968	5.8	316066	21.4
3Ant3Neu5Ac isomer 1	2956.0513	2956.0333	6.1	1559616	22.0
3Ant3Neu5Ac isomer 2	2956.0513	2956.0335	6.0	8191719	23.0
3Ant3Neu5Ac isomer 3	2956.0513	2956.0024	16.5	2474880	22.2
3Ant3Neu5Ac1Fuc isomer 1	3102.1093	3102.0796	9.6	680077	28.7
3Ant3Neu5Ac1Fuc isomer 2	3102.1093	3102.0745	11.2	4206234	22.5
4Ant3Neu5Ac isomer 1	3321.1837	3321.0361	10.3	510194	22.0
4Ant3Neu5Ac isomer 2	3321.1837	3321.1624	6.4	2900915	18.8
4Ant3Neu5Ac isomer 3	3321.1837	3321.1594	7.3	1714319	32.6
4Ant Neu5Ac isomer 4	3321.1837	3321.1634	6.1	175966	29.1
4Ant3Neu5Ac1Fuc isomer 1	3467.2414	3467.2179	6.8	1352964	20.7
4Ant3Neu5Ac1Fuc isomer 2	3467.2414	3467.2181	6.7	439372	22.8
4Ant4Neu5Ac isomer 1	3612.2791	3612.2499	8.1	1728006	20.7
4Ant4Neu5Ac isomer 2	3612.2791	3612.2335	12.6	1597215	17.7
4Ant4Neu5Ac isomer 3	3612.2791	3612.2494	8.2	467991	8.0
4Ant4Neu5Ac 1Fuc isomer 1	3758.3368	3758.3124	6.5	1004114	21.8
4Ant4Neu5Ac1Fuc isomer 2	3758.3368	3758.3110	6.9	917433	25.4

[a] M_{exp} and M_{theo}: experimental and theoretical monoisotopic molecular masses.

in the injection solution, which corresponds to a LOD of 0.03 μg of hAGP when injecting 150 nL sample volume. In our previous work, the reproducibility of labeling with [$^{12}C_6$] and [$^{13}C_6$]-aniline was evaluated [6]. However, the reproducibility of the over-all methodology was not yet estimated taking into account all steps from PNGase F digestion, glycan purification to glycan labeling. Moreover, the reproducibility was previously determined with 100 μg of glycoprotein (bovine AGP). In this work, we considered necessary to study the reproducibility of the established methodology with a much lower amount of glycoprotein, as the hAGP amounts in the samples purified and enriched from pathological and control sera were only around 5–6 μg. For this purpose, three independent samples ($n = 3$) of 5 μg of standard hAGP were labeled with [$^{12}C_6$]AN and another three with [$^{13}C_6$]AN. After derivatization, equimolar mixtures of hAGP-glycan-[$^{12}C_6$]AN and hAGP-glycan-[$^{13}C_6$]AN were prepared and analyzed by μZIC-HILIC-ESI-MS in negative mode by triplicate. Table 2 shows the molar ratios obtained experimentally for six *N*-glycans of standard hAGP, with standard deviations (±s) and relative standard deviations (RSDs). The RSD values obtained in this case were all below 10, though they were higher than those obtained in [6]. Such a trend was expected, first because here we evaluated the reproducibility of the over-all methodology and, second, because the amount of glycoprotein was lower giving lower glycan signals and making quantitation of peak areas more difficult in terms of reproducibility. *In toto*, the reproducibility obtained for 5 μg of hAGP was acceptable and it demonstrates that the methodology is still adequate for this amount of glycoprotein.

3.3. Analysis of hAGP released N-glycans isolated from serum samples

Once demonstrated the reliability of the method with the standard glycoprotein, we proceeded to the relative quantitation of hAGP glycans isolated from serum samples from patients with chronic pancreatitis and different stages of pancreatic cancer (initial, stage III and IV). First, hAGP from control and pathological serum samples were purified by IAC. IAC-separated fractions F1 and F2 taken for our experiments were controlled for purity by SDS/PAGE analysis and silver staining. Single bands of hAGP were obtained in all cases of control and patient sera. Additional quantitative (proteomic type) experiments comparing IAC-purified AGP amounts with analogous amounts of commercial standard AGP on the level of tryptic peptides, gave an agreement between both proteins in the range of about 95%. We thus assume that traces of concomitant proteins which might potentially still present after IAC purification will not significantly bias the over-all results (it is worth to keep in mind that the isotope-coded labeling procedure applied here would help keeping the bias resulting from such an co-enrichment effect small, as the amounts of identical glycan species are compared between samples. Any bias introduced by the IAC purification, favoring or suppressing a particular species, would happen in control and patient sera to a more or less comparable extent).

The aim of this work was to evaluate the established methodology when applied to biological/clinical samples and to study the glycosylation pattern of this protein in diseases with a high inflammatory component like pancreatitis or pancreatic cancer, each compared to healthy controls. Different authors have described an altered glycosylation of acute-phase proteins in different types of cancers. It has been reported an increase in sialylation, branching and/or fucosylation of their *N*-glycans [1,2,4]. However, the analytical methodologies established up to now for glycan analysis do not provide reliable results when small differences in glycan expression are observed between samples. Relative quantitation by stable isotope labeling followed by μZIC-HILIC-MS analysis is an excellent tool to carry out a trustworthy quantitation of the glycans present in a sample. In addition, this method also allows to distinguish between isobaric isomers (*e.g.*, linkage isomers). To attain meaningful results, we took care that the samples that we compare contain approximately the same amount of protein in order to be sure that the amount of glycan detected is due to an over-expression or under-expression of the glycan of interest, and not because the amount of protein in both samples is not comparable. For this reason, we checked the amount of hAGP isolated from pathological and control sera by UV quantitation using the Nanodrop spectrophotometer. For this purpose, 4 μg of standard hAGP were submitted to glycan release and labeling with [$^{12}C_6$]AN and 4 μg of hAGP from the control serum pool for labeling with [$^{13}C_6$]AN. After derivatization, an equimolar mixture of standard hAGP-glycan-[$^{12}C_6$]AN and control-pool hAGP-glycan-[$^{13}C_6$]AN was analyzed by μZIC-HILIC-ESI-MS in negative ion mode by triplicate. Fig. 2 shows the EICs for the glycans 2Ant2Neu5Ac and 3Ant3Neu5Ac in both samples (standard hAGP, in orange and control-pool hAGP, in blue). It can be observed from the EICs and also from the MS spectra of Fig. 2a(i) and b(i), that the signals of the glycans in both samples were practically the same. This result confirmed that the quantification with Nanodrop was excellent and reliable enough to do a relative quantitation by our established GRIL method.

For the analysis of the pathological samples, 5–6 μg of purified hAGP was used, depending on the amount of pathological sample available. The amount of hAGP from the control pool was always taken equivalently to the pathological sample amount. After PNGase F digestion and purification, glycans were labeled with [$^{12}C_6$]AN and [$^{13}C_6$]AN, respectively. Then, both samples were mixed (1:1) and analyzed by μZIC-HILIC-ESI-MS. Fig. 3 shows as an example two hAGP glycans (3Ant3Neu5Ac and 3Ant3Neu5Ac1Fuc) in one of the samples with pancreatitis (CP-2) compared to one sample from pancreatic cancer stage IV patient (PaC-6). 3Ant3Neu5Ac was less abundant in the PaC-6 sample compared to control; in the CP-2 sample the expression of this glycan was also decreased, although to a lesser extent compared to PaC-6. In the

Table 2
Reproducibility of the methodology (PNGase F digestion, glycan purification and labeling). Experimental molar ratios obtained from a 1:1 mixture of *N*-glycans released from 5 μg of standard hAGP, labeled with [$^{12}C_6$]AN and [$^{13}C_6$]AN and analyzed by μZIC-HILIC-ESI-MS in negative ion mode ($n = 3$).

	Glycan-[$^{12}C_6$]AN		Glycan-[$^{13}C_6$]AN		Exp. ratio[b] ($n = 3$)	RSD of exp. ratio
	M_{exp}[a]	Peak area	M_{exp}[a]	Peak area		
2Ant2Neu5Ac isomer 2	2299.8761	214311	2305.8970	270110	0.87 (±0.09)	10.0
2Ant2Neu5Ac1Fuc	2445.9449	10253	2451.9441	10767	1.02 (±0.09)	8.9
3Ant3Neu5Ac isomer 2	2956.1170	463078	2962.1414	570989	0.86 (±0.03)	3.3
3Ant3Neu5Ac1Fuc isomer 2	3102.1746	171674	3108.1978	208963	0.85 (±0.06)	7.5
4Ant4Neu5Ac isomer 3	3612.3589	109139	3618.3778	139707	0.83 (±0.07)	8.1
4Ant4Neu5Ac1Fuc isomer 2	3758.3947	27297	3764.4097	32441	0.89 (±0.08)	9.0

[a] M_{exp}: experimental monoisotopic molecular mass.
[b] Average experimental ratio ($n = 3$). Experimental ratios obtained from 3 independent binary mixtures of [$^{12}C_6$]AN and [$^{13}C_6$]AN hAGP *N*-glycans. Each experimental ratio was calculated as: EIC peak area glycan-[$^{12}C_6$]AN/peak area glycan-[$^{13}C_6$]AN.

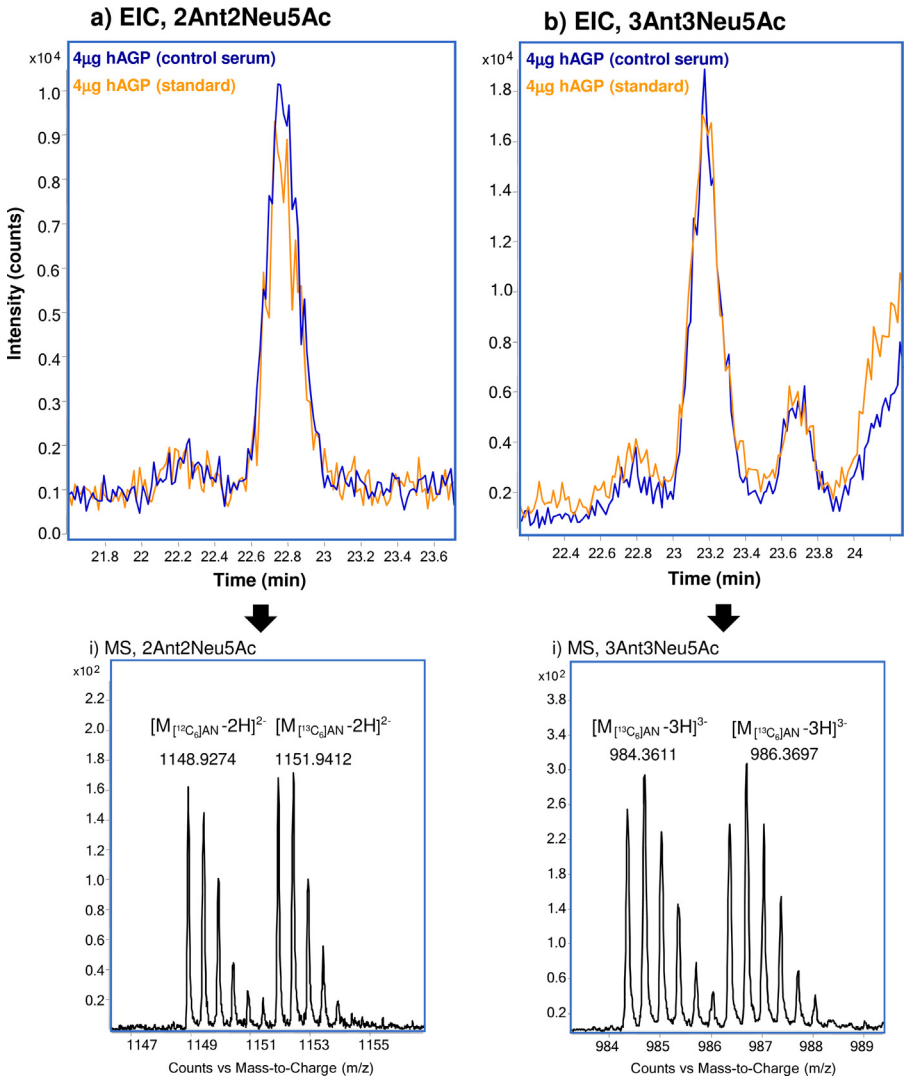

Fig. 2. µZIC-HILIC-ESI-MS analysis of a mixture (1:1) of aniline-labeled *N*-glycans released from 4 µg of standard hAGP and 4 µg of hAGP in the pool control serum: (a) EICs of 2Ant2Neu5Ac glycan; (b) EICs of the 3Ant3Neu5Ac glycan (hAGP control, in blue, and hAGP standard, in orange) with the negative ion mass spectra over the time windows corresponding to the indicated peaks (a(i) and b(i)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

case of the fucosylated glycan (3Ant3Neu5Ac1Fuc), this was higher expressed in the cancer sample (compared to control), whereas in CP-2 its signal was slightly lower compared to the control. Fig. 4 shows the peak areas measured from the EICs of all glycans detected in the control and in the two pathological samples (CP-1 and PaC-4). We can indeed observe that the abundance of most hAGP glycans depends on the state (healthy *vs.* pathological) and differ between the two pathologies (CP *vs.* PaC). It seems that the abundance of fucosylated glycans increases in PaC, but not in CP, and it is likely that the increase in the abundance observed for the fucosylated species corresponds to the decrease observed for the non-fucosylated ones. Interestingly, an increase in branching of AGP glycans in pathological samples was not observed. The sum of all tetraantennary glycans always showed lower peak areas in PaC and CP samples compared to the healthy control, unlike reported by [12]. To achieve consistent results, relative quantitation was performed for all hAGP glycosylation variants, and Table 3 shows the experimental molar ratios obtained in each type of pathology ($n = 2$): CP and different stages of PaC (initial PaC, PaC stage III and PaC stage IV). The relative quantitation of the pathological samples compared to the control confirmed that there is a decrease in the non-fucosylated glycans and an increase of the fucosylated glycans in PaC samples, and this effect becomes more pronounced as the stage of PaC increases. In contrast, the results obtained with the two pancreatitis samples indicate that the expression of the glycans does not substantially vary compared to control. The average molar ratios obtained in CP samples for all hAGP glycans were around 0.8 which could suggest that glycans were slightly under-expressed in CP compared to control. However, it is important to keep in mind that molar ratio values of 0.8 are still within the confidence limit (*i.e.*, not yet significantly changes). Hence, we conclude that the expression of most of hAGP glycans in CP samples cannot be seen as being significantly different from those in the healthy control pool.

When comparing different isomers of isobaric glycans, in most instances we did not observe significant differences in the abundance pattern of these isomers. Commonly, if one isomer was over-expressed compared to the control, the rest of isomers of the glycan had a similar behavior, and vice-versa. However, in the case of fucosylation of the glycan species 3Ant3NeuAc, we did detect significant differences in the isomer pattern comparing pathological samples between each other and between control. As can be seen in Fig. 3 and from the data in Table 3, while isomer 1 and 2 of the non-fucosylated species decreased in PaC samples, the abundance of isomer 3 was similar to the control. This alteration was observed in all PaC samples with the exception of

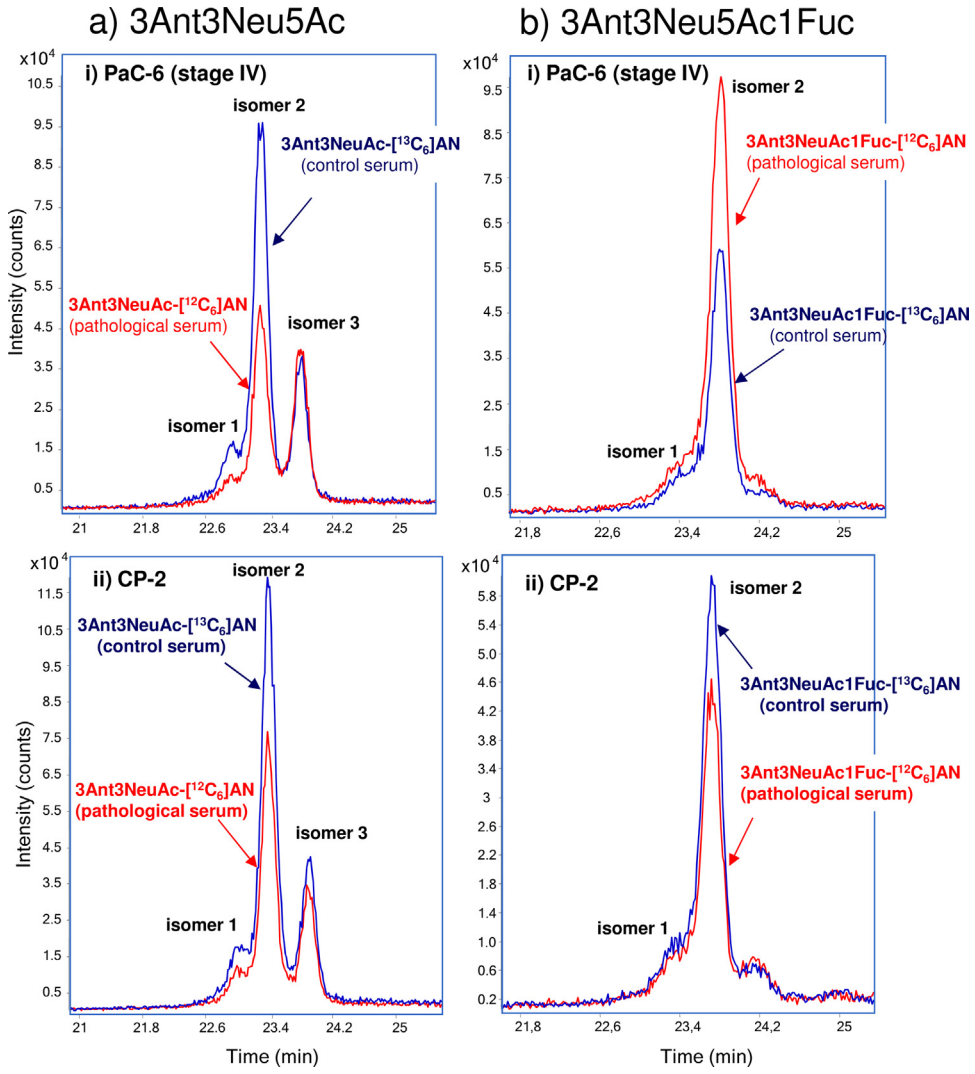

Fig. 3. μZIC-HILIC-ESI-MS analysis of a mixture (1:1) of aniline-labeled *N*-glycans released from 5 μg of control hAGP and 5 μg of pathological hAGP: (a) EICs of 3Ant3Neu5Ac glycan; (b) EICs of the 3Ant3Neu5Ac1Fuc glycan (hAGP control, in blue, and hAGP pathological, in red). (i) Sample with pancreatic cancer with stage IV (PaC-6) and (ii) sample with chronic pancreatitis (CP-2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

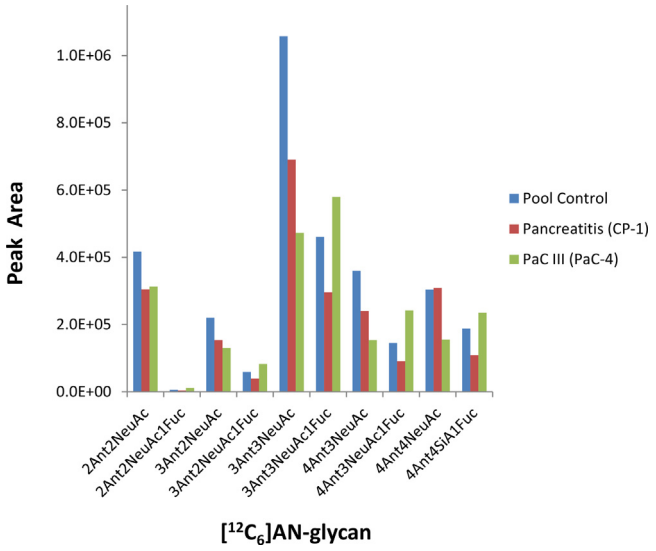

Fig. 4. Peak areas measured from the EICs of all the detected *N*-glycans of hAGP in three different samples: pool control (blue), pancreatitis (CP-1, red) and pancreatic cancer with stage III (PaC-4, green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PaC-1 (sample with an initial PaC). As the HILIC separated isomers are assumed to correspond to linkage variants of the sialic acids (2,6- or 2,3-linked), one can interpret these data such that the extent of fucosylation of the mentioned species (which results in a decrease in the abundance of the non-fucosylated species) is influenced by the linkage position of the sialic acids. Hypothesizing that the activities of fucosyl-transferases are influenced by sialic acid linkage positions primarily when dealing with fucosylation in the antennas (not in the core), one can see these data as an indication for the presence of antenna-fucosylation. This hypothesis, however, has to be corroborated by MS^2 data.

Fig. 5 shows relative abundances (compared to control) for fucosylated and non-fucosylated species of the most relevant glycans in all pathological samples. We can observe that PaC samples clearly show an increase of all fucosylated glycans as the stage of the disease increases, and this is unlike to CP samples. These findings do not support previous studies which found fucosylation of triantennary and tetraantennary glycans of hAGP being also over-expressed in CP samples [12]. 2Ant2Neu5Ac1Fuc expression was more difficult to evaluate in the different stages of PaC because this minor glycan showed higher uncertainties in the relative quantitation, as peak areas were more difficult to integrate. Nevertheless, this fucosylated glycan was over-expressed only in

Table 3
Experimental molar ratios obtained from a 1:1 mixture of *N*-glycans released from 5 μg of control hAGP labeled with [$^{13}C_6$]AN and 5 μg of pathological hAGP labeled with [$^{12}C_6$]AN and analyzed by μZIC-HILIC-ESI-MS in negative ion mode.

	Pancreatitis ($n=2$) Ratio (±s)	PaC initial ($n=2$) Ratio (±s)	PaC III ($n=2$) Ratio (±s)	PaC IV ($n=2$) Ratio (±s)
2Ant2Neu5Ac isomer 1	0.82 (±0.15)	0.81 (±0.38)	0.99 (±0.25)	0.63 (±0.08)
2Ant2Neu5Ac isomer 2	0.83 (±0.11)	0.71 (±0.26)	1.06 (±0.29)	0.63 (±0.09)
2Ant2Neu5Ac1Fuc	0.73 (±0.32)	0.75 (±0.19)	2.29 (±0.52)	1.42 (±0.48)
3Ant2Neu5Ac isomer 2	0.71 (±0.10)	0.61 (±0.51)	0.72 (±0.22)	0.46 (±0.12)
3Ant2Neu5Ac isomer 3	0.80 (±0.08)	0.71 (±0.32)	0.73 (±0.16)	0.59 (±0.15)
3Ant2Neu5Ac1Fuc isomer 1	0.77 (±0.13)	0.95 (±0.27)	1.31 (±0.03)	1.62 (±0.20)
3Ant2Neu5Ac1Fuc isomer 2	0.73 (±0.18)	1.06 (±0.31)	1.33 (±0.43)	1.50 (±0.43)
3Ant3Neu5Ac isomer 1	0.78 (±0.15)	0.82 (±0.49)	0.65 (±0.30)	0.48 (±0.07)
3Ant3Neu5Ac isomer 2	0.76 (±0.10)	0.64 (±0.41)	0.60 (±0.24)	0.43 (±0.10)
3Ant3Neu5Ac isomer 3	0.80 (±0.11)	0.80 (±0.08)	0.95 (±0.19)	0.86 (±0.25)
3Ant3Neu5Ac1Fuc isomer 1	0.89 (±0.20)	1.10 (±0.28)	1.21 (±0.29)	1.64 (±0.41)
3Ant3Neu5Ac1Fuc isomer 2	0.70 (±0.16)	0.82 (±0.12)	1.39 (±0.08)	1.45 (±0.30)
4Ant3Neu5Ac isomer 1	0.69 (±0.13)	0.86 (±0.73)	0.65 (±0.31)	0.52 (±0.24)
4Ant3Neu5Ac isomer 2	0.74 (±0.11)	0.70 (±0.63)	0.62 (±0.33)	0.53 (±0.20)
4Ant3Neu5Ac isomer 3	0.79 (±0.12)	0.75 (±0.51)	0.65 (±0.35)	0.64 (±0.27)
4Ant3Neu5Ac isomer 4	0.81 (±0.12)	0.89 (±0.17)	1.15 (±0.51)	1.00 (±0.47)
4Ant3Neu5Ac1Fuc isomer 1	0.74 (±0.17)	0.95 (±0.14)	1.58 (±0.15)	1.42 (±0.40)
4Ant3Neu5Ac1Fuc isomer 2	0.75 (±0.21)	1.11 (±0.68)	1.44 (±0.28)	1.92 (±0.59)
4Ant4Neu5Ac isomer 2	0.67 (±0.12)	0.67 (±0.69)	0.64 (±0.34)	0.45 (±0.13)
4Ant4Neu5Ac isomer 3	0.77 (±0.08)	0.76 (±0.63)	0.79 (±0.32)	0.60 (±0.27)
4Ant4Neu5Ac1Fuc isomer 1	0.75 (±0.15)	0.86 (±0.09)	1.20 (±0.30)	1.39 (±0.62)
4Ant4Neu5Ac1Fuc isomer 2	0.82 (±0.17)	1.03 (±0.25)	1.53 (±0.06)	1.54 (±0.61)

PaC samples (like the rest of fucosylated glycans) but not significantly changed in abundance in CP. Based on these results, the two glycan species 3Ant3NeuAc1Fuc and 4Ant3NeuAc1Fuc could be seen as potential biomarkers for diagnosing the stage of PaC and, further, to differentiate between cancer (PaC) and inflammatory (CP) pathologies of the pancreas. In early stages of cancer, however, the difference between cancer and inflammatory glycan pattern is not so clear. Fig. 5 indicates that, whereas one of the initial PaC samples (PaC-2) already showed an increase of the fucosylated glycans, the glycosylation pattern of PaC-1 was similar to those obtained for the two CP samples. Hence, to confirm if relative quantitation of hAGP *N*-glycans followed by μZIC-HILIC-ESI-MS analysis could be used in the future for (early) diagnosis of pancreatic cancer and to distinguish cancer from chronic pancreatitis, it will be necessary to extent this preliminary study to more clinical cases, especially those with chronic pancreatitis and initial PaC.

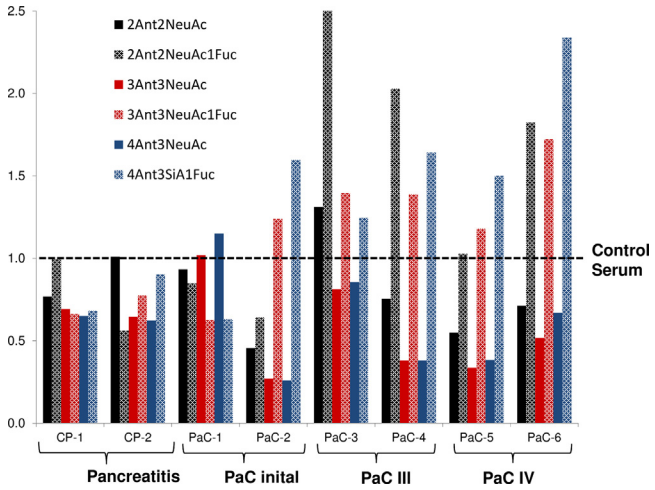

Fig. 5. Experimental molar ratios for the most significant *N*-glycans of hAGP in all pathological samples. A molar ratio value of 1 is obtained when the glycan in the pathological sample have the same peak area as the glycan detected in the pool control.

4. Conclusions

In the first part of this paper we could show that the use of SDS and β-ME prior to PNGase F digestion significantly improved the yield of the glycan release from hAGP increasing in this way the sensitivity of the method. We further demonstrated that the reproducibility of the over-all methodology, covering all steps from PNGase F digestion to glycan labeling and separation, was adequate for the amount of glycoprotein present in our samples (about 5 μg) after purification and enrichment from pathological and control sera.

Carrying out relative quantitation of hAGP released glycans after isolation from serum samples originating from individuals with chronic pancreatitis (CP) and different stages of pancreatic cancer (PaC), our preliminary results indicate that on the basis of the relative abundance of certain selected glycan species (particularly fucosylated glycans like 3Ant3NeuAc1Fuc and glycan isomers like 3Ant3NeuAc isomers 1 and 2 *vs.* 3), it seems feasible to distinguish between hAGP of healthy *vs.* pathological sera and between inflammatory diseases of the pancreas (CP) and pancreatic cancer (PaC). The isotope coding permitted to attain an enhanced reliability of the quantitative results which makes even small differences between samples more relevant. It is evident that the limited sample number analyzed up to now by the method established here does not yet allow answering the question of aberrant hAGP glycosylation associated with the mentioned types of diseases on a statistically sound level. To go further in this direction, this study will be extended to a higher number of probands at each stage of disease, especially those with CP and initial PaC. Nevertheless, these preliminary but relevant results concerning hAGP glycosylation demonstrate that the presented methodology involving isotope coding for relative quantitation of *N*-glycan species (and isobaric isomers thereof) could be extremely useful in patho-glycomics, particularly for finding novel glycoprotein based biomarker structures related to cancer and/or other diseases.

Acknowledgments

M.B. acknowledges University of Girona for a pre-doctoral fellowship. Part of this study was supported by the Spanish

Ministry of Science and Innovation (CTQ2011-27130 awarded to V. S-N and BIO 2010-16922 awarded to R.P.). We thank Drs. Angel Puerta and Mercedes de Frutos from Spanish National Research Council (CSIC, Madrid) for their helpful assessment in the preparation and use of the immunoaffinity column.

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Annex 2

Glycomic approaches for the discovery of targets in gastrointestinal cancer

Review

This review has been published in the journal *Frontiers in Oncology*.
Mereiter S, **Balmaña M**, Gomes J, Magalhaes A, Reis CA.
Front Oncol. 2016 Mar 9;6:55 doi: 10.3389/fonc.2016.00055

frontiers in Oncology

REVIEW
published: 09 March 2016
doi: 10.3389/fonc.2016.00055

Glycomic Approaches for the Discovery of Targets in Gastrointestinal Cancer

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Gastrointestinal (GI) cancer is the most common group of malignancies and many of its types are among the most deadly. Various glycoconjugates have been used in clinical practice as serum biomarker for several GI tumors, however, with limited diagnose application. Despite the good accessibility by endoscopy of many GI organs, the lack of reliable serum biomarkers often leads to late diagnosis of malignancy and consequently low 5-year survival rates. Recent advances in analytical techniques have provided novel glycoproteomic and glycomic data and generated functional information and putative biomarker targets in oncology. Glycosylation alterations have been demonstrated in a series of glycoconjugates (glycoproteins, proteoglycans, and glycosphingolipids) that are involved in cancer cell adhesion, signaling, invasion, and metastasis formation. In this review, we present an overview on the major glycosylation alterations in GI cancer and the current serological biomarkers used in the clinical oncology setting. We further describe recent glycomic studies in GI cancer, namely gastric, colorectal, and pancreatic cancer. Moreover, we discuss the role of glycosylation as a modulator of the function of several key players in cancer cell biology. Finally, we address several state-of-the-art techniques currently applied in this field, such as glycomic and glycoproteomic analyses, the application of glycoengineered cell line models, microarray and proximity ligation assay, and imaging mass spectrometry, and provide an outlook to future perspectives and clinical applications.

Keywords: gastric cancer, colorectal cancer, pancreatic cancer, glycomics, glycan biomarkers, microarray, proximity ligation assay, imaging mass spectrometry

OPEN ACCESS

Edited by:
Leonardo Freire-de-Lima, Federal University of Rio de Janeiro, Brazil

Reviewed by:
Stephan Von Gunten, University of Bern, Switzerland
Ali Mobasheri, University of Surrey, UK

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Specialty section:
This article was submitted to Molecular and Cellular Oncology, a section of the journal Frontiers in Oncology

Received: *03 February 2016*
Accepted: *24 February 2016*
Published: *09 March 2016*

Citation:
Mereiter S, Balmaña M, Gomes J, Magalhães A and Reis CA (2016) Glycomic Approaches for the Discovery of Targets in Gastrointestinal Cancer. Front. Oncol. 6:55. doi: 10.3389/fonc.2016.00055

GLYCOBIOLOGY IN CANCER

The cells' glycocalix constitutes an important interface with the extracellular milieu and plays critical roles in physiological and pathological conditions. This glycan-rich coating of the cells' plasma membrane is composed by different classes of glycoconjugates, including glycoproteins, glycolipids, and proteoglycans, which participate in key regulatory events for cellular and organ homeostasis. Alterations in glycosylation can interfere with normal molecular functions such as cell–cell recognition, communication, and adhesion, leading to acquisition of malignant features.

Moreover, the shedding of aberrant glycoconjugates, uniquely expressed by tumor cells, into circulation provides one valuable source of biomarkers for cancer diagnosis and prognosis (1).

Substantial advances in the frontiers of cancer glycobiology have been possible in the recent past due to the combination of novel tumor cell biology concepts with cutting-edge glycomic technologies. Specific glycosylation alterations have been identified in tumors and some of the molecular pathways underlying these modifications have been disclosed (2). In addition, aberrant glycoforms have been demonstrated to be molecularly associated with more aggressive cancer cell and tumor features, including increased migration, invasion, and metastization potential, providing novel targets for therapeutic intervention (3–5).

This review describes the recent progress in gastric cancer, colorectal cancer (CRC), and pancreatic ductal adenocarcinoma (PDAC) glycobiology and discusses the clinical value of aberrant glycosylation as a source of screening biomarkers and therapeutic targets. A comprehensive overview of the advances in glycomic and glycoproteomic tools is also provided and their possible applications for tumor glycan-profiling and discovery of novel targets for improving gastrointestinal (GI) tumors' clinical management are discussed.

GLYCOSYLATION ALTERATION IN GASTROINTESTINAL CANCERS

Despite the large amount of glycan epitopes that can be found in the human GI tract and the complex and manifold alterations of the glycosylation machinery during the process of carcinogenesis and cancer progression, the current knowledge makes it possible to group the most common glycan alterations. Expression of truncated simple *O*-glycans, changes in *N*-glycan branching, and increase in sialylation and fucosylation are three major *N*- and *O*-glycosylation events involved in GI cancer that will be described in detail (**Figure 1**). Furthermore, we give an overview on other common glycosylation alterations in cancer, such as changes in *O*-GlcNAcylation, modified glycosphingolipids, and glycosaminoglycans (GAGs) and proteoglycans.

Truncated Simple *O*-Glycans

One common feature observed in GI tumors is the overexpression and exposure of short, truncated *O*-glycans. Mucin-type *O*-glycans are found on most transmembrane and secreted proteins. A single *O*-glycan oligosaccharide chain can present more than 20 monosaccharide constituents (6). In malignancy, *O*-glycans are often shortened resulting in an increase of the monosaccharide Tn antigen (GalNAcα1-Ser/Thr), the disaccharide T antigen (also known as Thomsen–Friedenreich antigen or core 1 structure, Galβ1-3GalNAcα1-Ser/Thr) and their sialylated forms, STn (Neu5Acα2-6GalNAcα1-Ser/Thr), and ST (Neu5Acα2-3Galβ1-3GalNAcα1-Ser/Thr), respectively (**Figure 1**) (7, 8).

Polypeptide GalNAc transferases (ppGalNAcTs), which are the initiating enzymes of the mucin-type *O*-glycosylation (9, 10), show often altered expression in cancer (11–13). A total of 20 different ppGalNAcTs are known in human and their expression profile and subcellular localization determine *O*-glycosylation sites and densities (9, 14). In CRC, for example, the ppGalNAc-T3 is associated with tumor differentiation, disease aggressiveness, and prognosis (12). In gastric cancer, the expression of ppGalNAc-T6 is associated with venous invasion and the downregulation of ppGalNAc-T2 increases the cancer cell proliferation, adhesion, and invasion (11, 15).

In addition, enzymes competing for the same substrate can also induce expression of truncated glycans and exposure of protein epitopes that would be hidden otherwise. For instance, the relative enzymatic activities of C2GnT (*N*-acetylglucosaminyltransferase) and ST3Gal-I (sialyltransferase), two glycosyltransferases that compete for the same substrate, have been shown to determine the *O*-glycan structure in cancer cells (16).

STn is expressed in most GI carcinomas correlating with decreased cancer cell adhesion, increased cancer cell invasion, and poor prognosis of the patients (17–23). The terminal STn epitope is synthesized by the sialylation of Tn by the ST6GalNAc-I sialyltransferase (17, 18). In cancer, the formation of STn may occur due to ST6GalNAc-I upregulation, early sialylation caused by glycosyltransferase misslocalization in the secretory pathway, or the impairment of the elongation of the Tn antigen (14, 17, 18, 24).

In gastric cancer, expression of STn is a common feature associated with more malignant phenotypes. The overexpression of ST6GalNAc-I has been shown to induce migration and invasion in gastric carcinoma cells *in vitro* (20).

In this regard, another gene that can underlie the synthesis of truncated *O*-glycans is *COSMC*, which encodes for a C1GalT1 dedicated chaperone (25). The galactosyltransferase C1GalT1 is responsible for the elongation of the Tn antigen to form the core 1 structure also known as the T antigen. The absence of a functional *COSMC* entails the dysfunction of C1GalT1. In PDAC, it has been shown that hypermethylation of *COSMC*, and not somatic mutations, is the prevalent cause of truncated *O*-glycans (23). In addition, the downregulation of C1GalT1 in combination with the upregulation of ST6GalNAc-I has been associated with increased STn expression in CRC cell lines and epithelial cells derived from resected CRC tumor tissue (26). In contrary, the overexpression of C1GalT1 is associated with invasion, metastization, and poor survival in CRC. In C1GalT1 overexpressing CRC cells, the knockdown of C1GalT1 suppresses the malignant phenotype *in vitro* and *in vivo* (27). Increased levels of C2GnT, a glycosyltransferase responsible for the biosynthesis of core 2 structures, are also frequent in CRC (28). This enzyme has also a critical role in the biosynthesis of terminal sialylated Lewis antigens on *O*-glycans that will be further discussed in Section "Increased Sialylation and Fucosylation."

Normal pancreas does not express the Tn antigen and its corresponding sialylated epitope STn (21). The Tn antigen is detected in 75–90% of PDACs and up to 67% in precursor lesions (24). The appearance of the STn in mucins, on the other hand, is a late event in PDAC disease progression (29). These truncated *O*-glycans are associated with cancer cell growth and tumor invasion in PDAC (23, 24). The situation is slightly different in CRC, where the overexpression of T antigen is associated with early events in cancer progression and both Tn and STn antigens are frequently overexpressed in advanced and poorly differentiated adenocarcinomas

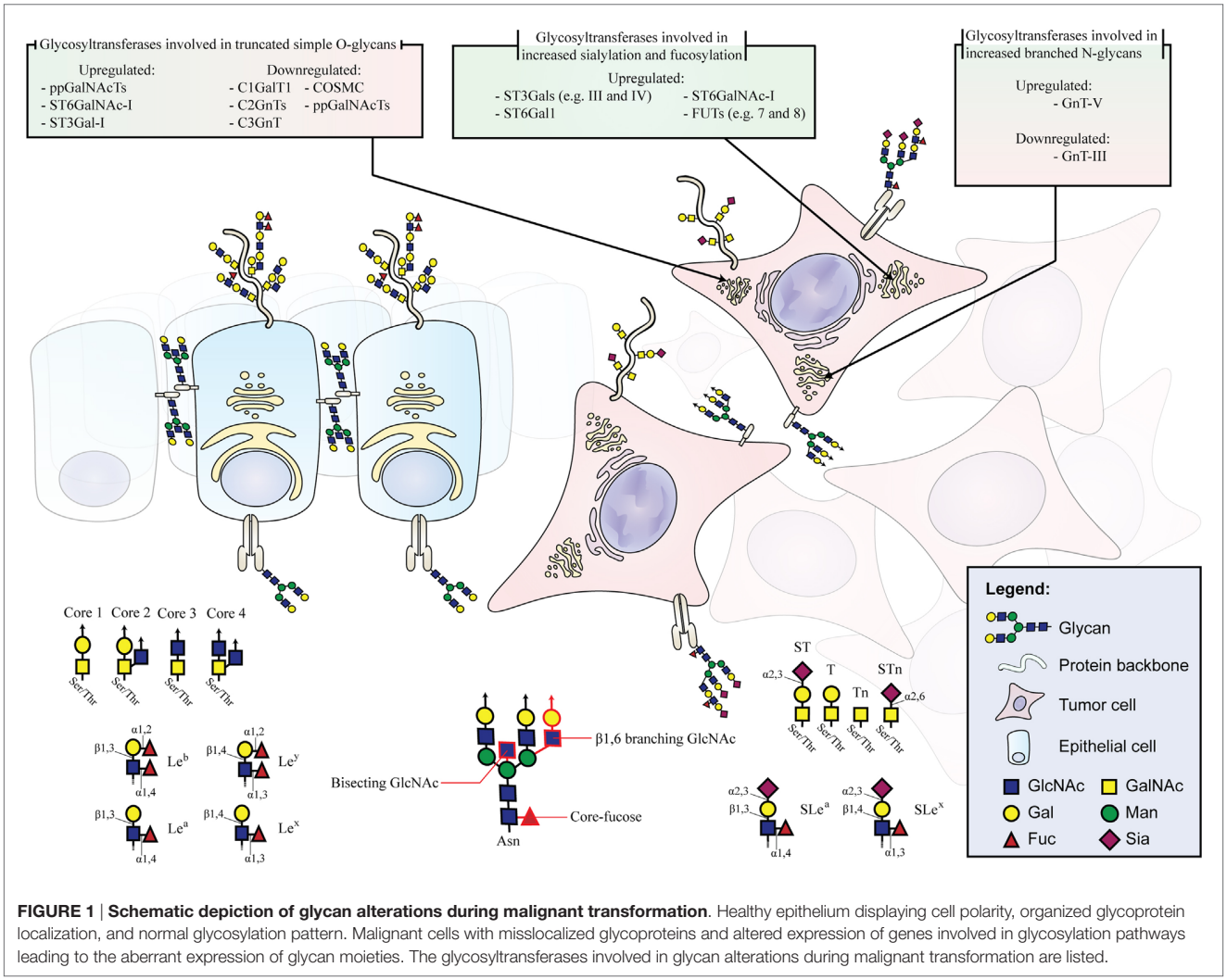

FIGURE 1 | Schematic depiction of glycan alterations during malignant transformation. Healthy epithelium displaying cell polarity, organized glycoprotein localization, and normal glycosylation pattern. Malignant cells with misslocalized glycoproteins and altered expression of genes involved in glycosylation pathways leading to the aberrant expression of glycan moieties. The glycosyltransferases involved in glycan alterations during malignant transformation are listed.

and also in mucinous carcinomas. Therefore, these antigens are considered useful markers for poor outcome (22).

Besides cores 1 and 2, *O*-glycans with cores 3 and 4 are also often expressed in normal GI epithelia, especially in colon, but are significantly decreased in malignancy due to downregulation of the core 3 and 4-synthetase (8, 30–32).

The *de novo* expression of truncated *O*-glycans in GI cancers is of avail in the search of specific cancer biomarkers. It leads to the expression of unique glycopeptide structures and, because of the small steric size of these truncated *O*-glycan moieties, to the exposure of protein regions that would otherwise be masked, and, therefore, not detected by specific antibodies (29, 33).

Branched *N*-Glycans

The biosynthesis and maturation of *N*-glycan structures is defined by a complex interplay of numerous glycosidases and glycosyltransferases in the endoplasmic reticulum and Golgi. Among *N*-glycan types, the complex *N*-glycans display the largest structural diversity. Two structural features of complex *N*-glycans are the β1,6-branching, catalyzed by the glycosyltransferase GnT-V, and the bisecting-GlcNAc, added by the glycosyltransferase GnT-III. GnT-V is known to be upregulated in gastric carcinoma (**Figure 1**) (34), leading to the increased branching of *N*-glycans and contributing to cancer cell invasion and metastases (35, 36). Analogically, normal colon epithelium presents high levels of bisecting-GlcNAc, due to high expression levels of GnT-III, which is associated with suppression of the tumor progression. However, during cancer progression, these bisecting structures are decreased (37) and it has been described a general increase of β1,6-branched in complex *N*-linked glycans that are also associated with tumor invasion and metastasis (38). Histochemical studies using specific lectins for the detection of β1,6-branched structures showed increased staining concomitant with tumor CRC staging (39), and an association with lymph node metastasis and decreased survival rates in CRC patients (40). GnT-V, the enzyme responsible for the synthesis of β1,6-branched *N*-glycans, is commonly upregulated in CRC correlating to the metastatic potential and consequently

considered an important prognosis factor to detect poor CRC patients' outcome (41). A recent study demonstrated that GnT-V levels modulate CRC stem cells and tumor formation by Wnt signaling (42). Increased extension of β1,6-branched complex *N*-glycans by long polymers of *N*-acetyllactosamine (LacNAc) due to upregulation of β3GnT8 has also been described in CRC cells (43).

Regarding PDAC progression, little has been described about bisecting structures, although the increase in highly branched *N*-glycans is well established. The number of tri- and tetra-antennary glycans is significantly increased in both pancreatic cancer cells and PDAC patients' serum and correlate with cancer progression (44, 45).

Another mechanism leading to increased branching is the downregulation of GnT-III and the addition of bisecting-GlcNAc. Bisected structures cannot be further modified by GnT-V and, therefore, preclude the formation of branched *N*-glycans under healthy conditions.

The interplay of GnT-III and GnT-V modulates cell adhesion and migration in a gastric cancer context (46, 47). This has been shown to be particularly important for cell–cell and cell–matrix interactions in gastric cancer by altering the functionality of E-cadherin and integrins in malignant transformation. E-cadherin promotes adherence junction formation and, thus, maintains intercellular adhesion. E-cadherin is stabilized by bisected *N*-glycans delaying its endocytosis and turnover (47–49). Furthermore, bisecting-GlcNAc on E-cadherin is gastric tumor suppressive by downregulating signaling pathways involved in cell motility and the EMT process (50–54). Conversely, E-cadherin is dysregulated when glycosylated with branched *N*-glycans by GnT-V in the context of gastric cancer (34, 52, 53). GnT-V is commonly upregulated in gastric carcinomas contributing to cell invasion and metastases (35, 36). The overexpression of GnT-V leads to destabilization of adherence junctions, delocalization of E-cadherin into the cytoplasm, and mesenchymal appearance of the cells with increased metastatic capability (34, 52, 55).

Integrins convey adhesion to extracellular matrix components and are often altered in GI carcinomas. In gastric cancer, the modification of α3β1 integrin with branched *N*-glycans increases cell migration (56). The modification of α3β1 integrin with bisecting-GlcNAc has the opposite effect by inhibiting cell migration (56). Consistently, the overexpression of GnT-III resulted in the inhibition of α5β1 integrin-mediated cell migration and reduced binding to fibronectin due to a specific *N*-glycosylation site on the α5 integrin (57, 58).

Increased Sialylation and Fucosylation

Sialic acids are the largest and the only intrinsically negatively charged monosaccharides present in human glycosylation. As a terminal event, sialylation caps glycosylation chains usually resulting in exposed locations of the negative charge at the forefront of the oligosaccharides and first encounter point for adjacent glycans, proteins, and cells. Sialylation has, therefore, been shown to play important roles in modulating cellular recognition, cell adhesion, and cell signaling (59). Moreover, cancer cell sialylation patterns define sialic acid-binding lectins (Siglecs) interactions and modulate immune response (60, 61).

An increase in global sialylation, especially in α2,6- and α2,3-linked sialylation, owing to altered glycosyltransferases expression, has been closely associated with cancer and commonly described as one of the main modifications in GI cancers (62, 63). For example, ST6Gal-I, the enzyme that adds α2,6-linked sialic acid to lactosamine chains (Neu5Acα2,6Galβ1,4GlcNAc), is commonly overexpressed in GI cancers correlating with poor prognosis (59, 64, 65). Additionally, α2,3-sialyltransferases, such as ST3Gal-III and ST3Gal-IV, are often upregulated in the course of gastric cancer and PDAC progression leading to a more invasive and metastatic phenotypes of the cancer cells (65–69). Furthermore, sialylation, in particular α2,3 and α2,6-linked, can modulate the ECM adhesion and migration. Specifically, it has been described that while the overexpression of terminal α2,6-linked sialic acid leads to increased ECM adhesion, the overexpression of α2,3-linked terminal sialic acid epitopes in PDAC cancer cell lines results in a more migratory phenotype (70). Similarly, in gastric cancer cells, the overexpression of α2,3-linked terminal sialic acid epitopes causes a more invasive phenotype *in vitro* and *in vivo* (67).

The major α2,3-sialylated antigens associated with cancer are SLe^a and SLe^x (**Figure 1**). Although these structures can also be present in non-neoplastic cells, SLe^a and SLe^x have been demonstrated to be highly expressed in many malignant tissues, including GI tumors, both in glycoproteins and glycosphigolipids (71–74). SLe^x-increased expression levels are associated with advanced stages and have been correlated with poor survival in GI cancer patients (75–77). SLe^x is the well-known ligand for selectins (78). During inflammation, selectins mediate the initial attachment of leukocytes to the endothelium during the process of leukocyte extravasation. In cancer, SLe^x interactions with selectins favor metastasis by forming emboli of cancer cells and platelets and promoting their arrest on endothelia (77).

The overexpression of SLe^x in a gastric carcinoma cell line transfected with *ST3GAL4* has shown to increase the cells invasive potential both *in vitro* and *in vivo* due to the activation of the oncogenic c-Met receptor tyrosine kinase (67). Moreover, overexpression of *ST3GAL4* has been shown to result in RON receptor tyrosine kinase activation and co-expression of RON and SLe^x is observed in gastric tumors (79). This is of particular biological relevance since it has been described that RON activation contributes to tumor progression, angiogenesis, and therapy resistance and correlates with bad prognosis (80–84).

Sialylated Lewis epitopes are potential good markers for prognosis due to their high incidence of recurrence or presence in metastasis and correlation with the tumor stage. For example, a recent work described the increase of the SLe^x epitope on ceruloplasmin in PDAC. The increased ceruloplasmin with the SLe^x epitope in chronic pancreatitis was lower, suggesting good specificity for pancreatic malignancy (85). Moreover, studies using high-density antibody microarray also detected increased levels of SLe^x and SLe^a antigens on glycoproteins in serum or plasma of CRC patients (86).

Overexpression of the enzyme β-galactoside α2,6-sialyltransferase I (ST6Gal-I), especially in *N*-glycans and not in *O*-glycans, has been associated with CRC progression, increased invasion, and metastization and consequently poor prognosis in

CRC patients (64, 87). Further studies taking into consideration the low levels of ST6Gal-I in healthy individuals and upregulation in CRC patients could lead to the development of new diagnostic and therapeutic targets.

Fucosylation is also an important modification involved in cancer and inflammation (88). The attachment of fucose to *N*-glycans, *O*-glycans, and glycolipids has been reported in cancer tissues, regulating different biological processes, and being also responsible for the increased expression of Lewis antigens (89) (and sialylated-Lewis antigens, as previously described). Different studies link the presence of fucosylated epitopes on specific glycoproteins with cancer. In particular, research performed on some acute phase proteins suggest the suitability of fucosylated epitopes for cancer management. It has been demonstrated that fucosylated alpha-fetoprotein (AFP) is more specific as a hepatocellular carcinoma biomarker than AFP itself. Nowadays fucosylated AFP (AFP-L3) is used for hepatocellular carcinoma risk assessment (90, 91). Acute phase proteins, such as AFP, are proteins synthesized by hepatocytes and have shown clinical value as markers for liver and pancreatic-related diseases. For example, haptoglobin and AGP have revealed an increase in fucosylated epitopes that could help to improve PDAC diagnosis (89, 92).

Increased activity in α1,3 and α1,4 fucosyltransferases (FUTs) was described in CRC patients, resulting in the synthesis of SLe^x and SLe^a epitopes, respectively (89). Particularly, FUT6 was more recently reported as the major regulator of SLe^x biosynthesis in CRC (93). Increased levels of fucosylation in plasma samples of CRC patients compared to normal controls were also described using methods for *N*-glycoproteomics analysis to identify plasma markers (94). In addition, increased levels of α1,2-FUT1 and FUT2, which add fucose to terminal galactose and are essential for the synthesis of Lewis Y and B antigens, were shown in CRC tumors (95). Alterations of FUT expression have also been described in the process of gastric carcinogenesis (96). In particular, the downregulation of FUT3 and FUT5 changes the Lewis antigens expression and reduces the adhesion capacities of gastric cancer cells (97). This is contrary to what is observed in gastric inflamed mucosa, where FUT3 is upregulated (98).

Increased core-fucosylation of *N*-glycans catalyzed by α1,6-FUT8 has been described in CRC patients and is associated with tumor aggressiveness (37, 99). The core-fucosylation of E-cadherin enhances the cellular adhesion of CRC cells (100). However, in gastric cancer, the decrease of core-fucosylation has been demonstrated to be a common event contributing to cancer cell proliferation (101).

Other Relevant Glycosylation Alterations

In addition to the mucin-type *O*-glycosylation, there are further forms of protein *O*-glycosylation, including the modification of nuclear and cytoplasmic proteins with *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc). Noteworthy, increased *O*-GlcNAcylation is a general feature of cancer and the modification of proteins with *O*-GlcNAc has been shown to play key regulatory roles in tumor cell signaling (102). The addition of *O*-GlcNAc to nuclear and cytosolic proteins is mediated by the *O*-GlcNAc transferase (OGT), whereas the enzyme *O*-GlcNAc-selective *N*-acetyl-beta-D-glucosaminidase (*O*-GlcNAcase) removes the *O*-GlcNAc, returning the protein to its basal state (103). *O*-GlcNAcylation has been shown to have extensive crosstalk with phosphorylation and to antagonize phosphorylation-mediated cell signaling (104).

In the pancreas, beta-cells are characterized by expressing high levels of the OGT enzyme. This allows these cells to dynamically respond to physiological increases in the extracellular glucose levels by converting glucose to UDP-GlcNAc, which is the OGT substrate, and therefore modulating intracellular *O*-linked protein glycosylation (105). In PDAC, hyper-*O*-GlcNAcylation has been associated with increased expression of the OGT enzyme and reduction of the *O*-Glc-NAcase glycosidase and has been demonstrated to block cancer cell apoptosis and to lead to the oncogenic activation of the NF-kB signaling pathway (106). Similarly, increased *O*-GlcNAcylation in colon has been demonstrated to contribute for the development of colitis and colitis-associated cancer by enhancing NF-kB-mediated signaling (107).

Along with aberrant protein glycosylation, cancer cells also display major glycosylation alterations on other classes of glycoconjugates, including the proteoglycans and the glycosphingolipids. Proteoglycans consist of a core protein with one or more covalently attached large GAG chains, and can be either located at the cell membrane or secreted. The syndecans are a family of transmembrane proteoglycans that carry heparan sulfate GAG chains and that can also be additionally modified with chondroitin sulfate chains (108). The heparan sulfate-rich proteoglycan syndecan-4, a critical partner of integrins for the establishment of focal adhesion complexes, has been shown to be upregulated in gastric mucosa in response to the oncogenic bacteria *Helicobacter pylori*. However, its functional role in the gastric carcinogenesis process remains to be disclosed (109, 110). Another syndecan family member, the syndecan-1, has been reported to be differently regulated and expressed in GI tumors. Loss of syndecan-1 expression has been described in gastric adenocarcinomas of higher stages (111), while in CRC and PDAC the expression of this proteoglycan has been shown to be upregulated, suggesting its possible application as a biomarker (112, 113).

Heparan sulfate GAG chains can also be carried by glypicans, a family of glycosylphosphatidylinositol (GPI)-anchored proteoglycans. Glypicans have been shown to bind a wide range of signaling molecules and to regulate the signaling of the Wnt, Hedgehog, fibroblast growth factor, and bone morphogenetic protein (BMP) pathways (114). Glypican-1 has been shown to be overexpressed in PDAC cell models and patient tumors (115). Moreover, the key role of glypican-1 in PDAC progression has been well documented using mouse models (116, 117). Recently, glypican-1 has been shown to be specifically expressed by cancer circulating exosomes and, therefore, to have potential to be used as a minimal-invasive diagnostic and screening tool to detect early PDAC stages (118).

The CD44 proteoglycan has also been on the focus of tumor biology research because the expression of specific splice variants is strongly associated with malignancy. Specifically, the exon v6-containing CD44 isoform (CD44v6) is highly expressed in premalignant and malignant gastric lesions (119). Modification of CD44v6 with STn was demonstrated in gastric mucosa and serum of cancer patients, indicating its potential as a biomarker

for early diagnosis of gastric tumors (120). Different strategies aiming the impairment of CD44-dependent cancer cell migration have been proposed. The ceramide nanoliposome (CNL) was shown to induce anoikis and to limit metastasis by inducing lysosomal degradation of CD44 in PDAC cells (121).

Another glycosylation modification frequently observed in cancer is the altered sialylation of glycosphingolipids that can lead to the appearance of tumor-associated antigens. The human plasma membrane-associated sialidase NEU3, which catalyzes the removal of sialic acids from glycoproteins and glycolipids, is a key enzyme for ganglioside degradation. NEU3 has been shown to be overexpressed in many tumors, including CRC (122). Modulation of ganglioside expression by increased NEU3 activity has been proposed as a mechanism of protection against programed cell death and has, therefore, a critical implication in therapeutic strategies (123). Recently, NEU3 was demonstrated to regulate the Wnt signaling pathway, therefore contributing for the malignant transformation of CRC cells (124). These findings suggest NEU as a relevant target for diagnosis and therapy of CRC. During CRC progression, besides reduced expression of sialylated gangliosides, overall alteration in glycosphingolipids glycosylation includes increased fucosylation, decreased acetylation and sulfation, and reduced expression of globo-series glycans (125).

GLYCAN CANCER BIOMARKERS

Glycosylation changes on glycoconjugates either expressed on the cell surface or secreted by cancer cells are potential sources of cancer biomarkers. The overexpression of these altered glycosylated structures and the loss of polarity of carcinoma cells lead to the shedding of glycoconjugates with altered glycosylation into the circulation. Currently, several serological assays used in the clinics are based on the quantification of glycoconjugate levels in the serum of cancer patients. Most of these biomarkers have been useful for prognostic and monitoring purposes. These include well-established serological biomarkers, such as the CA15-3 assay, detecting mucin MUC1 glycoprotein used for breast cancer (126–130), the CA125 assay, which detects the circulating mucin MUC16 in ovarian cancer (131, 132), and the prostate-specific antigen (PSA), which is used to detect prostate diseases (133, 134).

Regarding GI cancer, one of the most used serological assay detects the SLe^a carbohydrate antigen. SLe^a is present on circulating glycolipids and glycoproteins and is detected by the CA19-9 assay. This serological assay is applied in patients with a previously established diagnosis of PDAC, CRC, gastric, or biliary cancers and used to monitor their clinical response to therapy (135–138).

Another important serological test used in the clinics for GI tumors is the carcinoembryonic antigen (CEA) assay, which detects the CEA glycoprotein produced by carcinoma cells. In GI cancer, CEA is expressed at high levels and shed into the bloodstream being useful for prognosis evaluation and follow-up of these patients (129, 137, 139, 140).

In general, most of these serological assays have primarily been useful for prognosis and patients' monitoring applications. Unfortunately, some of these biomarkers can also be detected due to benign lesions or other factors, such as smoking, which has limited their use in cancer screening strategies for diagnostic purposes. Given the usually late diagnosis of GI cancer, highly specific serum markers for cancer detection and screening are highly needed. Recent developed strategies and advanced technologies are contributing to the definition of novel and more specific glycoconjugate targets. Several of these new targets are currently evaluated and hold potential for improving the cancer detection and early diagnosis.

INNOVATIVE GLYCOBIOLOGICAL STRATEGIES

The difficulty of glycobiological research lies in the intrinsic complexity of glycosylation and its versatile conjugates. Whereas genomic and proteomic analysis made a leap forward by DNA sequencing and mass-spectrometric protein sequencing, respectively, that enabled the reading of a linear code with limited number of variabilities; for the more complex glycans, no comparable tool exists.

Nevertheless, the recent years have brought up many innovative approaches and methods that enable the unraveling of glycobiological challenges. With the development of glycoengineered cell strategies, glycan complexities have been reduced and the effects of specific glycan epitopes have been pinpointed. On the other hand, analytical methods and protocols for glycomic and glycoproteomic analyses have improved and new approaches, such as the adaptation of the array technology on glycans and lectins or novel antibody-based assays, have accelerated the acquisition of glycobiological knowledge. The following sections discuss several promising strategies in the glycobiology field.

Glycoengineered Cell Line Models

The characterization of the function of glycans in cancer has been a major challenge in the field due to technical difficulties related to the complexity and heterogeneity of glycans synthesized in eukaryotic cells.

Genetic engineered cell models have been developed to study the functions of specific glycan epitopes in cancer. Some of these models include the overexpression of glycosyltransferases, which has allowed the characterization of the biosynthesis and function of simple cancer-associated carbohydrate epitopes, such as Tn, STn, T, and ST (17, 18, 20, 141, 142). Similar strategies have used stably transfected cell lines with glycosyltransferases to characterize the function of branched glycan structures (52, 56) as well as terminal sialylated/fucosylated structures frequently overexpressed by cancer cells, as previously explained in Section "Increased Sialylation and Fucosylation" (67, 143, 144).

Another major challenge in the field was related to the identification of structures at individual glycosylation sites. Major efforts have been done in this discipline with the generation of site-specific mutants of important proteins in cancer. One example is the use of site-specific mutants of *N*-glycosylation sites of the human epidermal growth factor receptor. This strategy has allowed the demonstration that Asn-420-linked oligosaccharide chain in this receptor interferes with its activation in cancer cell lines (145). Another cell line model has addressed the role of

E-cadherin *N*-glycosylation sites in gastric cancer (146–148). The use of E-cadherin constructs engineered to lack specific *N*-glycosylation sites has demonstrated the effect of specific *N*-glycosylation structures on cell adhesion (149, 150).

The recent use of genomic editing tools has allowed the development of isogenic cell systems that along with extensive application of mass spectrometry (MS) methods is utilized for high-throughput site-specific *O*-Glycosylation (*O*-GalNAc and *O*-Mannose) proteomics. These technologies have enabled the precise determination of protein *O*-glycosylation sites in cells (151, 152). These strategies have greatly evolved in the past years and are showing vast potential in the glycobiology field. One approach has used the zinc-finger nucleases targeting the knockout of *COSMC* gene and has been applied in several human cancer cell lines originated from different organs (152). These so-called SimpleCell models produce stable cells expressing homogeneous truncated *O*-glycosylation with Tn and/or STn *O*-glycans (24, 120, 151, 153).

These cell models have provided a source of unlimited material for isolation and identification of GalNAc *O*-glycopeptides from cell lysates or secretomes using lectin chromatography followed by advanced MS, enabling the identification of hundreds of unique *O*-glycoproteins and *O*-glycosylation sites in several cell line models from different tissues (120, 153, 154). In addition, this approach has provided a versatile method for the functional analysis of different ppGalNAc-Ts (153, 155). Furthermore, similar strategies have been applied targeting the *O*-mannose glycoproteome. To reduce the structural heterogeneity of *O*-mannosylation (*O*-Man), the nuclease-mediated gene editing of a human cell line was performed by zinc-finger nuclease targeting of the *POMGNT1* gene. This gene encodes for the enzyme POMGnT1 that controls the first step in the elongation of *O*-Man glycans. The *O*-Man glycoproteome has been characterized using both chromatography and advanced MS (156).

The knowledge of *O*-glycosites in specific cancer cell types allows for the analysis of novel biological functions of glycosylation and for potential cancer cell-specific *O*-glycosites. This is particularly important given the complexity of *O*-glycosylation and that the various ppGalNAc-Ts that control the protein *O*-glycosylation sites may determine large variation at protein, cell, and tissue levels (9).

Glycomic Strategy

Glycomics is the study of all glycan structures of a given cell, tissue, or organism. The intrinsic complexity of glycan structures and their versatile conjugates render this field particularly challenging. Due to the constant advancement of analytical instruments and methods, the *N*- and *O*-glycomic characterization of cancer cell lines, tumors, and cancer patients' body fluids has rendered possible. Still, there is no single ideal method for this analysis and, thus, today a large variety of analytical methods is available for the glycomic characterization, resulting from different combinations of initial sample preparation, derivatization, glycan separation, and detection. Each method bares advantages and disadvantages.

For the glycomic analysis of cells or tumors, the sample is usually homogenized and proteins are denatured, followed by the release of glycans (**Figure 2A**). The study of the glycans of serum or plasma is more challenging and requires often purification steps for glycoproteins prior to the release of their glycan structures. There are several methods to release glycans from the protein backbone to facilitate their characterization. The release of glycans is not a prerequisite as the analysis of whole glycopeptides is also possible (covered in Section "Glycoproteomic Strategy"). The most prominent technique to release *N*-glycans is by Peptide-*N*-Glycosidase F (PNGase F). The release of *N*-glycans via PNGase F is robust, fast, and efficient and is capable of liberating all types of human *N*-glycan structures. PNGase F-released glycans can be chemically labeled. On the other hand, no enzyme has so far been characterized that enables the efficient release of all types of *O*-glycans. For instance, the enzyme *O*-glycanase releases only core 1 *O*-glycans from their peptide backbone. Therefore, chemical techniques have to be utilized for whole *O*-glycomic analyses, such as reductive β-elimination.

Released glycans can be analyzed after derivatization or in their native form (underivatized). The derivatization of glycans bares several advantages, such as adding fluorescent tags for the photometric detection or chemical modification of side groups to stabilize glycan constituents. Despite these advantages, it is preferred in some cases to work with the native glycan, avoiding several time-consuming preparation steps and sample losses.

Complex mixtures of glycans, as they arise from clinical samples or even cell lines are usually separated by chromatography or capillary electrophoresis [reviewed in Ref. (157)] and detected by fluorescence detector (FLD) or MS. The sensitive and quantitative fluorescence detection requires fluorescently tagged glycans, and gives on its own only limited structural information derived from chromatographic or electrophoretic retention times. MS, on the other hand, can be applied on both native and derivatized oligosaccharides and may yield detailed structural information of the glycans. Since the analytes are not consumed by FLD, a sequential setup with MS is possible and often advantageous.

Three successful glycomic workflows that have revealed in the past years several findings in GI cancer are porous graphitized carbon separation with electrospray ionization and tandem MS (PGC-ESI-MS/MS), hydrophilic interaction ultra/high performance liquid chromatography with FLD (HILIC-FLD-UPLC/HPLC), and matrix-assisted laser desorption/ionization MS (MALDI-MS).

Porous graphitized carbon separation with electrospray ionization and tandem MS is a workflow used for both *N*- and *O*-glycomic analyses. First, the *N*-glycans are liberated from the glycoproteins with PNGase F, followed by reductive β-elimination of the glycoproteins to release the remaining O-glycans. The *N*- and *O*-glycans are separated by liquid chromatography with a PGC column, which resolves most isomeric structures and complements, therefore, ideally the subsequent MS and MS/MS structural analysis. A recent glycomic study by PGC-ESI-MS/MS has described structural glycan alterations in CRC, including several unique glycans found solely in the tumor region and indicated a correlation between EGFR expression and sialylation in CRC (158). This method has lately been further utilized for the *N*- and *O*-glycomic characterization of CRC cell lines and tumors, revealing great *O*-glycomic differences between tumors and all tested cell line models (26).

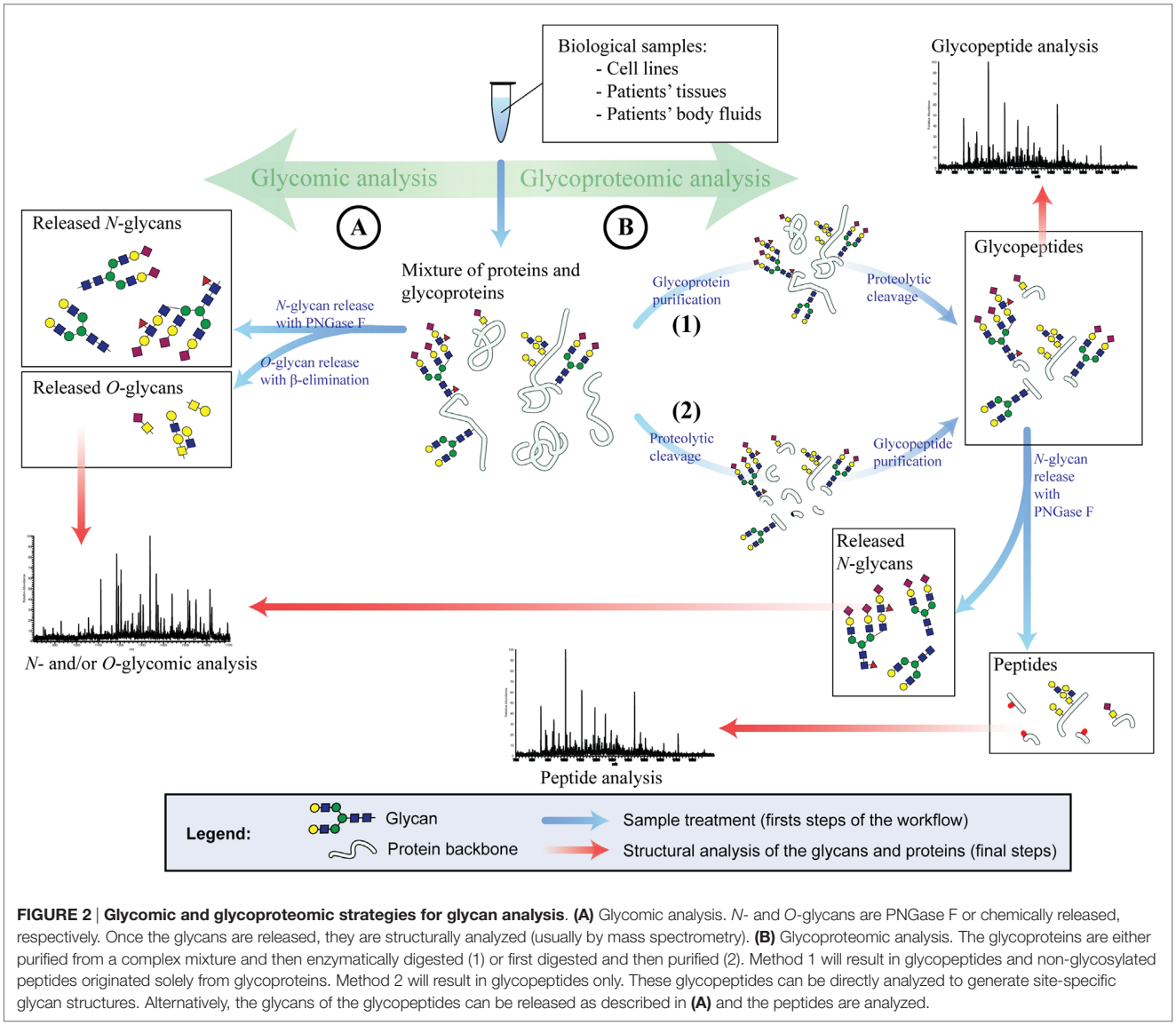

FIGURE 2 | Glycomic and glycoproteomic strategies for glycan analysis. **(A)** Glycomic analysis. *N*- and *O*-glycans are PNGase F or chemically released, respectively. Once the glycans are released, they are structurally analyzed (usually by mass spectrometry). **(B)** Glycoproteomic analysis. The glycoproteins are either purified from a complex mixture and then enzymatically digested (1) or first digested and then purified (2). Method 1 will result in glycopeptides and non-glycosylated peptides originated solely from glycoproteins. Method 2 will result in glycopeptides only. These glycopeptides can be directly analyzed to generate site-specific glycan structures. Alternatively, the glycans of the glycopeptides can be released as described in **(A)** and the peptides are analyzed.

Hydrophilic interaction ultra/high performance liquid chromatography with FLD is used for the quantitative profiling of *N*-glycans. The *N*-glycans are released by PNGase F and reductively aminated with a fluorophore. The labeled *N*-glycans are applied on a HILIC-ultra performance chromatography (UPLC), which separates the glycans according to their size and monosaccharide composition. The retention time can be converted to glucose units (GU) by comparing it with a dextran ladder, yielding reproducible results. Hence, due to the few sample preparation steps, the high recovery of the HILIC column, the quantitative detection via the fluorescent tag, and the possibility of multiplexing, this analysis can be applied for large-scale *N*-glycomic studies. The HILIC-FLD-UPLC *N*-glycomic analysis has recently been applied in a large-scale discovery study on serum of gastric cancer patients revealing an increase in certain SLe^x carrying *N*-glycan structures that correlated with disease progression. Furthermore, in this study other structures, such as bisected *N*-glycans, have been shown to decrease with disease progression (159, 160).

Recently, the combination of HILIC-FLD-UPLC and PGC-ESI-MS/MS has been used for *N*- and *O*-glycomic analysis of a gastric cancer cell line overexpressing the sialyltransferase ST3Gal-IV (79). This cell line has previously been shown to present a more invasive phenotype (67). The glycomic analysis revealed a broad range of cancer-associated alterations, such as decreased bisected and increased branched structures, truncation of *O*-glycans, and a shift from α2,6- to α2,3-sialylated *N*-glycans (79).

The use of MALDI-MS is another very successful approach of analyzing the *N*-glycome of clinical samples, such as body fluids. MALDI is based on a laser impulse that excites a solid matrix in which the analytes are embedded which in turn desorbs and ionizes the analytes for MS analysis. MALDI is relatively tolerant

to salt and other contaminants, which allows uncomplicated sample preparation after the release of *N*-glycans. This method has recently been applied on the serum of gastric cancer patients and control groups and has been able to identify *N*-glycomic differences between the serum of gastric cancer patients and that of non-atrophic gastritis patients (161). In a large-scale study on sera of PDAC patients, a tendency toward higher branched and fucosylated *N*-glycans has been observed when compared to sera from healthy individuals. The major part of the significantly altered *N*-glycan structures were specifically increased in patients with distant metastases and the ratio of the quantity of two glycans has been proposed as a robust diagnostic marker for PDAC (162). Another recent study utilizing MALDI-MS has revealed in pancreatic cyst fluids, of which certain subtypes bare a high risk of undergoing malignant transformation, the hyperfucosylation of *N*-glycans (163).

A broad range of alterations in CRC tissues versus controls have been identified by sequential analyses of fluorescently tagged *N*-glycans by HILIC-FLD-HPLC and MALDI-MS. Additionally, multivariate statistical evaluation and further MS-based structure elucidation have been applied and revealed among others the decrease of bisected structures and the increase of glycans with sialylated lewis epitopes. Furthermore, abnormal core-fucosylated high mannose *N*-glycans have been uniquely found in cancer tissue (37).

Glycoproteomic Strategy

Glycoproteomics is the study of proteins that carry glycan modifications. It usually focuses on the identification and quantification of glycoproteins and the characterization of protein glycosylation sites. Given that most clinical cancer biomarkers are glycoproteins, this field is particularly promising for the identification of new biomarker targets in cancer. Biological samples, such as cell lines, tissues, and body fluids, can be analyzed. However, the glycoproteomic analysis of complex biological samples, such as tissues or sera, is analytically challenging due to the large complexity and vast dynamic range of concentrations of glycoproteins.

The glycoproteomic pipeline typically consists of numerous steps, such as glycoprotein or glycopeptide enrichment, isotopic labeling (optional), multidimensional protein or peptide separation, tandem mass-spectrometric analysis, and bioinformatic data interpretation (**Figure 2B**). In cancer, the vast majority of glycoproteomic findings are based on bottom-up analysis of peptides ("shotgun proteomics"). For this purpose, glycoproteins are proteolytically cleaved into glycopeptides before or after the enrichment step. The enrichment of glycoproteins or glycopeptides is a critical step of the glycoproteomic analysis. Even though this field is rapidly evolving, so far no method has been established that captures unbiased every glycoprotein or glycopeptide and enables full glycoproteomic coverage. Currently, most popular enrichment methods are based on lectins (164–166) or on hydrazide solid-phase extraction (167, 168) and sometimes applied in combination to increase the glycoproteomic coverage (168). Alternative strategies are boronic acid functionalized beads (169), size exclusion chromatography (170), hydrophilic interaction (171), and graphite powder micro column (172). Due to the difficulties of covering the whole glycoproteome many cancer studies pursue a different strategy of enriching specifically glycoproteins and glycopeptides carrying cancer-relevant glycan epitopes, such as sialic acids or sialylated Lewis epitopes. These methods are usually based on lectins [such as SNA, WGA, and MAL (173)], antibodies (159), enrichment by titanium dioxide (79, 174), or affinity purification of metabolic labeled glycoproteins (175–177). After the enrichment and proteolytic digestion (not necessarily in this order), glycopeptides may be deglycosylated and are multidimensional separated via chromatography and/or electrophoresis and analyzed by tandem MS. The deglycosylation is a requirement of some enrichment methods, such as hydrazide solid-phase extraction, but may be also applied for all *N*-glycoproteomic analysis. The PNGase F release of *N*-glycans leads to the conversion of the *N*-glycan carrying asparagine to aspartic acid and can, thus, be spotted on the peptide backbone by MS. For the generation of site-specific structural information of *N*- and *O*-glycans, whole glycopeptides are analyzed utilizing a combination of different MS fragmentation methods or collision energies that either fragment peptides or glycans (178–180). This strategy is being optimized in recent years and bares great potential for the discovery of new cancer biomarkers because it unravels site-specific glycan alterations in cancer.

Glycoproteomic analyses have been applied in GI cancer mainly for the identification of biomarkers, such diagnostic biomarkers or biomarkers for multidrug resistance in gastric cancer (181, 182). Glycoproteomics in combination with glycoengineered cell line models was in recent years able to increase the coverage of *O*-glycosylated proteins and to identify numerous novel *O*-glycosylation sites in gastric cancer and PDAC, generating several new potential biomarkers (24, 120).

Other Glycoanalytical Techniques

MS-based glycomic and glycoproteomic analyses require expensive equipments and a fair amount of expertise. MS-independent methods, such as glycoprotein, antibody-lectin-sandwich, and lectin arrays, are capable of rapid data acquisition of glycomic alterations in cancer samples. Glycan arrays, on the other hand, enable a screening for specificities of glycan-binding proteins, improving the data interpretation of antibody and lectin-based research. Regarding tumor biology, it is very relevant to determine not only the glycosylation modifications harbored by tumor cells but also to disclose the topographic distribution of these alterations within the tumor and adjacent tissue. Novel approaches for the identification of *in situ* glycan modification of specific proteins include proximity ligation assay (PLA) and imaging mass spectometry (IMS).

Arrays

The binding of biological molecules to solid matrixes was an idea first described by Chang in 1983 (183). This technology initially consisted of coating glass cover slips with different antibodies in close proximity forming a matrix-like array. Arrays recognize partners from large amounts of biological material using high-throughput screening miniaturized, multiplexed and parallel processing, and detection methods based on multiple probes covalently attached to a solid substrate. Depending on the molecule that is deposited on the surface,

different microarrays exist. To analyze glycan-containing structures, the most common classification is glycan, glycoprotein, or lectin microarray, and also a variant of the latter called antibody-lectin sandwich array (**Figure 3**) (184). The advantages that the microarray technology offers are the small volume of sample required for the analyses, the high reproducibility, and the reduced cost and time to process many samples. Therefore, microarray platforms have been highlighted by its extensive application in the field of biomarker validation, where a large number of samples must be analyzed multiple times (185). Moreover, depending on the type of microarray assay performed, information about the glycan-linkage configuration can be obtained.

Glycan microarrays are used mainly to characterize the binding specificities and affinities of proteins (mostly antibodies and lectins) toward glycans (**Figure 3A**) (186–188). However, they can also be applied for the screening of inhibitors of carbohydrate-mediated interactions and of sugar interactions of an entire organism, such as a whole cell or virus (185, 189, 190). Current available platforms consist of approximately 20,000 microspots of antigens reaching the capacity to include most known human microbial pathogens, autoantigens, and tumor-associated antigens (191–193). The diversity and scope of glycan arrays are continuously increasing allowing a better characterization of glycan-binding proteins but leading to more complex data. Different software tools are currently available for data interpretation (194–196). Glycan arrays present oligosaccharides that were either purified from a biological source or *de novo* synthesized. Regarding the latest ones, it is important to highlight recent works describing new methodologies that allow sialic acid (197, 198) and GAG synthesis (199).

As an alternative to the direct binding of glycans to the array surface, glycans can be presented on proteins or peptides that are attached to the array. A recent advancement in this approach is the coiled coil-based technology, which allows the presentation of the antigens at high densities while mimicking the *in vivo* orientation attached to a fiber-forming peptide. This platform showed increased sensitivity for the identification of antibodies against parasitic glycan antigens and might be adapted in the future for cancer diagnostic (200).

Glycoprotein microarrays are based on printing purified or enriched glycoproteins onto the slides and screening these proteins for glycan epitopes using different lectins or glycan-recognizing antibodies (**Figure 3B**). This approach is usually followed by analytical techniques to identify the spotted proteins and to verify the glycan epitopes found by the array analysis. A recently performed glycoprotein array analysis of lectin-enriched sera from PDAC patients, chronic pancreatitis patients (benign pancreatic disease), and healthy individuals has correctly clustered these three groups, being the PDAC group significantly different from the other two (201). In addition, the glycoprotein microarray may use synthesized peptides and recombinant protein fragments that have been *in vitro* glycosylated for the detection of human autoantibodies (202, 203).

Lectin microarrays, where different lectins are spotted onto the slide, enable a rapid and high-sensitivity profiling of glycan

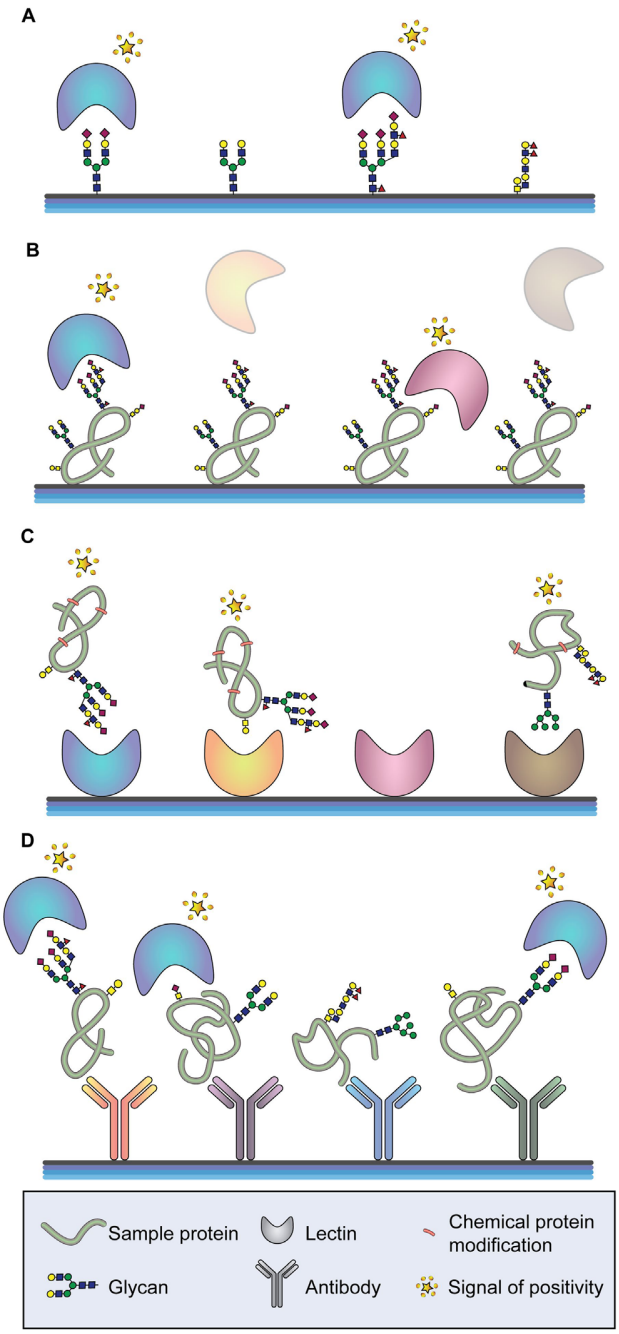

FIGURE 3 | Arrays for glycan analysis. **(A)** Glycan arrays. Different glycan moieties are spotted onto the array to determine specificities of labeled glycan-binding molecules (typically lectins or antibodies). **(B)** Glycoprotein arrays. Glycoproteins are enriched from a sample and spotted onto the array. The glycosylation moieties of the spotted glycoproteins are determined by screening with different glycan-binding molecules. **(C)** Lectin arrays. A series of lectins with well-defined glycan-binding properties are spotted onto the array and different labeled proteins are tested. **(D)** Antibody-lectin sandwich arrays. Multiple antibodies for a series of proteins of interest are spotted onto the array, and the glycan epitopes of the captured proteins are probed using labeled lectins and glycan-binding antibodies.

features found in complex samples, such as cells, tissues, body fluids, and synthetic glycans and their mimics (**Figure 3C**) (204, 205). Lectin arrays offer a general view of the glycan structures on a complex sample and integrate the information from all proteins with the disadvantage that no information about specific glycan changes of the respective protein constituents will be obtained (206). A recent work displayed different glycopatterns in gastric cancer compared to gastric ulcer applying Cy3-labeled proteins extracted from tissues to lectin microarrays (207). Another recent lectin array approach has identified differences in α2-macroglobulin glycosylation between healthy individuals and patients with CRC. The spotted serum purified α2-macroglobulin has displayed, among other changes, significant differences in the content of branched *N*-glycans and α2,6 sialylation (208).

A variant of the lectin array is the antibody-lectin sandwich array. Antibodies to known glycoproteins are spotted on a solid support, and complex glycoprotein samples, which can be crude or prefractionated, are bound to the microarray (**Figure 3D**) (184, 185). The glycosylation of the captured target proteins are then screened by labeled lectins and glycan-specific antibodies. Antibody-lectin sandwich arrays are highly effective for profiling variation in specific glycans on multiple target proteins. Performing this technology, specific glycoforms of MUC5AC and endorepellin glycoproteins in the cyst fluid of patients with precancerous pancreatic cysts have been found (209). In these assays, the glycoprotein nature of the antibodies must be considered and different approaches to prevent glycan recognition of the antibodies by the secondary antibody or lectin applied exist. The chemical derivatization of the glycans of the spotted antibodies also prevents their ability to be recognized by glycan-binding molecules, both antibodies and lectins. One efficient method to study glycans on individual proteins from complex mixtures uses chemically derivatized capture antibodies and tests the glycosylation of captured target proteins by lectins and glycan-binding antibodies. Applying this approach, cancer-associated glycan alterations on the proteins MUC1 and CEA in the serum of PDAC patients have been identified (210). Another strategy consists on producing recombinant antibodies in organisms that do not carry out post-translational modification, such as glycosylation. This approach has been performed for the detection of glycans linked to CEA by ELISA coating the microplate with recombinantly scFv expressed in *Escherichia coli* and using lectins as detection probes (211).

Regarding GI cancer research, arrays have been widely used to discover new biomarkers consisting of proteins bearing aberrant glycosylation that could lead to a more accurate diagnostic.

In situ Proximity Ligation Assay

The association of the glycan expression and location with clinical and molecular characteristics of cancer tissues has rendered possible by histochemistry techniques using glycan-binding antibodies or lectins (205). However, one major limitation of this technique is the lack of capacity to identify the proteins *in situ* on which these glycan motifs are localized. This limitation has been surpassed by the development of the *in situ* proximity ligation assay (PLA) (**Figure 4**) (212, 213).

This sensitive antibody-based method reveals the colocalization between specific proteins and specific glycan structures in tissues and cell samples. The identification of protein glycoforms is of utmost importance for the understanding of glycobiological cellular processes in cancer. PLA could also detect other post-translational modifications of proteins in tissue samples with subcellular resolution. The PLA technology is based on the binding of two specific PLA probes, each containing a unique oligonucleotide, to two targets of interest (**Figure 4A**). Antibodies, lectins, and other binding proteins can act as probes. A ligation solution, containing bridging oligonucleotides and a ligase, will hybridize the oligonucleotides of the PLA probes if they are in close molecular proximity to form a closed circle (**Figure 4B**). This closed nucleotide circle will be amplified by a DNA polymerase generating repeated copies of the circular DNA strands. Finally, fluorescent or chromogenic oligonucleotides hybridize to the amplification product and can be detected as individual spot by microscopy (**Figure 4C**) (213). The first study using this innovative PLA strategy applied to glycobiology has showed that the mucin MUC2 is a major carrier of the cancer-associated STn glycan antigen both in intestinal metaplasia and gastric carcinoma (214). The use of *in situ* PLA for the identification of a mucin glycosylation profile in cancer lesions is being extended, opening new opportunities for the development of novel diagnostic and prognostic markers. One recent study screened for tissue-specific aberrant mucin glycoforms in mucinous adenocarcinomas from different organs (stomach, ampulla of Vater, colon, lung, breast, and ovary). In GI tissues mucins carrying a set of truncated, simple O-glycans and sialylated Lewis antigens have been detected by this approach (215).

More recently, PLA has been used in combination with different glycoproteomics strategies to identify specific glycoforms as potential biomarkers in gastric cancer, leading to the identification of CD44v6/STn (120) and RON/SLe^x (79).

The PLA technique will further improve our understanding of specific protein glycosylation changes that occurs in cancer tissues and that could be applied in clinic as new markers for GI cancer progression (216).

Imaging Mass Spectrometry

Imaging mass spectrometry is a very novel and promising technology that was first developed in 1997 by Caprioli and colleagues for the analysis of proteins (217). This method is based on MALDI-MS and utilizes the laser ionization of a localized area for the two-dimensional screening of a tissue sample. IMS generates for each ionization point of the tissue a spectra that yields structural information and, thus, reveals the spatial distribution of analytes (**Figure 5**). Recently, this technique has been adapted for glycomic analysis and has allowed to create *N*-glycosylation maps of several different frozen tissue (218). Following, *N*-glycan IMS has been also applied on formalin-fixed paraffin-embedded tissue (219). The *N*-glycan IMS workflow consists of four steps. First, *N*-glycans are liberated by PNGase F incubation of the deparaffinized or thawed tissue slide. Second, a thin layer of MALDI matrix is sprayed on top of the tissue slide. Third, the slide is two-dimensionally screened by multiple MALDI-MS analysis. Lastly, each identified *N*-glycan structure

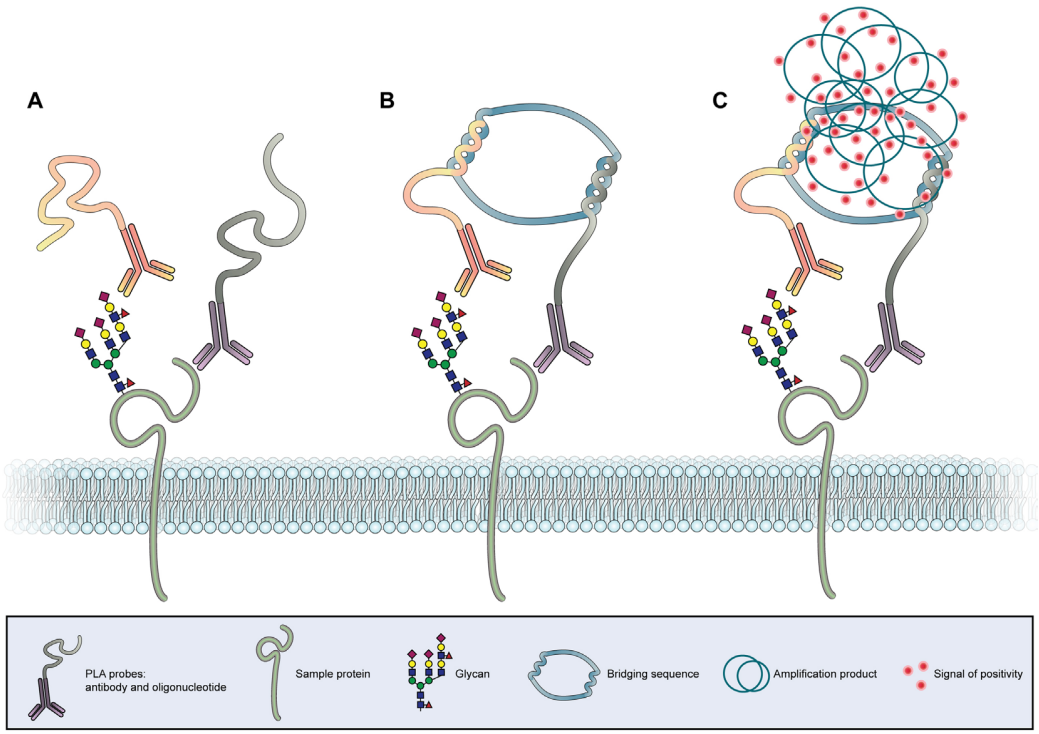

FIGURE 4 | ***In situ*** **proximity ligation assay**. **(A)** Two antibodies, one specific for the protein backbone and another specific for a glycan epitope, are conjugated with two different oligonucleotide chains (PLA probes). **(B)** In case the antibodies bind to molecules in close proximity a bridging sequence links the two oligonucleotide sequences. **(C)** A subsequent polymerase induced amplification in combination with labeled nucleotides leads to the formation of a fluorescent or chromogenic signal at the co-expression site of the protein and glycan, allowing the *in situ* detection of the PLA signal.

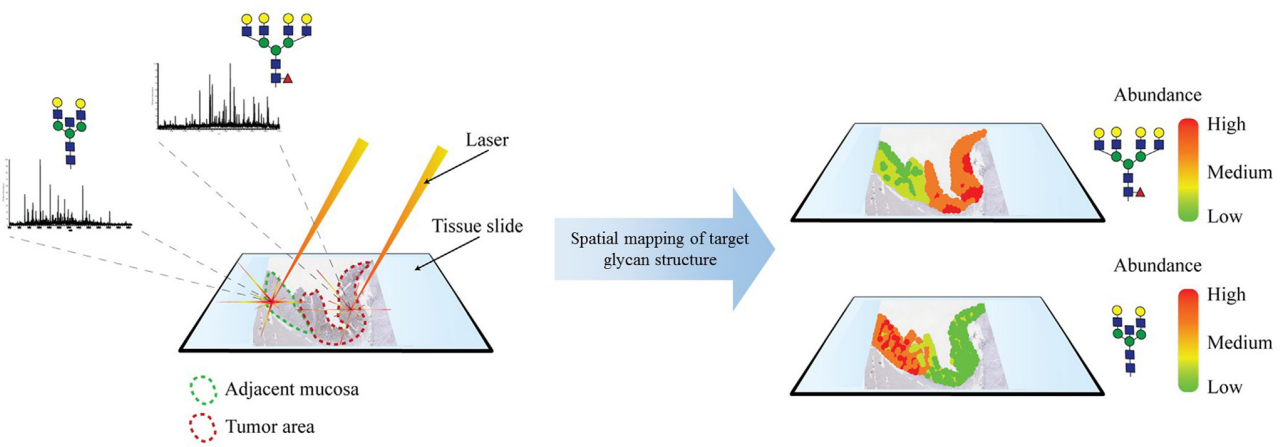

FIGURE 5 | **Imaging mass spectrometry**. MALDI-based ionization generates an MS glycan spectrum of a selected spot of a tissue section. This process is repeated throughout the tissue. Identified glycan structures are mapped on the tissue and can be correlated with histopathological features.

can be computationally visualized on the tissue, generating an epitope map.

IMS applied on hepatocellular carcinomas has shown to be capable of spatially defining glycan compositions and distinguishing malignant tissue from healthy tissue (220). Preliminary IMS results on other tumors, such as PDAC, have been able to differentiate between histopathological areas, such as fibroconnective tissue (220).

The IMS application on *N*-glycan analysis is still in its early stages of development, but bares enormous potential as a next-generation *N*-glycomic tumor characterization tool.

FUTURE PERSPECTIVES AND CLINICAL APPLICATIONS

The recent advances in the glycomic and glycoproteomic fields are currently providing crucial information on the understanding of the role that glycans play in the biology of cells, tissues, and organisms, both in physiological and pathological conditions. However, many issues still remain to be understood, particularly in complex diseases, such as cancer. Advances in the glycobiology field could contribute to disclose key information regarding cancer biological properties, including the identification of prognostic and therapeutic response biomarkers.

In addition, the recent developments in this field could contribute to overcome the limitations of the current serological assays. The set of novel strategies presented in this review provide a clear view for future validation of potential biomarkers and points toward the translation of these strategies in the clinical setting.

AUTHOR CONTRIBUTIONS

All authors have contributed to the conception, drafting, and revision of the manuscript. SM and MB designed the figures. All authors approved the review final form.

FUNDING

We acknowledge the support from the European Union, Seventh Framework Programme, Gastric Glyco Explorer initial training network: grant number 316929. IPATIMUP integrates the i3S Research Unit, which is partially supported by FCT, the Portuguese Foundation for Science and Technology. This work is funded by FEDER funds through the Operational Programme for Competitiveness Factors-COMPETE (FCOMP-01-0124-FEDER028188) and National Funds through the FCT-Foundation for Science and Technology, under the projects: PEst-C/SAU/LA0003/2013, PTDC/BBB-EBI/0786/2012, and PTDC/BBB-EBI/0567/2014. AM acknowledges the grant received from FCT, POPH (Programa Operacional Potencial Humano), and FSE (Fundo Social Europeu) (SFRH/BPD/75871/2011). MB acknowledges the University of Girona for pre-doctoral fellowship.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.