Universitat de Girona

GLYCAN ALTERATIONS OF SERUM PROTEINS AS TUMOUR MARKERS. PROSTATE-SPECIFIC ANTIGEN IN PROSTATE CANCER AND ACUTE-PHASE PROTEINS IN PANCREATIC CANCER

Ariadna SARRATS CARBÓ

ISBN: 978-84-694-5968-3
Dipòsit legal: GI-693-2011
http://hdl.handle.net/10803/31943

Universitat de Girona

DOCTORAL THESIS

GLYCAN ALTERATIONS OF SERUM PROTEINS AS TUMOUR MARKERS. PROSTATE-SPECIFIC ANTIGEN IN PROSTATE CANCER AND ACUTE-PHASE PROTEINS IN PANCREATIC CANCER

ARIADNA SARRATS CARBÓ

2010

Universitat de Girona

Rosa Peracaula Miró, professora titular del Departament de Biologia de la Universitat de Girona,

CERTIFICO:

Que aquest treball, titulat "**Glycan alterations of serum proteins as tumour markers. Prostate-specific antigen in prostate cancer and Acute-phase proteins in pancreatic cancer**", que presenta **Ariadna Sarrats Carbó** per a l'obtenció del títol de doctor/a, ha estat realitzat sota la meva direcció i que compleix els requeriments per poder optar a **Menció Europea**.

Girona, 30 de Juliol de 2010

Dra. Rosa Peracaula Miró

Als Meus Pares
Josep i Fina

If you would be a real seeker after truth, you must at least once in your life doubt, as far as possible, all things.

René Descartes, in *Discours de la Methode*

Acknowledgments

A.S. acknowledges University of Girona for a pre-doctoral fellowship (BRUdG 2006-2010), for short-term mobility fellowships (2007 and 2008) and for a publication grant (2008-2009).

This work was supported by the Spanish *Ministerio de Ciencia e Innovación* (grants BIO 2004-0438 and BIO 2007-61323), La *Marató de TV3* foundation (grant 050932), the Government of Catalonia (grant 2005SGR00065), Roche Diagnostics (Barcelona, Spain), EUROCarbDB RIDS Contract (No. 011952) and the Irish National Institute for Bioprocessing Research and Training.

Personal Thanks (*Agraïments*)

Aquesta tesi doctoral no hauria estat possible sense l'ajut d'un munt de gent, a la que m'agradaria dir-vos un GRÀCIES de tot cor acompanyat d'un gran somriure.

En primer lloc, voldria agrair a la Rosa i en Rafa i haver-me ofert la possibilitat de realitzar la tesi doctoral en el seu grup així com haver-me proporcionat els mitjans per formar-me a nivell científic i professional. També voldria agrair a tots els membres i ex-membres de Bioquímica del Càncer el seu suport a diferents nivells. A la Sílvia, per haver-me introduït en el món del laboratori i per haver estat al meu costat per donar-me tan bons consells com bones crítiques. A l'Eva, per la seva agradable companyia i el seu bon humor. A la Marta, per haver estat la millor companya amb qui compartir aquest dur i llarg camí que és la tesi doctoral. I gràcies a la resta de bioquímics 105 Anna, Sònia, Clara, Montse, Dolors, Cristina i MªGràcia per estar sempre amb mi.

També voldria agrair a la resta de membres del Departament de Biologia el seu suport i molt especialment al grup de d'Enginyeria de Proteïnes per haver-me ajudat en les primeres etapes de la meva formació al laboratori, així com també al llarg de la meva estada al departament. Gràcies Toni, Maria i Marc.

Gràcies a la gent de d'Institut de Biomedicina i Biotecnologia (IBB), especialment a l'Enrique per permetre'm realitzar una estada al seu laboratori i a en Miquel per introduir-me en el món de la biologia molecular.

I would also like to thank the Dublin-Oxford Glycobiology Lab without which the development of this Doctoral Thesis would not have been possible. Thanks Pauline for accepting me in your lab to perform the glycan analysis. Thanks Radka for your help and kindness. Thanks to the rest of the members of the Conway Institute. My stay in Dublin has been one of the greatest experiences of my PhD.

Moltíssimes gràcies als companys becaris, perdó, investigadors en formació, per acollir-me des del primer dia i fer-me sentir com a casa. Gràcies Olaya per introduir-me al grup i per la teva gran amistat. Gràcies Anna pel teu somriure de *Gaxona* i pels teus ànims. Gràcies Marçal pel teu pragmatisme i la teva ironia. Gràcies Marc per la teva estadística i per la teves classes de filosofia pràctica. Gràcies Roger per les teves masurques i els teus xotis. Gràcies Olga per la teva serenor i els teus consells. Gràcies Alexandra per la teva mirada i el teu caràcter. Gràcies Roger per la teva música i la teva companyia. Gràcies Àlex per fer-me riure tan amb les teves imitacions. Gràcies Joan per la teva calma i el teu bon rotllo. Gràcies Pere pels bons moments.

Gràcies també als companys del Departament d'Ecologia pel vostre agradable tracte. Gràcies Josep per les excursions i les pescades. Gràcies Pere per les teves converses.

Voldria agrair als meus amics de sempre el seu suport incondicional al llarg d'aquests d'anys i especialment en els moments més durs. Gràcies Guillem Q, Agnès, Lluís, Marta, Guillem F, Joan, Cristina i Cris. A les meves companyes de la llicenciatura, Xènia i Elisa, merci per les vostres xerrades, la vostra companyia i la vostra ajuda. Gràcies Rosó i MªÀngels per preocupar-vos tan per mi. Gràcies als *Modernos* gironins per les estones de lleure tan necessàries. Gràcies Mike, Santi, Marc i Joan. Moltes mercès als companys del Qrambla, del Nou espiral i de La Perla per compartir tanta dansa amb mi!

Per últim voldria agrair a la meva família el seu suport incondicional. Aquesta tesi és vostra. Em considero molt afortunada de sentir-vos al meu costat. Gràcies Pare pel teu rigor però també la teva comprensió. Gràcies Mare per les teves abraçades. Gràcies Martí per escoltar-me sempre. Gràcies iaia Natàlia pel teu ordre i experiència. Gràcies avi Josep pels teus contes i per introduir-me als experiments. Gràcies iaia Lola per la teva dolçor. Gràcies Tima per pensar sempre amb mi. Gràcies Andreu per la teva alegria.

Gràcies Albert per donar-me la perfecte combinació l'aire fresc i calent necessari per acabar aquesta tesis. Gràcies per fer-me sentit tan bé.

I gràcies a tothom que ha estat important per mi en algun moment i que no he pensat a anomenar.

Diuen els anglesos que un doctor en qualsevol camp és un *Philosophy Doctor* (PhD). Qui ho vulgui comprendre no tindrà més remei que realitzar la seva pròpia tesi doctoral.

Summary

Cancer is one of the leading causes of death worldwide. The survival rate of cancer patients is substantially increased when they are diagnosed at localized stage, for which the availability of adequate tumour markers is crucial.

Prostate cancer (PCa) is the most common male cancer in Western Countries. PCa screening and monitoring is performed by a combined approach of digital rectal examination and the evaluation of Prostate-Specific Antigen (PSA) serum levels. This protein is specifically produced by the prostate and it is secreted into the prostate lumen, but it may reach the peripheral circulation in the event of a prostate cancer, due to the characteristic loss of basal cell layer of prostate tumour glands. However, PSA may also show increased serum levels in other prostate diseases such as benign prostatic hyperplasia (BPH), which results in unnecessary biopsies and discomfort for a number of non-cancer patients. Therefore, the specificity or PSA to distinguish between PCa and BPH patients urges to be improved.

Pancreatic cancer (PaC) is the cancer with the lowest 5-Year Survival Rate (about 5%). This low survival is due to the intrinsic aggressiveness of this type of cancer, together with the inadequateness of existing biomarkers, such as CA19-9. CA19-9 is the tumour marker currently used for pancreatic cancer but its use has been only approved for monitoring because it is unable to differentiate PaC from benign pancreaticobiliary disorders such as chronic pancreatitis (CP). For that, the research of new pancreatic cancer tumour markers is considered of major interest.

Cancer cells show altered glycosylation of their cell surface and secreted glycoconjugates. In particular, prostate and pancreatic cancer tissues have been described to display altered glycosylation, such as increased levels of the glycan epitope Sialyl-Lewis X (SLe^x). The determination of specific tumour-associated glycoforms of proteins secreted by cancer cells that reach the bloodstream may either improve the specificity of known cancer biomarkers (e.g. PSA) or allow the discovery of new tumour markers. Thus, this work has investigated PSA subforms and their glycosylation with the aim to improve the capacity of this serum marker to differentiate between prostate cancer (PCa) and benign prostatic hyperplasia (BPH). In addition it has also evaluated serum glycoproteins with increased SLe^x in pancreatic cancer (PaC) patients, compared to chronic pancreatitis (CP) patients and healthy controls (HC).

PSA subforms were obtained by two-dimensional electrophoresis (2-DE). This technique allowed the separation of 5 different main subforms of approximately 33 kDa, which were named F1 to F5 from the more to the less acidic subform. The determination of the relative percentages of the main subforms F2-F4 in a cohort of 20 PCa patients and 20 BPH patients showed that the relative percentage of F3 (%F3) was significantly decreased in PCa compared to BPH and this decrease was more marked at advanced stages of the cancer. %F4 behaved oppositely and correlated positively with the stage of the cancer.

Characterization of PSA 2-DE subforms was performed in order to understand which their biochemical differences were in order to quantify them using "friendly" laboratory assays that could be easier implemented in Clinics. *N*-glycan characterization of PSA 2-DE subforms from serum of a prostate cancer patient and from seminal plasma of a healthy donor revealed that subforms F1, F2 and F3 had an almost identical *N*-glycan NP-HPLC profile containing mono and disialylated complex biantennary structures. Subform F4 contained mainly monosialylated complex biantennary structures. No glycans were detected in F5. Thus, the different proportion of F3 and F4 observed between PCa and BPH patients was attributed to different content of sialic acid in PSA. The biochemical differences between F1, F2 and F3 remain unknown as no other PTM such as deamidations, phosphorylations or O-glycosylation were detected. The main 2-DE subform F3 showed a decrease of core fucosylation when comparing the *N*-glycans released after the digestion with ABS from the serum PSA of the PCa patient and the PSA from seminal plasma of a healthy donor.

These results have shown that PSA decreased sialylation and core fucosylation may aid in the detection of tumour related PSA and should be evaluated in a larger cohort of patients to determine their potential clinical use.

In an attempt to find serum glycoproteins with increased SLe^x that could be used as pancreatic cancer markers, the acute-phase protein alpha-1-acid-glycoprotein was identified after serum depletion of albumin and IgG. It showed enhanced SLe^x immunoreactivity in the sera of pancreatic cancer (PaC) and some chronic pancreatitis (CP) patients compared to healthy controls. Acute-phase proteins are proteins synthesized by the liver hepatocytes that show changes in their plasma concentration as part of the acute phase response. We subsequently performed *N*-glycan sequencing to investigate glycosylation of other major APP including alpha-1-acid-glycoprotein (AGP), haptoglobin (HPT), fetuin (FET), alpha-1-antitrypsin (AT) and transferrin (TRF) in PaC and CP patients and healthy controls, which were isolated from the sera of the patients by 2-DE. An increased SLe^x was observed in AGP, HPT and TRF in advanced PaC patients but also in the CP patients. This last group of patients showed in addition

increased SLe^x in FET and AT. *N*-glycan sequencing allowed the study of other *N*-glycan modifications such as branching, which was increased in most of the studied APP in advanced PaC and/or CP patients, and core fucosylation which was only increased in the advanced PaC patients in AGP and HPT.

In order to search for other proteins with increased SLe^x only in the PaC patients, we depleted PaC, CP and HC serum samples of the twelve most abundant serum proteins, which include all APP previously found to bear increased SLe^x in both PaC and CP patients. We therefore identified other glycoproteins with increased SLe^x immunoreaction only in the PaC patients and not in the CP patients which corresponded to alpha-2-macroglobulin (A2M), ceruloplasmin (CERU), inter-alpha-trypsin inhibitor heavy chain H4 (ITH4), complement C3 (C3), complement component C6 (C6), complement C4-A (C4A), complement C4-B (C4B). All of them were again glycoproteins produced by the liver and not by the tumour cells. Since changes in SLe^x and core fucosylation of the studied APP have also been described in other cancer types, their evaluation in a larger cohort of patients including different types of malignancies is required to establish the clinical utility of APP core fucosylation or SLe^x changes in pancreatic adenocarcinoma. The serum levels of these increased SLe^x proteins range between 0.1-2 mg/mL, which precludes the detection of potential minor proteins with increased SLe^x derived from the pancreatic tumour. The discovery of PaC specific serum markers should be oriented either on the serum depletion of more liver APP or in the evaluation of proteins glycoforms specifically expressed by the pancreatic tumour tissues that may reach the bloodstream.

In summary, the results presented in this work and those reported by other groups have shown that serum glycoproteins in cancer patients sera may show altered glycosylation either if their biosynthesis takes place in the cancer cell itself (tumor-derived glycoforms), as in the case of PSA and prostate cancer, or if they are produced by another healthy organ (host-response glycoforms), which would be the case of some APP and pancreatic cancer. Tumor-derived glycoforms are ideally candidates for the development of diagnostic biomarkers whereas host-response glycoforms are easily found in other types of cancers and body disturbances and their use could be more restricted to prognostic or monitoring purposes. The findings presented in this work related to decrease of PSA core fucosylation and sialylation in Prostate cancer and increase in some APP core fucosylation and SLe^x in pancreatic cancer should be evaluated in a larger cohort of patients to determine their possible role in the screening, diagnosis or monitoring of prostate and pancreatic cancer patients, respectively.

Resum

El càncer és una de les principals causes de mort a tot el món. La taxa de supervivència dels

El càncer és una de les principals causes de mort a tot el món. La taxa de supervivència dels pacients de càncer augmenta quan es diagnostiquen a estadis inicials, per la qual cosa és indispensable disposar de marcadors tumorals adequats.

El càncer de pròstata (PCa) és el càncer masculí més comú a Occident. El cribratge i monitorització d'aquest càncer es realitza mitjançant una estratègia combinada d'examen rectal digital i d'avaluació dels nivells sèrics d'Antigen prostàtic específic (PSA). Aquesta proteïna està produïda específicament per la pròstata i se secreta al lumen prostàtic, però pot arribar a la circulació perifèrica en el càncer de pròstata a causa de la pèrdua de la capa de cèl·lules basals característica de les glàndules prostàtiques tumorals. Tot i així, es poden observar nivells elevats de PSA sèric en altres patologies prostàtiques com la hiperplàsia benigna de pròstata (BPH), cosa que porta a biòpsies innecessàries i malestar a un elevat nombre de pacients sense càncer. És per aquest motiu que és urgent augmentar l'especificitat del PSA per diferenciar els pacients de PCa i BPH.

El càncer de pàncrees (PaC) és el càncer amb la taxa relativa de supervivència als 5 anys més baixa (al voltant d'un 5%). Aquesta baixa supervivència és deguda a l'agressivitat intrínseca d'aquest tipus de càncer, juntament amb la inadequació dels biomarcadors existents, com el CA19-9. Aquest últim és el marcador tumoral utilitzat actualment pel càncer de pàncrees, però el seu ús només ha estat aprovat per a la monitorització ja que no permet diferenciar el PaC d'alteracions pancreatobiliars benignes, com la pancreatitis crònica (CP). Per aquest motiu, es considera d'interès capital la recerca de nous marcadors de càncer de pàncrees.

Les cèl·lules de càncer mostren una glicosilació alterada de la seva superfície cel•lular i dels seus glicoconjugats de secreció. En concret, s'ha descrit que els teixits de càncer de pròstata i de pàncrees presenten glicosilació alterada com ara nivells elevats de l'epítop glucídic Sialyl-Lewis X (SLe^x). La identificació de glicoformes de proteïnes específiques secretades per cèl·lules tumorals que arribin al torrent circulatori podria millorar l'especificitat de biomarcadors de càncer coneguts, com el PSA, o bé permetre el descobriment de nous marcadors tumorals. Així, en aquest treball s'han investigat les subformes del PSA i la seva glicosilació amb l'objectiu de millorar la capacitat d'aquest marcador per diferenciar entre pacients amb càncer de pròstata i amb hiperplàsia benigna prostàtica. A més, també s'han avaluat glicoproteïnes

sèriques amb nivells augmentats de SLe^x en pacients de càncer de pàncrees, comparat amb pacients amb pancreatitis crònica i controls sans.

Les subformes de PSA es van obtenir per electroforesis bidimensional (2-DE). Aquesta tècnica va permetre la separació de 5 subformes principals d'aproximadament 33 kDa i que es van anomenar F1 a F5, de major a menor acidesa. La determinació dels percentatges relatius de les subformes principals F2-F4 en un cohort de pacients de 20 PCa i 20 BPH va mostrar que el percentatge relatiu de F3 (%F3) era significativament menor en els PCa respecte dels BPH i que aquesta disminució era més marcada a estadis avançats del càncer. %F4 va mostrar un comportament oposat i es correlacionà positivament amb l'estadi del càncer.

Per tal de comprendre les diferències bioquímiques de les subformes de PSA es va procedir a la seva caracterització, que hauria de permetre desenvolupar assajos diagnòstics fàcilment implementables a la pràctica clínica. La caracterització N-glicosídica de les subformes de PSA procedents del sèrum d'un pacient PCa i de plasma seminal d'un donant sa va revelar que les formes F1, F2 i F3 tenien un perfil de N-glicans quasi idèntic amb estructures complexes, biantenàries, tan mono com disialidades. En canvi, la forma F4 contenia majoritàriament estructures monosialidades també complexes i biantenàries. No es van detectar sucres a la subforma F5. Per tant, les diferents proporcions de F3 i F4 observades entre els pacients de PCa i BPH es va atribuir a diferent contingut d'àcid siàlic en el PSA. Continuen desconegudes les diferències bioquímiques entre F1, F2 i F3, ja que no s'han pogut detectar altres modificacions postraduccionals com desamidacions, fosforilacions o O-glicosilació. La subforma majoritària, F3, va mostrar una menor fucosilació core si es comparaven els N-glicans alliberats després de la digestió amb ABS del PSA sèric d'un pacient de PCa i del PSA de plasma seminal d'un donant sa.

Aquests resultats han mostrat que la disminució de la sialilació de la fucosilacó core del PSA podria ajudar en la detecció de PSA relacionat amb la presència de processos tumorals i que, per tant, s'haurien d'avaluar en un cohort de pacients més gran per tal de determinar el seu potencial ús clínic.

En la cerca de proteïnes sèriques amb nivells augmentats de SLe^x que es poguessin utilitzar com a marcadors de càncer de pàncrees, després d'eliminar dels sèrums l'albúmina i les immunoglobulines, es va identificar la proteïna de fase aguda alpha-1-acid-glycoprotein (AGP). Aquesta va mostrar immunoreactivitat augmentada per SLe^x en els sèrums de pacients de càncer de pàncrees i en alguns procedents de pacients amb pancreatitis crònica (CP) comparat amb pacients sans. Les proteïnes de fase aguda són proteïnes sintetitzades pel fetge que

mostren canvis de la seva concentració plasmàtica com a part de la resposta de fase aguda. Es va procedir, doncs, a la seqüenciació N-glucídica d'altres APP, inclosa l'AGP, l'haptoglobina (HPT), la fetuina (FET), l'alpha-1-antitripsina (AT) i la transferrina (TRF), en pacients amb PaC, pacients amb CP i controls sans. Les APP estudiades es van aïllar dels sèrums mitjançant 2-DE. Es va observar un augment de SLe^x en AGP, HPT i TRF en pacients avançats amb PaC però també en patients amb CP. Aquest últim grup de pacients va mostrar, a més, un augment de SLe^x en FET i AT. La seqüenciació glucídica va permetre estudiar altres modificacions N-glucídiques com el grau de ramificació, que es va veure augmentat en la majoria de les APP estudiades en els pacients de PaC i/o CP, i la fucosilació core, que es va mostrar incrementada únicament en els pacients de PaC en AGP i HPT.

Per tal de buscar altres proteïnes amb augment de SLe^x únicament en PaC, es van eliminar les dotze proteïnes majoritàries (incloent totes les APP amb SLe^x augmentat estudiades anteriorment) del sèrum de PaC, CP i HC. D'aquesta manera es van identificar altres glicoproteïnes sèriques amb augment de SLe^x en PaC i no en CP corresponents a alpha-2-macroglobulina (A2M), ceruloplasmina (CERU), inter-alpha-trypsin inhibitor heavy chain H4 (ITH4), complement C3 (C3), complement C6 (C6), complement C4-A (C4A), complement C4-B (C4B). Totes elles van resultat ser, altra vegada, glicoproteïnes produïdes pel fetge i no per les cèl·lules tumorals.

Atès que canvis en els nivells de SLe^x i de fucosilació core de les APP estudiades també s'han descrit en altres tipus de càncers, és necessari avaluar aquestes modificacions en un cohort de pacients major incloent altres tipus de càncers per tal d'establir la utilitat clínica dels nivells de fucosilació core i de Sle^x de les APP en el càncer de pàncrees.

Els nivells sèrics de les proteïnes amb nivells elevats de SLe^x són de 0.1-2 mg/mL, la qual cosa impedeix la detecció de proteïnes minoritàries amb SLe^x augmentat que provinguin dels tumors de pàncrees. La recerca de marcadors específics de càncer de pàncrees s'hauria d'orientar ja sigui cap a l'eliminació de més APP dels sèrums o bé cap a l'avaluació de glicoformes de proteïnes específicament secretades pels tumors de pàncrees que arribin a la circulació sanguínea.

En resum, els resultats mostrats en aquest treball i els presentats per altres grups indiquen que les glicoproteïnes sèriques en pacients de càncer poden mostrar glicosilació alterada tant si la seva biosíntesi té lloc en la cèl·lula tumoral (glicoformes derivades de tumor), com és el cas del PSA en el càncer de pròstata, com si són produïdes per un altre òrgan sa (glicoformes de resposta al tumor), com seria el cas de les APP i el càncer de pàncrees. Les glicoformes

derivades del tumor són candidats ideals per al desenvolupament de biomarcadors de diagnòstic mentre que les glicoformes de resposta al tumor són fàcilment detectades en altres diversos tipus de càncers i alteracions de l'organisme i el seu ús estaria més restringit a prognosi i monitorització. Les troballes presentades en aquest treball, referents a la disminució de la fucosilació core i sialilació del PSA en el càncer de pròstata i a l'augment de la fucosilació core i SLe^x d'algunes APP en càncer de pàncrees, s'haurien d'avaluar en un cohort de pacients més gran per determinar el seu paper en el cribratge, diagnòstic o monitorització del càncer de pròstata i de pàncrees, respectivament.

Resumen

El cáncer es una de las principales causas de muerte en todo el mundo. La tasa de supervivencia de los pacientes de cáncer aumenta cuando se diagnostican en los estadios iniciales, por lo que es indispensable disponer de marcadores tumorales adecuados.

El cáncer de próstata (PCa) es el cáncer masculino más común en occidente. El cribaje y monitorización de este cáncer se realiza mediante una estrategia combinada de examen rectal digital y de evaluación de los niveles séricos de antígeno prostático específico (PSA). Esta proteína está producida específicamente por la próstata y se secreta en el lumen prostático, pero puede llegar a la circulación periférica en el cáncer de próstata debido a la pérdida de la capa de células basales características de las glándulas prostáticas tumorales. No obstante, se pueden observar niveles elevados de PSA sérico en otras patologías prostáticas como la hiperplasia benigna de próstata (BHP), lo que conduce a biopsias innecesarias y malestar a un elevado número de pacientes sin cáncer. Por este motivo es urgente aumentar la especificad del PSA a fin de diferenciar los pacientes de PCa y BPH.

El cáncer de páncreas (PaC) es el cáncer con la tasa relativa de supervivencia a los 5 años más baja (alrededor de un 5 %). Esta baja supervivencia es debida a la agresividad intrínseca de este tipo de cáncer, junto con la inadecuación de los biomarcadores existentes, como el CA19-9. Este último es el marcador tumoral utilizado actualmente para el cáncer de páncreas, pero su uso sólo ha estado aprobado para la monitorización ya que no permite diferenciar el PaC de alteraciones pancreatobiliares benignas, como la pancreatitis crónica (CP). Por este motivo, se considera de interés capital la búsqueda de nuevos marcadores de cáncer de páncreas.

Las células de cáncer muestran una glicosilación alterada de su superficie celular y de sus glicoconjudados de secreción. En concreto, se ha descrito que los tejidos de cáncer de próstata de páncreas presentan glicosilación alterada, como niveles elevados del epítopo glucídico Sialyl-Lewis X (SLe^x). La identificación de glicoformas de proteínas específicas secretadas por células tumorales que lleguen al torrente circulatorio podría mejorar la especificidad de biomarcadores de cáncer conocidos, como el PSA, o bien permitir el descubrimiento de nuevos marcadores tumorales. En este trabajo se han investigado las subformas del PSA, así como su glicosilación, con el objetivo de mejorar la capacidad de este marcador para diferenciar entre pacientes con cáncer de próstata y con hiperplasia benigna prostática. Asimismo, se han evaluado glicoproteínas séricas con niveles aumentados de SLe^x en pacientes de cáncer de páncreas, comparado con pacientes con pancreatitis crónica y controles sanos.

Las subformas del PSA se han obtenido por electroforesis bidimensional (2-DE). Esta técnica ha permitido la separación de 5 subformas principales de aproximadamente 33 kDa y que se han nombrado F1 a F5, de mayor a menor acidez. La determinación de los porcentajes relativos de las subformas principales F2-F4 en un cohorte de pacientes de 20 PCa y 20 BHP ha mostrado que el porcentaje relativo de F3 (%F3) era significativamente menor en los PCa respecto de los BPH y que esta disminución era mayor en estadios avanzados del cáncer. %F4 mostró un comportamiento opuesto y se correlacionó positivamente con el estadio del cáncer.

A fin de comprender las diferencias bioquímicas de las subformas de PSA se procedió a su caracterización, lo que debería permitir el desarrollo de ensayos diagnósticos fácilmente implementables en la práctica clínica. La caracterización *N*-glicosídica de las subformas de PSA procedentes del suero de un paciente PCa y de plasma seminal de un donante sano reveló que las formas F1, F2 y F3 tenían un perfil de *N*-glicanos casi idéntico con estructuras complejas, biantenarias, tan mono como disialidadas. En cambio, la forma F4 contenía mayoritariamente estructuras monosialidades también complejas y biantenarias. No se detectaron azúcares en la subforma F5. Por tanto, las diferentes proporciones de F3 y F4 observadas entre los pacientes de PCa y BPH se atribuyeron a diferente contenido de ácido siálico en el PSA. Continúan desconocidas las diferencias bioquímicas entre F1, F2 y F3 ya que no se han podido detectar otras modificaciones postraduccionales como desamidaciones, fosforilaciones, o *O*-glicosilación. La subforma mayoritaria, F3, mostró una menor fucosilacó core si se comparaban los *N*-glicanos liberados después de la digestión con ABS del PSA sérico de un paciente de PCa y del PSA de plasma seminal de un donante sano.

Estos resultados han mostrado que la disminución de la sialilación de la fucosilacó core podrían ayudar en la detección de PSA relacionado con la presencia de procesos tumorales y que, por tanto, se deberían evaluar en un cohorte de pacientes mayor a fin de determinar su potencial uso clínico.

En la búsqueda de proteínas séricas con niveles aumentados de SLe^x que se pudieran utilizar como marcadores de cáncer de páncreas, después de eliminar de los sueros la albúmina y las inmunoglobulinas, se identificó la proteína de fase aguda *alpha-1-acid-glycoprotein* (AGP). Ésta mostró inmunoreactividad aumentada por SLe^x en los sueros de pacientes de cáncer de páncreas y en algunos procedentes de pacientes con pancreatitis crónica (CP) comparado con pacientes sanos. Las proteínas de fase aguda (APP) son proteínas sintetizadas por el hígado que muestran cambios de su concentración plasmática como parte de la respuesta de fase aguda. Se procedió, entonces, a la secuenciación *N*-glucídica de otras APP, incluida la AGP, la haptoglobina (HPT), la fetuína (FET), la alpha-1-antitripsina (AT) y la transferrina (TRF), en

pacientes con PaC, pacientes con CP y controles sanos. Las APP estudiadas se aislaron de los sueros mediante 2-DE. Un aumento de SLe^x se observó en AGP, HPT y TRF en pacientes avanzados con PaC pero también en pacientes con CP. Este último grupo de pacientes mostró, además, un aumento de SLe^x en FET y AT. La secuenciación glucídica permitió el estudio de otras modificaciones *N*-glucídicas como el grado de ramificación, que se vio aumentado en la mayoría de las APP estudiadas en los pacientes de PaC i/o CP, y la fucosilación core, que se mostró incrementada únicamente en los pacientes de PaC en AGP y HPT.

A fin de buscar otras proteínas con aumento de SLe^x únicamente en PaC, se eliminaron las doce proteínas mayoritarias (incluyendo todas las APP con SLe^x aumentado estudiadas anteriormente) de sueros de PaC, CP y HC. De este modo, se identificaron otras glicoproteínas séricas con aumento de SLe^x en PaC y no en CP correspondientes a alpha-2-macroglobulina (A2M), ceruloplasmina (CERU), *Inter.-alpha-trypsin inhibitor heavy chan H4* (ITH4), complemento C3 (C3), complemento C6 (C6), complemento C4-A (C4A), complemento C4-B (C4B). Todas ellas resultaron ser, otra vez, glicoproteínas producidas por el hígado y no por las células tumorales.

Dado que cambios en los niveles de SLe^x y de fucosilación core de las APP estudiadas también se han descrito en otros tipos de cáncer, es necesario evaluar estas modificaciones en un cohorte de pacientes mayor incluyendo otros tipos de cáncer a fin de establecer la utilidad clínica de los niveles de fucosilación core y de SLe^x de las APP en el cáncer de páncreas.

Los niveles séricos de las proteínas con niveles elevados de SLe^x son de 0.1-2 mg/mL, lo que impide la detección de proteínas minoritarias con SLe^x aumentado que provengan de tumores de páncreas. La búsqueda de marcadores específicos de cáncer de páncreas se debería orientar ya sea hacia la eliminación de más APP de los sueros o bien hacia la evaluación de glicoformas de proteínas específicamente secretadas por los tumores de páncreas que lleguen a la circulación sanguínea.

En resumen, los resultados mostrados en este trabajo y los presentados por otros grupos indican que las glicoproteínas séricas en pacientes de cáncer pueden mostrar glicosilación alterada tanto si su biosíntesis tiene lugar en la célula tumoral (glicoformas derivadas de tumor), como es el caso del PSA en el cáncer de próstata, como si son producidas por otro órgano sano (glicoformas de respuesta al tumor), como sería el caso de las APP y el cáncer de páncreas. Las glicoformas derivadas del tumor, son candidatos ideales para el desarrollo de biomarcadores de diagnóstico mientras que las glicoformas de respuesta al tumor son fácilmente detectadas en diversos tipos de cáncer y alteraciones de el organismo y su uso

estaría más restringido a la prognosis y monitorización. Los descubrimientos presentados en este trabajo referentes a la disminución de la fucosilación core y sialilación del PSA en el cáncer de próstata y el aumento de la fucosilación core y SLe^x de algunas APP en cáncer de páncreas, se deberían evaluar en un cohorte de pacientes mayor para determinar su papel en el cribaje, diagnóstico o monitorización del cáncer de próstata y de páncreas, respectivamente.

Abbreviations

α	alpha
β	beta
1-DE	one-dimensional electrophoresis
2-AB	2-aminobenzamide
2-AP	2-aminopyridine
2-DE	two-dimensional electrophoresis
AAL	*Aleuria aurantia* lectin
ABS	*Arthrobacter ureafaciens* sialidase
ACT	alpha 1-antichymotrypsin
AFP	Alpha-fetoprotein
AFP-L	Alpha-fetoprotein L3 subfraction
AGP	alpha-1-acid glycoprotein
AMARC	alpha-methylacyl coenzyme A racemase
APP	acute-phase proteins
Asn	asparagine
AT	alpha-1-antitrypsin
AUC	area under the curve
BKF	bovine kidney alpha-fucosidase
BPH	benign prostatic hyperplasia
BPH	benign prostatic hyperplasia
BPSA	BPH associated PSA
BTA	bladder tumour-associated antigen
BTG	bovine testes β-galactosidase
CA	cancer antigen
CE	capillary electrophoresis
CEA	carcinoembryonic antigen
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CI	confidence interval
ConA	concanavalin A
CP	chronic pancreatitis
Do	dolichol
DTT	dithiothreitol
EPCA-2	early prostate cancer antigen-2
ER	endoplasmic reticulum
ESI	electrospray ionization
FACE	fluorophore-assisted carbohydrate electrophoresis
FDA	Food and Drug Administration
FDP	fibrin and fibrinogen degradation products
FET	fetuin
FISH	fluorescent in-situ hybridization
fPSA	free PSA
%fPSA	percentage of fPSA to tPSA
Fuc	L-fucose
FUT	fucosyltransferase
Gal	D-galactose
GalNAc	*N*-acetyl-D-galactosamine
GISA	glycosylation immunosorbent assay
GIST	gastrointestinal stromal tumour
Glc	D-glucose
GlcA	D-glucuronic acid
GlcNAc	*N*-acetyl-D-glucosamine
GnT	*N*-acetylglucosaminyltransferase
GSTP1	Glutathione S-transferase π
GU	glucose units
GUH	*Streptococcus pneumonia* β-N-acetylglucosaminidase
HC	healthy control
HCC	hepatocellular carcinoma
HGPIN	high-grade prostatic intraepithelial neoplasia
HPLC	high-performance liquid chromatography
HPT	haptoglobin
IdoA	L-iduronic acid
IEF	Isoelectrofocusing
IHC	immunohistochemistry
IL	interleukin
kDa	kilodalton
LacNAc	*N*-acetyllactosamine
LGPIN	low-grade prostatic intraepithelial neoplasia
MAA	*Maackia amurensis* agglutinin

MALDI	Matrix-assisted laser desorption/ionization
Man	D-mannose
Mgf	mascot generic file
MIC-1	macrophage inhibitory cytokine 1
MS	Mass spectometry
Mw	molecular weight
NANI	*Streptococcus pneumoniae* sialidase
NeuAc	*N*-acetylneuraminic acid
NMP22	nuclear matrix protein 22
NMR	nuclear magnetic resonance
NP-HPLC	normal phase high-performance liquid chromatography
OST	Oligosaccharyltransferase
PaC	pancreatic cancer
PAGE	polyacrylamide gel electrophoresis
PanINs	Pancreatic intraepithelial neoplasias
PAP	human prostatic acid phospahatase
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline
PCa	prostate cancer
pI	isolectric point
PNGaseF	*Flavobacterium meningosepticum* *N*-glycosidase
Pro	Proline
PSA	Prostate-specific antigen
PTM	post-translational modifications
PVDF	polyvinylidene difluoride
Q	quadrupole
ROC	Receiver operating characteristic
RT	room temperature
SDS	sodium dodecyl sulfate
Ser	Serine
Sia	Sialic acids
SLe^a	Sialyl-Lewis A
SLe^x	Sialyl-Lewis X
SNA	*Sambucus nigra* agglutinin
ST	Sialytransferases
TBST	0.1% Tween in Tris-buffered saline
Thr	Threonie
TNM	tumor-node-metastasis
TOF	time-of-flight
tPSA	total PSA
TRF	transferrin
WAX	weak anion exchange
Xyl	D-xylose

Outline

The present thesis has been divided into 8 different chapters.

The first and the second chapter explain the scientific background and motivation of the work performed in the thesis.
The **first chapter** is a general introduction to the fields of Glycobiology, Cancer and Tumour Markers.
The **second chapter** summarizes the main objectives of the thesis.

The body of the thesis consists of four different publications related to "Glycan alterations of serum proteins as tumour markers", two of them focus on glycan alterations of the prostate cancer serum marker Prostate-specific antigen (PSA) (third and fourth chapters) and two of them deal with glycan alterations of Acute-phase proteins in pancreatic cancer (fifth and sixth chapters).

The **third chapter** shows the analisis of PSA 2-DE subforms in prostate cancer and benign prostatic hyperplasia pacients and the evaluation of their potential diagnosis in the clinical practise. It corresponds to the publication:

> Sarrats, A., Comet, J., Tabares, G., Ramirez, M., Aleixandre, R. N., de Llorens, R. and Peracaula, R. (2010). Differential percentage of serum prostate-specific antigen subforms suggests a new way to improve prostate cancer diagnosis. *Prostate* 70(1):1-9.[1]

The **fourth chapter** shows the characteritzation of PSA 2-DE subforms namely at glycan level:

> Sarrats, A., Saldova, R., Comet, J., O'Donoghue, N., R, d. L., Rudd, P. and Peracaula, R. (2010). Glycan characterization of total PSA 2-DE subforms from serum and seminal plasma. *OMICS* 14(4):465-474[2]

The **fifth chapter** corresponds to the paper:

> Sarrats, A., Saldova, R., Pla, E., Fort, E., Harvey, D. J., Struwe, W. B., de Llorens, R., Rudd, P. M. and Peracaula, R. (2010). Glycosylation of liver acute-phase proteins in pancreatic cancer and chronic pancreatitis. *Proteomics-Clinical Applications* 4(4):432-448.[3]

The **sixth chapter** is a short communication to be sent for publication:

> Sarrats, A., Ferri, M.J., Figueras, J., Fort, E., de Llorens, R and Peracaula, R. (2010). Identification of potential pancreatic cancer serum markers: increased Sialyl-Lewis X glycoproteins.

The main contributions of the thesis are discussed in the seventh and eight chapters.
The **seventh chapter** summarizes de main findings of the thesis and gives a general disussion about them.
The **eighth chapter** highlights the main conclusions of the thesis.

The work performed in this thesis has given rise in addition to two review publications:

> Peracaula, R., Barrabes, S., Sarrats, A., Rudd, P. M. and de Llorens, R. (2008). Altered glycosylation in tumours focused to cancer diagnosis. *Dis Markers* 25(4-5):207-218.
>
> Peracaula, R., Sarrats, A. and Rudd, P. M. (2010). Liver proteins as sensor of human malignancies and inflammation. *PROTEOMICS - Clinical Applications* 4(4):426–431.

Journal citation reports 2009

[1] ***Prostate***. impact factor of 3.081; first quartile in Urology and Nephrology. This article was selected by the web "UroToday.com" (with more than 55,000 readers monthly, in September 2009, just after its publication on line in *Prostate* (August 2009) as the latest in cutting edge science and research relating to Urology.

[2] ***OMICS***. impact factor of 2.291.; second quartile in Biotechnology and Applied Microbiology.

[3] ***Proteomics-Clinical Applications*.** impact factor of 1.875; third quartile in Biochemical Research Methods. This Journal was published for the first time in 2007. *Proteomics*, in the same editorial group, have a 2009 impact factor of 4.426 for which the JCR 2010 of *Proteomics-Clinical Applications* is expected to improve).

Contents

Chapter 1

Introduction

1 Glycobiology

Glycobiology is the study of the structure, biosynthesis and biology of glycans (monosaccharides and sugar chains) that are widely distributed in nature as well as the proteins that recognize them (Varki and Sharon 2008). Glycobiology is considered quite a recent field of natural science and this is mainly due to the late development of methodologies for glycan analysis and the fact that sugars have traditionally been studied solely from the energetic point of view. The term *Glycobiology* was coined in 1988 by biochemist Raymond Dwek at the University of Oxford, UK who used the phrase to emphasize the importance of relating sugars back to basic biology rather than just isolating and examining them outside of their biological context (Rademacher *et al.* 1988). Nowadays, Glycobiology is one of the more rapidly growing fields in the natural sciences, with broad relevance to many areas of basic research, biomedicine and biotechnology.

Glycans have important roles in many aspects of biology but they are particularly important in the assembly of complex multicellular organs and organisms. The localization of glycans on the outer surface of cellular and secreted macromolecules gives them the position to modulate a variety of events in cell-cell, cell-matrix and cell-molecule interactions (Varki and Sharon 2008).

1.1 Monosaccharides and the glycosidic linkage

Monosaccharides are the basic structural unit of glycans. They consist of a chain of chiral hydroximetilene units, which terminates at one end with a hydroxymethyl group and at the other with either an aldehyde group or a ketone group. Monosaccharides in solution can adopt a cyclic form by reaction of one of the hydroxyl groups with the C-1 aldehyde or ketone which yields a hemiacetal or hemicetal group. This cyclation generates an additional, new asymmetric centre derived from the carbonyl carbon atom (anomeric carbon). Two stereoisomers are therefore formed because the anomeric hydroxyl group can assume two possible orientations. When the configurations are the same at the anomeric carbon and the stereogenic center furthest from the anomeric carbon, the monosaccharide is defined as the alpha (α) anomer. When the configurations are different, the monosaccharide is defined as the beta (β) anomer.

Monosaccharides can bear certain modifications such as esterification of hydroxyl groups (phosphate, acyl, aminoacyl or sulfate esters), deoxygenation of hydroxyl groups or oxidation of methyl groups to carboxyl groups.

Although several hundred distinct monosaccharides are known to occur in nature, only a small number of these are commonly found in animal glycans (Varki and Sharon 2008) (Figure 1). They can be classified in:

- Pentoses: Five-carbon neutral sugars, e.g., D-xylose (Xyl)
- Hexoses: Six-carbon neutral sugars, e.g., D-glucose (Glc), D-galactose (Gal), and D-mannose (Man).
- Hexosamines: Hexoses with an amino group at the 2-position, which can be either free or, more commonly, *N*-acetylated, e.g., *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-galactosamine (GalNAc).
- Deoxyhexoses: Six-carbon neutral sugars without the hydroxyl group at the 6-position (e.g., L-fucose [Fuc]).
- Uronic acids: Hexoses with a negatively charged carboxylate at the 6-position, e.g., D-glucuronic acid (GlcA) and L-iduronic acid (IdoA).
- Sialic acids: Family of nine-carbon acidic sugars (generic abbreviation is Sia), of which the most common is *N*-acetylneuraminic acid (NeuAc).

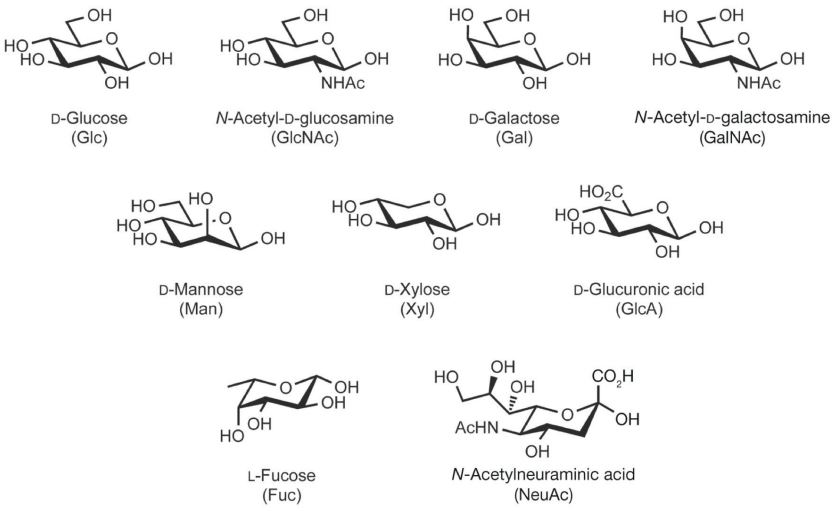


Figure 1. Most common monosaccharides found in vertebrates. Extracted from Bertozzi and Rabuka 2008.

A monosaccharide can be attached to another molecule by a glycosidic linkage, which is formed after the reaction of the hemiacetal group of the monosaccharide with and hydroxyl or amino group of another molecule to form *O*-glycosidic or *N*-glycosidic linkages, respectively. The glycosidic linkage is the fundamental linkage among sugars, to form sugar chains, and also between sugars and other molecules such as proteins or lipids, to form glycoconjugates. Glycosidic linkages can exist in two stereoisomeric forms, α or β, depending on the configuration of the anomeric carbon of the hemiacetal group.

The biosynthesis of glycans is primarily determined by the glycosyltransferases that assemble monosaccharide moieties into linear and branched glycan chains. They constitute a very large family of enzymes that catalyze a monosaccharide moiety-transfer reaction from a simple nucleotide sugar donor substrate to the acceptor substrate. They are classified according to their substrate specificity (Table 1).

Table 1. Main glycosyltransferase families. Classification according the International Union of Biochemistry and Molecular Biology.

Glycosyltransferase families	**Sugar donor substrate**
Sialyltransferases (ST)	CMP- sialic acid
Fucosyltransferases (FUT)	GDP-Fucose
Galactosyltransferases	UDP-Galactose
N-Acetylglucosaminyltransferases	UDP-GlcNAc
N-Acetylgalactosaminyltransferases	UDP-GalNAc
Manosyltransferases	GDP-Mannose

Contrary to DNA and protein sequences that are linear, glycans have heterogeneous structures that differ in composition, branching, linkage and anomericity. Thus, different aspects have to be borne in mind when depicting glycan structures. First of all, the nature of the different monosaccharides that compose the glycan. After that, the specific carbons that are involved in the glycosidic linkage (linkage position) and also the anomericity (α or β) of linkage. Different nomenclatures have been proposed to represent glycan structures (Harvey *et al.* 2009; Varki *et al.* 2009). The nomenclature used in the original figures of this work is summarized in Figure 2. It corresponds to the nomenclature attributed to Harvey *et al*. recently revised in Harvey *et al.* 2009. Other nomenclatures may be used throughout the text when figures are extracted from works by other authors.

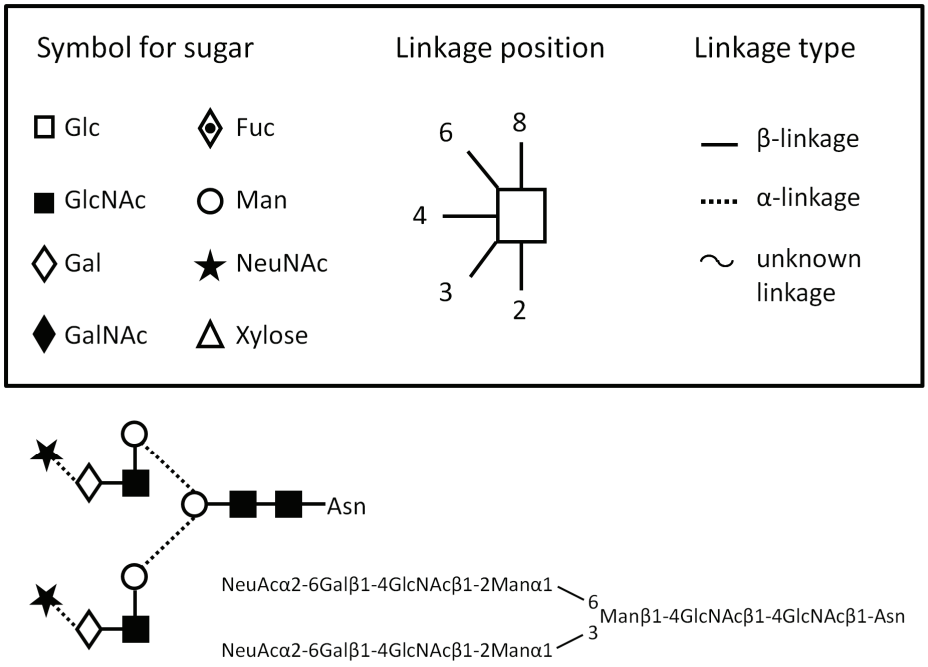


Figure 2. Nomenclature for *N*-glycan representation. The nomenclature attributed to Harvey *et al*. is the one used in original figures of this work (upper panel). An example of a complex, biantennary glycan structure using this nomenclature is shown (lower panel, extracted from GlycoBase; http://glycobase.ucd.ie).

1.2 Glycoconjugates

Glycans in covalent combination with other macromolecules (proteins and lipids) are known as glycoconjugates (glycoproteins and glycolipids).

In glycoproteins, glycans may be linked to the polypeptide backbone through an asparagine residue (*N*-glycans) or through a serine or threonine residue (*O*-glycans). *N*-glycans are considered the cornerstone of the understanding of mammalian glycosylation, because they are the best studied type. *O*-glycans are of especially relevant in the properties of mucins and proteoglycans.

Although the same glycosylation machinery is available to all proteins in a given cell, most glycoproteins emerge with characteristic glycosylation patterns and heterogeneous populations of glycans at each glycosylation site. Glycoproteins with a common polypeptide chain but bearing different glycans are known as glycoforms (Rudd and Dwek 1997).

Nearly all glycolipids in vertebrates are glycosphingolipids (glycans linked to lipids having ceramide as their core structure). Sialylated glycosphingolipids are known as gangliosides.

1.3 *N*-glycans

N-glycans are covalently attached to protein at asparagine (Asn) residue by an *N*-glycosidic bond between the amide nitrogen of asparagine and the hemiacetal group of a monosaccharide. In animal cells, the most common sugar linked to Asn is *N*-acetylglucosamine and it is always linked in β configuration (GlcNAcβ1-Asn) (Taylor and Drickamer 2003). Asn residues to which glycans can be attached must be located in a specific peptide sequence which is almost invariably Asn-X-Ser/Thr, where X can be any aminoacid except proline. *N*-glycans occur on many secreted and membrane-bound glycoproteins. They affect many properties of glycoproteins including their conformation, solubility, antigenicity, and recognition by glycan-binding proteins.

1.3.1 Types of *N*-glycans

All *N*-glycans share a common core sugar sequence, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn, and are classified into three types: (1) High-Mannose, in which only mannose residues are attached to the core; (2) Complex, in which only *N*-acetylglucosamines are linked to the core; and (3) Hybrid, in which only mannose residues are attached to the Manα1-6 arm of the core and one or two *N*-acetylglucosamines are on the Manα1-3 arm (Figure 3).

Complex type structures are subclassified according to the number of *N*-acetylglucosamines attached to the core (branches or antennae). Biantennary glycans are the most abundant ones and tri and tetrantennary structures are also common. Rare glycans containing five or more branches have also been documented (Taylor and Drickamer 2003).

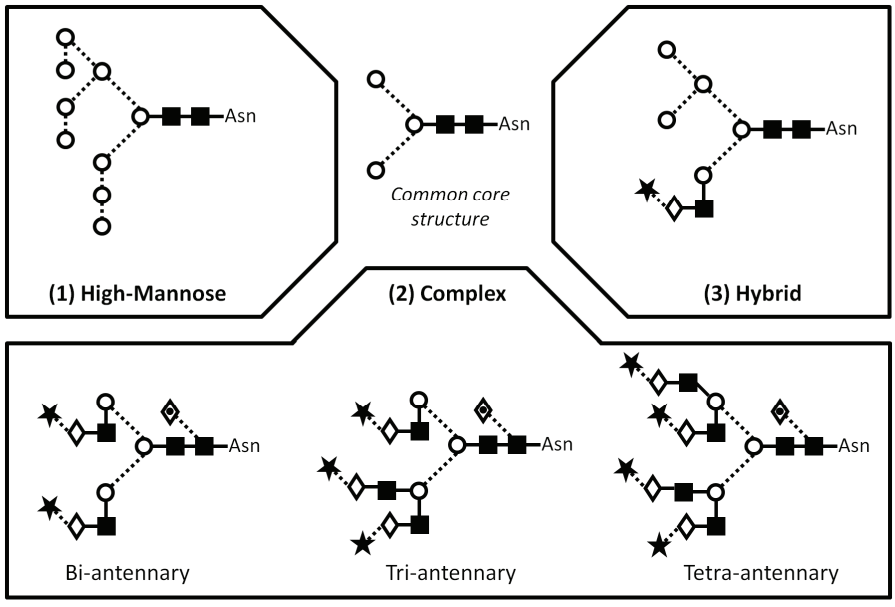


Figure 3. Types of *N*-glycans. (1) High-Mannose, (2) Complex and (3) Hybrid. Structures extracted from GlycoBase; http://glycobase.ucd.ie.

1.3.2 *N*-glycan synthesis

The biosynthesis of *N*-glycans can be divided into three stages: (1) Formation of the lipid-linked precursor oligosaccharide; (2) Transfer of the dolichol-linked precursor to nascent proteins; and (3) Processing of the oligosaccharide (Stanley *et al.* 2008).

(1) Formation of the lipid-linked precursor oligosaccharide. The first step of the *N*-glycan synthesis is the formation of the precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ attached to the lipid Dolichol (Dol) by a pyrophosphate linkage ($Glc_3Man_9GlcNAc_2$-P-P-Dol). This synthesis is initiated on the cytoplasmic face of the Endoplasmic reticulum (ER) by the transfer of GlcNAc-P from UDP-GlcNAc to membrane-bound Dol-P, forming GlcNAc-P-P-Dol. Subsequent GlcNAc and mannose transfer reactions generate $Man_5GlcNAc_2$-P-P-Dol which is then translocated across the ER membrane bilayer so that the glycan becomes exposed to the lumen of the ER. After that, it is extended by the addition of four mannose and three glucose residues that generate the mature *N*-glycan precursor $Glc_3Man_9GlcNAc_2$-P-P-Dol.

(2) Transfer of the Dolichol-linked Precursor to Nascent Proteins. Oligosaccharyltransferase (OST) catalyzes the transfer of $Glc_3Man_9GlcNAc_2$-P-P-Dol to Asn-X-Ser/Thr sequon (where X ≠ Pro) in newly synthesized proteins as they emerge from the translocon in the ER membrane. This transfer occurs in approximately 70% of the candidates' sequons (Apweiler *et al.* 1999).

(3) Processing of the oligosaccharide. The protein-bound *N*-glycan is subsequently remodeled in the ER and Golgi by a complex series of reactions catalyzed by membrane-bound glycosidases and glycosyltransferases to give rise to the different types of *N*-glycans.

Early processing steps occur in the ER and cis-golgi. The sequential removal of first glucose (glycosidases I and II) and later α1-2 linked mannose residues (α-mannosidase I) originates High-Mannose structures from 9 to 5 mannose residues (Man5-9GlcNAc2). These initial steps are known to have key roles in regulating glycoprotein folding via interactions with ER chaperones that recognize specific features of the trimmed glycan. They determine whether the glycoprotein continues to the Golgi or is degraded (Figure 4).

Late processing steps originate Hybrid and Complex *N*-glycans and are initiated in the medial-Golgi by the action of an *N*-acetylglucosaminyltransferase I (GnT-I), which adds a GlcNAc residue to C-2 of the mannose α1-3 in the core of Man5GlcNAc2. This structure originates the Hybrid type *N*-glycans. However, $GlcNAcMan_5GlcNAc_2$ is usually trimmed by α-mannosidase II which removes the terminal α1-3Man and α1-6Man residues to form $GlcNAcMan_3GlcNAc_2$ and a second GlcNAc residue is added to C-2 of the mannose α1-6 in the core by the action of GnT-II to yield the precursor for all biantennary, complex *N*-glycans

($GlcNAc_2Man_3GlcNAc_2$) (Figures 4 and 5). Additional branches can be initiated at C-4 of the core mannose α1-3 (by GnT-IV) and C-6 of the core mannose α1-6 (by GnT-V) to yield tri- and tetra-antennary *N*-glycans. Complex and hybrid *N*-glycans may carry a "bisecting" *N*-acetylglucosamine residue that is attached to the β-mannose of the core by GnT-III (Figure 5).

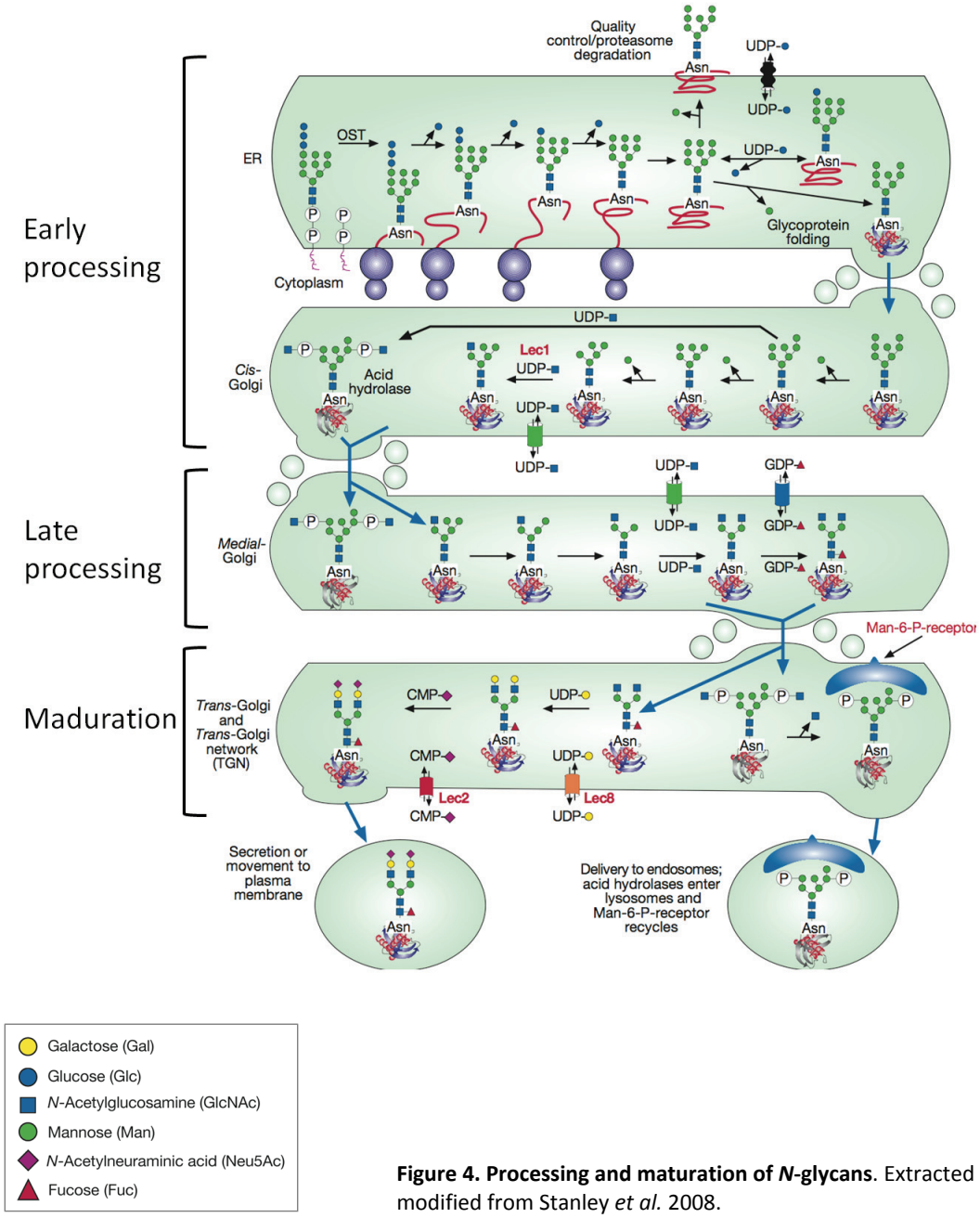


Figure 4. Processing and maturation of *N*-glycans. Extracted and modified from Stanley *et al.* 2008.

Maturation of *N*-glycans mostly occurs in the trans-Golgi and converts the limited repertoire of hybrid and complex *N*-glycans into an extensive array of structures. This part of the biosynthetic process can be divided into three components: *(a) Sugars addition to the core.*

In vertebrate *N*-glycans, the main core modification is the addition of fucose α1-6 linked to the *N*-acetylglucosamine adjacent to asparagine in the core. The fucosyltransferase involved in transferring fucose to this core (FUT VIII) requires the prior action of GnT-I; *(b) Elongation of branching.* Complex and hybrid *N*-glycans can be elongated by the addition of a β-linked galactose residue to the initiating GlcNAc in the C-3 (Type-1 structures) or in the C-4 (Type-2 structures). Antennae can be further lengthened by the sequential addition of *N*-acetylglucosamine and galactose residues, resulting in tandem repeats of LacNAc (-3Galβ1-4GlcNAcβ1-)n, termed poly-*N*-acetyllactosamine or polyLacNAc. These extensions preferentially occur on multi-antennary *N*-glycans and particularly on the β1-6GlcNAc branch; and (c) *"capping" or "decorating" reactions* involve the addition of sialic acid, fucose, galactose, GalNAc and sulfate to the branches.

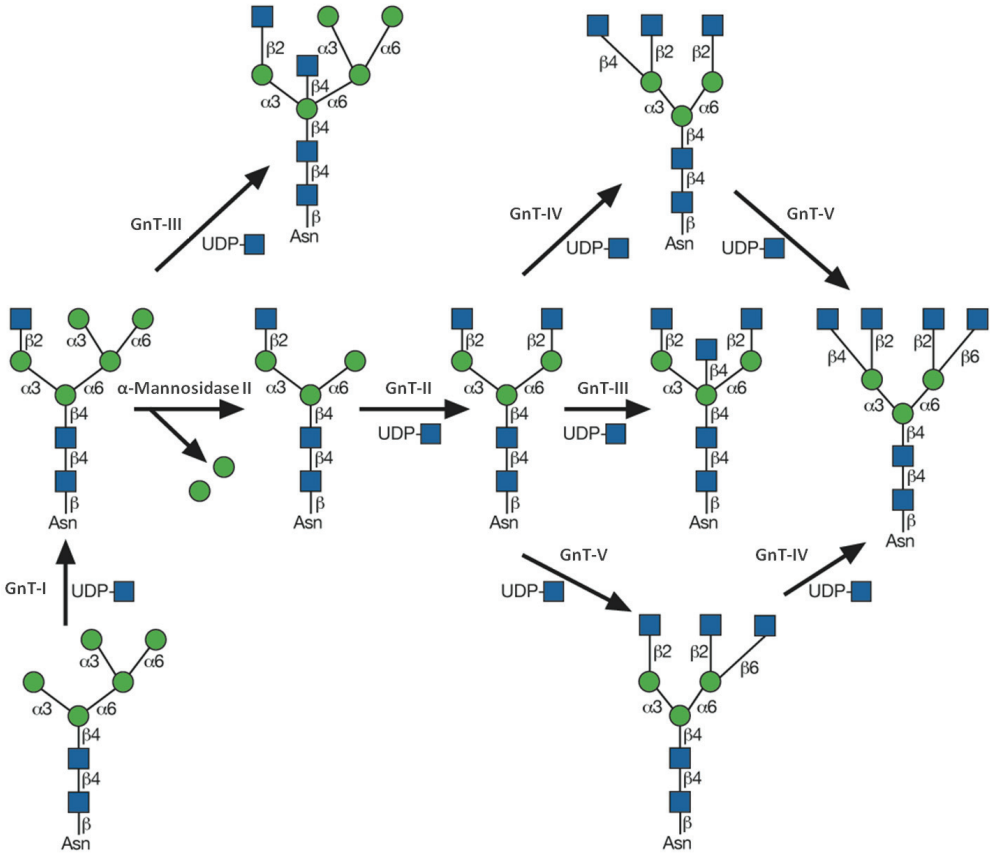


Figure 5. Branching of complex *N*-glycans. Extracted and modified from Stanley *et al.* 2008.

Type-1 and Type-2 structures can be subsequently fucosylated and sialylated at different positions to generate the different Lewis antigens; Lewis B (Le^b), Lewis A (Le^a), Sialyl-Lewis A (SLe^a), Lewis Y (Le^y), Lewis X (Le^x) and Sialyl-Lewis X (SLe^x). Different fucosyltransferases and sialyltransferases are involved in the synthesis of Lewis structures (Figure 6).

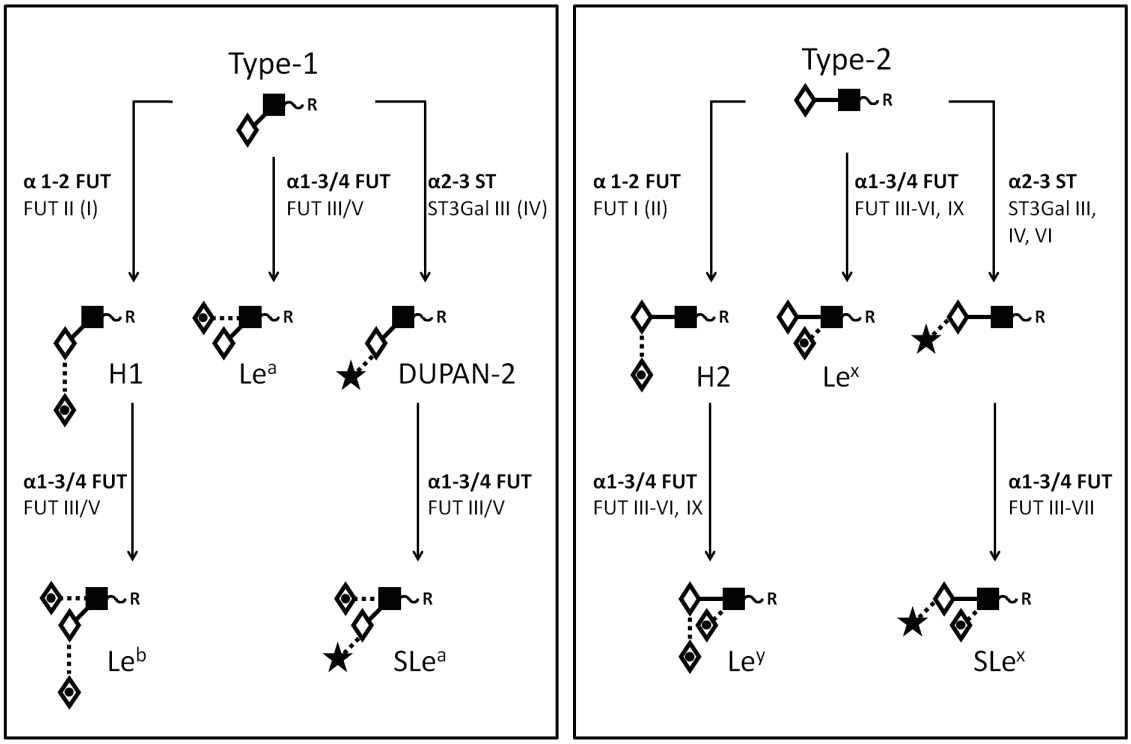


Figure 6. Synthesis of Lewis antigens. Different sialyltransferases (ST) and fucosyltransferases (FUT) are involved in the synthesis of Lewis antigens. α1-2 FUT, alpha 1-2 fucosyltransferases; α1-3/4 FUT, alpha 1-3/4 fucosyltransferases, α2-3 ST, alpha 2-3 sialyltransferases. In the synthesis of both SLe^a and SLe^x, sialylation must precede fucosylation of internal GlcNAc residues. Information extracted from Goelz *et al.* 1994; de Vries *et al.* 2001; Stanley and Cummings 2008; Carvalho *et al.* 2010; Perez-Garay *et al.* 2010.

In contrast to core glycan synthesis, which is constitutive in most cell types, the addition of branching and terminal sugars is often regulated in a tissue- or cell lineage-specific manner. In addition, many of these reactions are regulated in different cell conditions such as embryogenesis, differentiation, metabolic changes and malignancy. Regulation of outer-chain biosynthesis is largely a function of the expression levels of the relevant glycosyltransferases. However, the availability of sugar donors (nucleotide sugars) and transporters also plays an important role in the regulation of glycan synthesis.

1.4 Biological roles of Glycans

Glycans can mediate a wide variety of biological roles by virtue of their mass, shape, charge, or other physical properties. Glycans can also affect the intrinsic properties of proteins to which they are attached. However, many of their more specific biological roles are mediated via recognition by Glycan-Binding Proteins, which are classified broadly into two major groups, lectins (which include galectins and selectins) and glycosaminoglycan-binding proteins. A given glycan can have different roles in different tissues or at different times in development

(organism-intrinsic functions) or in different environmental contexts (organism-extrinsic functions). As a wide generalization, it can be stated that terminal sequences, unusual structures, and modifications of glycans probably mediate the more specific biological roles within the organism.

1.5 Altered glycosylation in cancer

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. Several examples have been found and correspond to either loss of expression or excessive expression of certain structures, the persistence of incomplete or truncated structures, the accumulation of precursors, and, less commonly, the appearance of novel structures. The most common glycan alterations are summarized below and in figure 7 (Varki *et al.* 2008).

(1) Increased branching of *N*-glycans, which results from enhanced expression of GnT-V (the enzyme responsible for the synthesis initiation of the β1-6 antennae). The change in expression of this enzyme results from increased transcription of its gene (MGAT5) and can be induced by various mechanisms, including viral and chemical carcinogenesis (Yamashita *et al.* 1985; Arango and Pierce 1988). The increase of β1-6 antennae and/or Gnt-V expression have been linked to a metastatic phenotype in cell lines and animal models (Demetriou *et al.* 1995; Seberger and Chaney 1999) and human cancer tissues (Takano *et al.* 1990; Seelentag *et al.* 1998; Handerson *et al.* 2005). Different mechanisms may be involved in the link between the increased GnT-V expression and the metastatic phenotype: (a) an increase in poly-*N*-acetyllactosamine-containing glycans (potentially recognized by galectins), (b) alterations in the cell-surface half-life of growth factor receptors caused by changes in galectin-mediated lattice formation; (c) increased outer-chain polyfucosylation and SLe^x production (potentially recognized by the selectins, as discussed below).

(2) Changes in sialic acid. The overall increase in cell-surface sialic acid content reduces attachment of metastatic tumour cells to the matrix, and may help protect cancer cells from recognition by the alternative pathway of complement activation (Pearlstein *et al.* 1980). The increase in sialylation is often manifested as specific increases in sialic acids attached to outer *N*-acetyllactosamine (Galβ1-4GlcNAc units) or to inner GalNAc-α1-*O*-Ser/Thr units on *O*-glycans. There is some evidence that the overexpression of Siaα2-6Galβ1-4GlcNAc units on *N*-

glycans may enhance β1-integrin action (Seales *et al.* 2005). Increase of Siaα2-3Galβ1-4GlcNAc may lead to an overexpression of Sialyl-Lewis antigens, as is discussed below.

(3) Increase of Lewis structures. Immunohistochemical studies on tumour specimens have shown that Lewis structures are frequently overexpressed in carcinomas, being carried on *O*-glycans as well as on *N*-glycans and glycosphingolipids. In particular, the expression of SLe^x and SLe^a in epithelial carcinomas correlates with tumour progression, metastatic spread, poor prognosis in humans, and metastatic potential in mice (Nakamori *et al.* 1997; Amado *et al.* 1998). These structures are ligands for the selectins and are normally involved in the extravasation of leucocytes from blood vessels to the inflammated tissues (Varki 1994). SLe^x and SLe^a located in the tumour cell surface serve as ligands for the E-selectin located on activated vascular endothelial cells and this interaction is been suggested to be crucial during the initial adhesion steps of hematogenous metastasis (Lowe *et al.* 1990; Takada *et al.* 1993; Kannagi 1997; Hosono *et al.* 1998; Izawa *et al.* 2000). In addition, the interaction between selectins and Sialyl-Lewis antigens may also be involved in primary tumour angiogenesis and tumour growth (Tei *et al.* 2002). Therefore, tumour cells presenting pathological selectin ligands that mediate interactions with endogenous selectins gain important selective advantages and those glycan antigens are critical in promoting the malignant behavior of cancer cells (Kannagi *et al.* 2004).

(4) Altered expression and glycosylation of mucins. Overexpression of mucins in carcinomas has been described for many years. Secreted mucins can be often found in the bloodstream of patients with cancer because of the loss of correct topology in malignant epithelial cells which allows mucins to be expressed on all aspects of the cells (Bhavanandan 1991). In the cancer cell, they are thought to act as “anti-adhesins” promoting displacement of a cell from the primary tumour in the initiation of metastasis. In carcinomas, mucins appear to be the major carriers of altered glycosylation. Carcinoma mucins usually show incomplete glycosylation, which leads to the accumulation of Tn and Sialyl-Tn antigens (type of *O*-glycans), which are barely found in normal tissues (Orntoft *et al.* 1990).

Other glycan alterations commonly found in cancer are increased expression of certain gangliosides which can be “shed” from cancer cell surface and found in body fluids, loss of glycophospholipid anchor expression in malignant and premalignant states involving the hematopoietic system, increased expression of galectins (especially galectin-3), increase of hyaluronan in the tumour-associated stroma, and changes in sulphated glycosaminoglycans (Varki *et al.* 2008).

These altered glycosylation patterns in tumours result from many factors including changes in the Golgi complex and the endoplasmic reticulum structure, in the availability of monosaccharide donors and in the expression of glycosyltransferases.

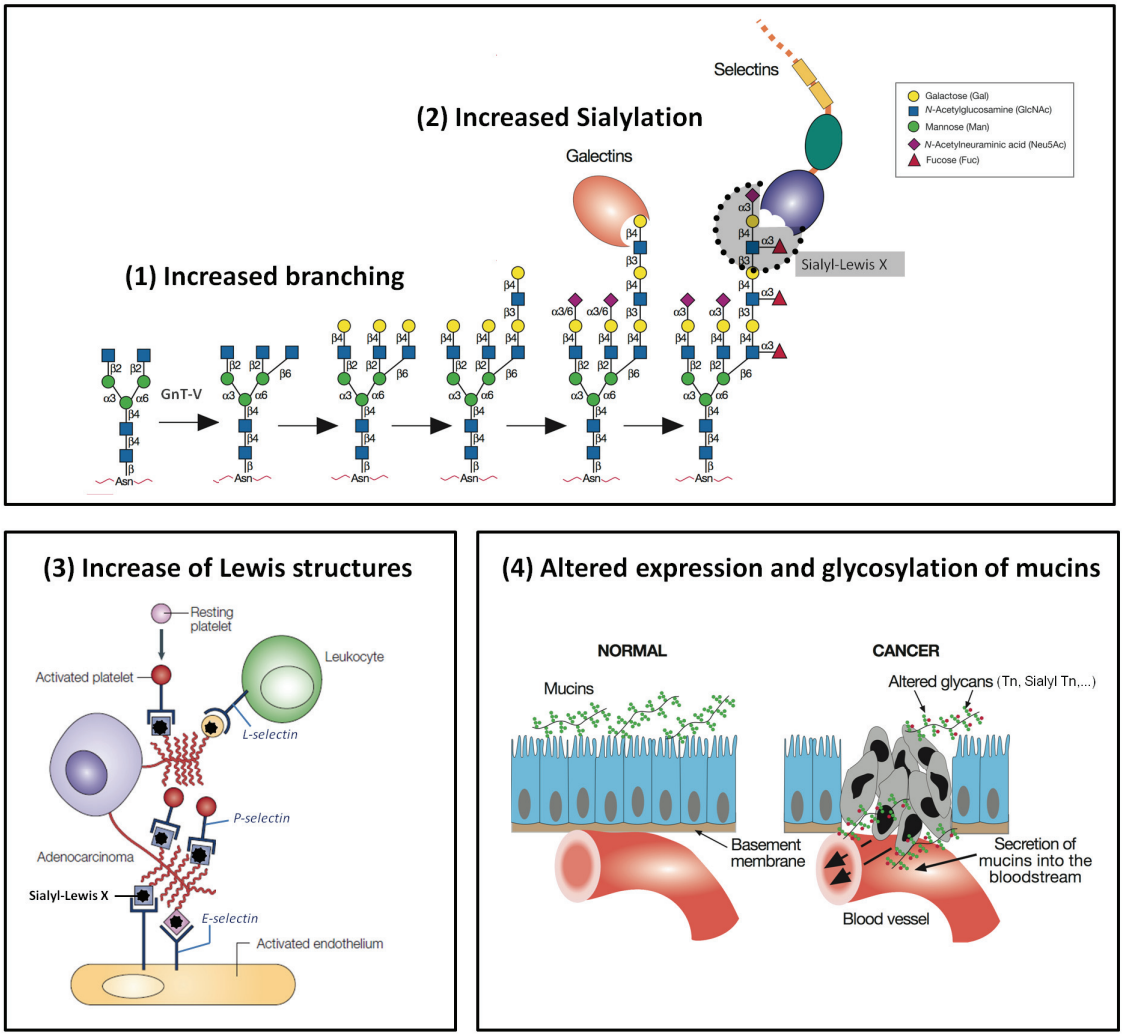


Figure 7. Some of the most common glycan alterations in cancer. (1,2) The increased size of *N*-glycans that occurs upon transformation can be explained by an elevation in *N*-acetylglucosaminyltransferase-V (GnT-V) activity, which catalyzes the β1-6 branching of *N*-glycans. This may lead to enhanced expression of poly-*N*-acetyllactosamines, which can also be sialylated and fucosylated. These structures are potentially recognized by galectins and selectins, respectively. (3) The overexpression of Lewis antigens in tumor cells promotes their dissemination through the bloodstream by the interaction with Selectins, which are important adhesion receptors expressed on activated platelets (P-selectin), leukocytes (L-selectin) and endothelial cells (E-selectin). Selectins bind to specific glycan receptors containing Sialyl-Lewis X or Sialyl-Lewis A. Interaction with P- and L-selectin promotes arrest of tumour cells at distant endothelial sites. Interaction with E-selectin mediates the initial adhesion steps of hematogenous metastasis of tumour cells. (4) Altered expression and glycosylation of mucins. Loss of normal topology and polarization of epithelial cells in cancer results in secretion of mucins with altered glycosylation into the bloodstream. The tumor cells invading the tissues and bloodstream also present such mucins on their cell surfaces. Upper panel and lower, right panel extracted and modified from Varki et al. 2008. Lower, left panel extracted and modified from Fuster and Esko 2005.

1.6 Glycan analysis

1.6.1 Detection of glycoproteins in a SDS-PAGE gel

During gel electrophoresis, a glycosylated protein typically presents one or more diffuse bands, which result from heterogeneity in its glycan chains. Visualized by protein-staining reagents, this phenomenon is often the first indication of the presence of glycans. Treatment of glycoproteins with endoglycosidases (such as *N*-glycosidase or *O*-glycosidase) is another option and if it results in a mobility change for one or more of the bands on the gel, the presence of glycan chains is indicated. A general method for detecting the presence of glycans on proteins in referred as periodic acid-Schiff (PAS) reaction, and involves periodate oxidation of vicinal hydroxyl groups followed by Schiff base formation with amine-or hydrazide-based probes. This method can be used to stain glycoproteins in polyacrylamide gels (Segrest and Jackson 1972) and on nitrocellulose membranes with a sensitivity of 0.1 μg of glycans (Wan and Van Huystee 1993). Antibody or Lectin overlays of blots of SDS-PAGE gels can also be used to detect the presence of specific glycans on glycoprotein bands or spots with a sensitivity ranging from 10 ng to 0.1 μg of glycoprotein (Wong *et al.* 2000; Peracaula *et al.* 2003a)

1.6.2 Antibodies and lectins

Antiglycan antibodies and lectins are widely used in glycan analysis. Certain glycans epitopes such as SLe^x and SLe^a may be detected using specific monoclonal antibodies against these antigens. Lectins may bind general determinants, such as α2-6 linked sialic acid (*Sambucus nigra* agglutinin, SNA) or α2-3 linked sialic acid (*Maackia amurensis* agglutinin, MAA). Fucose linked α1-2, -3 or -6 may be recognized by *Aleuria aurantia* lectin (AAL). Other lectins may recognize different structures with different affinities. For example Concanavalin A (ConA) binds tightly to high-mannose and hybrid *N*-glycans and weakly to biantennary complex *N*-glycans. However, it doesn't bind to tri and tetra-antennary complex type *N*-glycans (Cummings and Etzler 2008).

Some of antiglycan antibodies and lectins applications include cell separation and analysis by using flow-citometry, cytochemical characterization/staining of cells and tissues, purification and characterization of glyco-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 (Rosenfeld *et al.* 2007).

1.6.3 Glycan profiling and structural analysis

Glycans can be released enzymatically or chemically from glycoproteins. Once liberated, glycans can be labeled in their free reducing termini with fluorescent tags such as 2-aminobenzamide (2-AB), 2-aminopyridine (2-AP) among others to increase sensitivity in their detection. Free glycans may be subsequently analyzed by capillary electrophoresis (CE), fluorophore-assisted carbohydrate electrophoresis (FACE) or high-performance liquid chromatography (HPLC). The profiles obtained may provide glycan structural information based on their similarity to known standards. In addition, the use of sequential exoglycosidases (many of them specific for both monosaccharide residue and linkage type) treatments can supply more accurate structural information based on the measurement of the shifts in glycan elution times, which indicate glycan susceptibility to the enzyme. In this work, detailed structural analysis of *N*-glycans using HPLC combined with exoglycosidase array digestion have been used according to the methodology established by the group of Prof. PM. Rudd (Royle *et al.* 2006; Royle *et al.* 2008), which allows *N*-glycan characterization from 2 micrograms of glycoprotein (Royle *et al.* 2006)

Detailed characterization of purified glycans can be obtained by NMR spectroscopy and also by MS-based experiments. A simple ^{1}H-NMR can provide the entire primary structure of a glycan if ^{1}H-NMR spectra of well-characterized glycans of related structures are available for comparison. NMR spectroscopy also allows de novo full structural characterization of a glycan by assignment of both the ^{1}H- and ^{13}C-NMR spectra of a glycan. However, these techniques are considered relatively insensitive with respect to the amount of sample needed in order to obtain good-quality structural data, compared to other analytical techniques, such as mass spectrometry (Duus *et al.* 2000). MS experiments from released glycans may be used for glycomic profiling by assignment to each molecular ion based on the usually unique glycan composition for a given mass and prior knowledge of *N*- and *O*-glycan biosynthesis. These experiments are most conveniently carried out using MALDI because of its high sensitivity which allows important structural conclusions to be drawn on the basis of picomolar amounts of components (Morelle and Michalski 2007). Assignments can be confirmed in a second experiment employing ESI-MS/MS instrumentation by selecting each molecular ion for collisional activation and recording its fragment ion spectrum. If necessary, additional information can be provided by MS experiments on chemical and enzymatic digests, the choice of which is guided by the sequence information provided by mass mapping and MS/MS experiments (Harvey *et al.* 2008). Other MS approaches consist on the degradation of the polypeptide chain by proteases to generate glycopeptides which can be then analysed by MS.

1.6.4 Indirect analysis of sialic acid

Sialic acid is a negatively charge carbohydrate. Thus, glycoproteins bearing sialic acid in their glycan chain experience a decrease in their isoelectric point (pI), compared to their corresponding glycoforms with no sialic acid or to unglycosylated forms of the protein. Differences in sialic acid content may be therefore indirectly evaluated by techniques based on separation according to protein pI such as Chromatofocusing and Isoelectrofocusing (IEF).

Chromatofocusing is a protein-separation technique that combines the advantage of high-capacity ion-exchange procedures with the high resolution of isoelectric focusing into a single chromatographic focusing procedure. In this technique, proteins with different pI can be separated by being passed through a chromatofocusing column (packed with a specific medium) while a pH gradient is generated on the column by a specifically designed and matched amphoteric buffer. A second buffer is applied to elute the bound proteins roughly in order of their pI with peak widths in the range of 0.05 pH units (Li and Hutchens 1992).

In isoelectric focusing (IEF) an electric potential is applied across the gel, generating a pH gradient gel making one gel end more positive than the other. At all pHs other than protein isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel and if they are negatively charged they will be pulled to the more positive end of the gel. The proteins applied in this gel will move along the gel and will accumulate and focus at the gel pH corresponding to their isoelectric point; that is, the point at which the overall charge on the protein is 0 (a neutral charge). When after IEF, proteins are transferred to SDS-PAGE, a two-dimensional electrophoresis (2-DE) will take place. 2-DE gels are more resolving than IEF gels because proteins are separated by two properties, in the first dimension, by their pI (isoelectric focusing) and in the second dimension by their molecular weight.

Glycosylation immunosorbent assay (GISA) allows the detection of not fully sialylated glycoforms of a protein. It is based on the specificity of sialyltransferases and the immunosorbent technology (Poon *et al.* 2002). The glycoprotein of interest is captured using specific antibodies and then, a recombinant sialyltransferase transfers fluorescently labeled sialic acids onto nonsialylated terminal positions of *N*-glycans. The amount of sialic acid transferred is mostly dependent on the amount of nonsialylated *N*-glycan chains present in a glycoprotein.

2 Cancer

Cancer is one of the leading causes of death worldwide. The disease accounted for 7.4 million deaths (around 13% of all deaths worldwide) in 2004 and this number is projected to continue rising, with an estimated 12 million deaths in 2030 (World Health Organitzation 2009). In the developed countries, cancer is second to cardiovascular disease as a cause of death. Currently, in the United States one in four deaths in is due to cancer (Jemal *et al.* 2009). This illness represents a major public health concern not only for being a leading cause of death but also for its increasing high prevalence, which represents important clinical costs in diagnosis, treatment and follow-up procedures (Greenberg *et al.* 2010).

Cancer is a general term used to define a plethora of different pathologies that are characterized by uncontrolled cellular proliferation and growth, and under special conditions, tumour cell migration, invasion (intrusion and destruction of adjacent tissues), and metastasis (spreading to other organs and tissues). It results from multiple mutations that occur in several genes such as proto-oncogenes, tumour suppressor genes and/or DNA repair genes of somatic cells. These mutations alter the normal function of a wide spectrum of regulatory, apoptotic, and signal transduction pathways and give cancer cells selective advantages such as self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg 2000).

Tumours originating from epithelial cells are known as carcinomas and represent the most common type of human cancers, perhaps because most of the cell proliferation in the body occurs in epithelia, or because epithelial tissues are most frequently exposed to carcinogens. Adenocarcinomas are carcinomas with glandular organization and are the main sort of cancer found in colon, lung, ovarian, breast, prostate and pancreatic tissues among others.

Cancer follows a characteristic natural history. In an epithelial tissue, certain normal cells may become dysplastic showing subtle morphological abnormalities that suggest the beginning of cancerous transformation. Pathologists describe these changes as intraepithelial neoplasia, and classify them as low-grade (mild) and high-grade (moderate to severe). Increased cell proliferation may lead to a carcinoma *in situ*, which is the first malignant stage of cancer. Tumour may progress invading basement membrane and surrounding tissues by gaining the ability to break away from the primary tumour (reduction of cell adhesion) and to degrade and

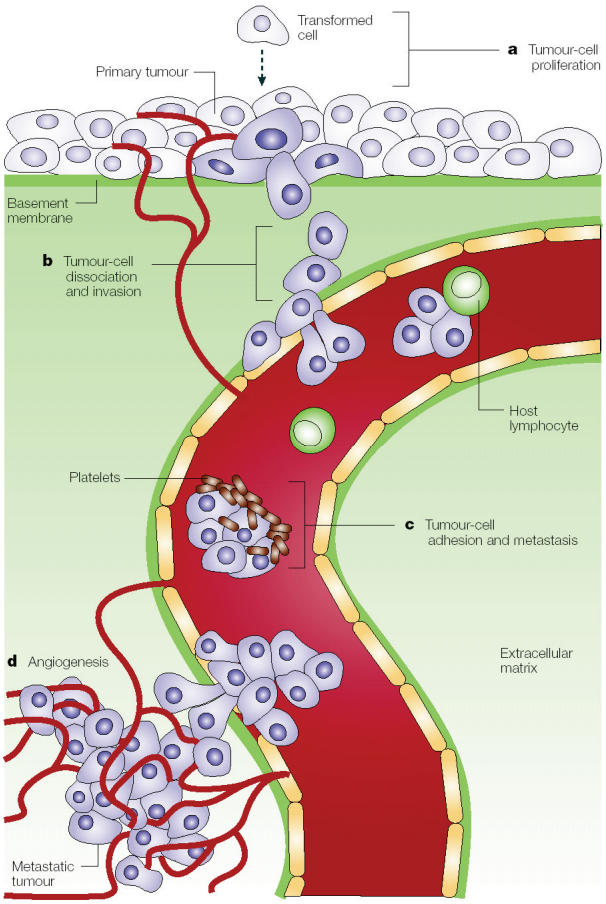


Figure 8. Stages of tumour progression. (a) Tumour proliferation, (b) Invasion, (c) Metastasis and (d) Angiogenesis. Extracted from Fuster and Esko 2005.

migrate through basement membranes and extracellular matrix (invasive carcinoma). Tumour cells may eventually reach local lymphatic or blood vessels and spread to the regional or distant lymph nodes and also to other organs. This last step, which may lead to the formation of a secondary tumour, is known as metastasis. During the dissemination of tumour cells through the bloodstream, they aggregate with host cells such as platelets and lymphocytes, which facilitates their extravasation from blood vessels into new tissues. In addition, both primary and secondary tumours must acquire new vasculature through a process called angiogenesis in other to achieve diameters greater than 2 mm (Figure 8). Glycans play an important role in the most of the above described steps of cancer (Fuster and Esko 2005).

Cancers are classified according to the anatomic extent of disease in the TNM system, which is based on the assessment of three components: T, the extent of the primary tumour; N, the absence or presence of regional lymph node metastasis, M; the absence or presence of distant metastasis. The use of numerical subsets of the TNM components indicates the progressive extent of the malignant disease (see Tables 2 and 3). Tumour grade is also included in this classification when it is intimately linked to prognosis, for example in prostate cancer.

In general, localized tumours can be removed effectively by surgery or radiotherapy, while metastatic disease requires relatively ineffective systemic therapy. The identification of patients with early stage disease is imperative to improve the chances of local control and cure. The availability of adequate biomarkers is of major importance in this early diagnosis of cancer.

This work has been focused on the serum biomarkers investigation of two different adenocarcinomas: prostate cancer and pancreatic adenocarcinoma.

2.1 Prostate cancer

Prostate cancer (PCa) is the most common male cancer in Western Countries, it has been estimated as the second leading cause of male cancer death in United States and the third in the European Union in 2008, after lung and colorectal carcinomas (Jemal *et al.* 2009; Ferlay *et al.* 2010b) (Figure 9). The incidence of PCa increases with age and about 80% cases are in men over 65.

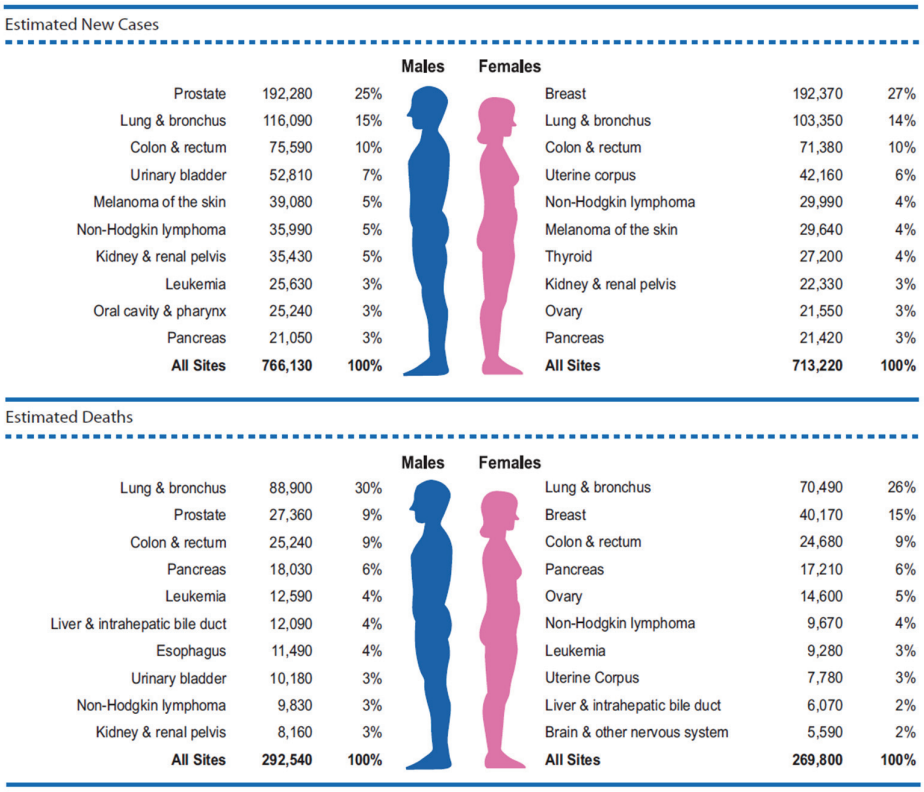


Figure 9. Ten leading cancer types for estimated new cancer cases and deaths, by Sex, United States 2009. Extracted from Jemal *et al.* 2009

The prostate is a complex tubulo-alveolar exocrine gland located directly beneath the bladder, surrounding the urethra in the male. It is composed of an epithelial parenchyma embedded within a connective tissue matrix. The epithelial cells are organized in glands that branch out from the urethra and terminate in secretory acini. The function of the prostate is to secrete and store a slightly alkaline fluid that constitutes part of the volume of the seminal plasma. It is divided in three anatomical zones: peripherical, central and transition zones.

Almost all prostate cancers (95%) are adenocarcinomas which are characteristically multifocal and heterogeneous. The majority of them (68%) arise in the peripherical zone, 24 % in the

transitional zone and 8% in the central zone. The malignant focus show glands with different architectural, nuclear, cytoplasmic and intraluminal features. They are more crowed and grow in a haphazard fashion. They also show a loss of basal layer (Liska *et al.* 2007). A high-grade prostatic intraepithelial neoplasia (HGPIN) may be associated with or precede prostate cancer in a high percentage of cases (Joniau *et al.* 2005) (Figure 10).

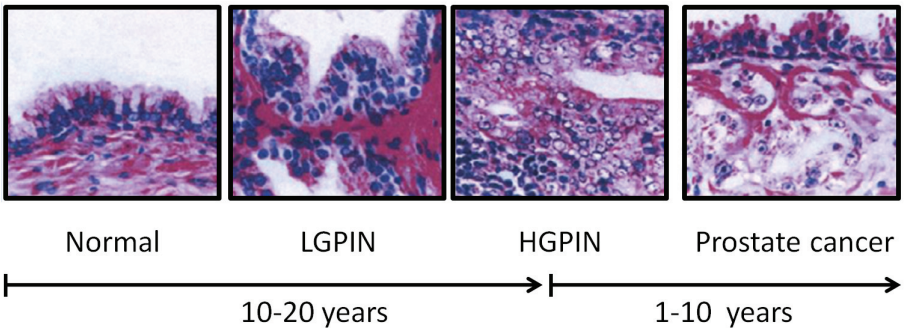


Figure 10. Histological images of benign prostate, prostatic intraepithelial neoplasia (PIN) and prostate cancer. Long period progression from normal tissue to low grade (LGPIN) and high grade (HGPIN) prostatic intraepithelial neoplasia, and then to prostate cancer. Extracted and modified from Sciarra *et al.* 2008

The Gleason system is the most widely used grading system for prostate adenocarcinomas. This method was developed by Dr Donald F Gleason (Gleason 1977) and is based entirely on the carcinoma histological pattern (glandular pattern and degree of differentiation).

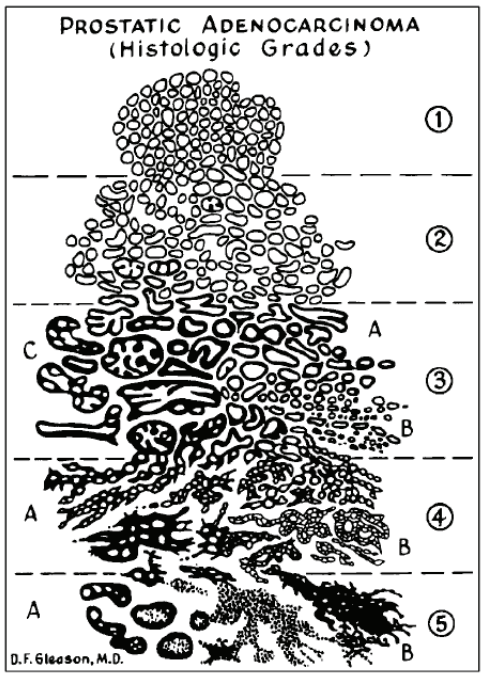


Figure 11. Gleason's histologic grades of prostate adenocarcinoma. Extracted from Gleason 1977.

Five histological patterns or grades (1-5) are defined (Figure 11). The most prevalent and the second prevalent pattern are identified; each is graded 1 to 5 and added to obtain Gleason score. Gleason score is used as prognostic factor. Increasing Gleason grade is directly related to lymphvascular invasion, tumour size, positive surgical margins, and pathological stage, including risk of extraprostatic extension and metastasis (Humphrey 2004).

Initially, small clumps of cancer cells remain confined to prostate glands. But later, prostate adenocarcinoma may spread locally, by direct invasion of seminal vesicles, urinary bladder or

surrounding tissues or distantly. Distant metastases can derive from an initial lymphatic spread or from a direct hematogenous spreading, mainly to the bones (Bracarda *et al.* 2005). The TNM system classifies prostate cancer according this anatomic extent (Table 2). This classification, together with the histological grade (Gleason) is used to stage prostate cancer.

Table 2. Classification of Prostate cancer*

TNM Clinical Classification

T - Primary tumour

TX Primary tumour cannot be assessed

T0 No evidence of primary tumour

T1 Clinically inapparent tumour, not palpable or visible by imaging
- T1a Tumour incidental histological finding in 5% or less of tissue resected
- T1b Tumour incidental histological finding in more than 5% of tissue resected
- T1c Tumour identified by needle biopsy (e.g., because of elevated PSA)

T2 Tumour confined within prostate (*Note:* Tumour found in one or both lobes by needle biopsy, but not palpable or reliably visible by imaging, is classified as T1c)
- T2a Tumour involves one-half of one lobe or less
- T2b Tumour involves more than one-half of one lobe but not both lobes
- T2c Tumour involves both lobes

T3 Tumour extends through the prostatic capsule (*Note:* Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is not classified as T3, but as T2)
- T3a Extracapsular extension (unilateral or bilateral)
- T3b Tumour invades seminal vesicle(s)

T4 Tumour is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphincter, rectum, levator muscles, and/or pelvic wall.

N - Regional Lymph Nods

Nx Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Regional lymph node or nodes metastasis

M - Distant Metastasis

MX Distant metastasis cannot be assessed
M0 No distant metastasis
M1 Distant metastasis
- M1a Non-regional lymph node(s)
- M1b Bone(s)
- M1c Other site(s)

G Histopathological Grading

Gx Grade cannot be assessed
G1 Well differentiated (slight anaplasia) (Gleason 2-4)
G2 Moderately differentiated (moderate anaplasia) (Gleason 5-6)
G3-4 Poorly differentiated/undifferentiated (marked anaplasia) (Gleason 7-10)

Stage Grouping

Stage	T	N	M	G
Stage I	T1a	N0	M0	G1
Stage II	T1a	N0	M0	G2, 3-4
	T1b,c	N0	M0	Any G
	T1,T2	N0	M0	Any G
Stage III	T3	N0	M0	Any G
Stage IV	T4	N0	M0	Any G
	Any T	N1	M0	Any G
	Any T	Any N	M1	Any G

*applies only to adenocarcinomas. Extracted from Sobin and Wittekind 2002.

In addition, clinically staging of prostate cancer is also used in the management of the disease. This classifies prostate cancer in localized (T1-2, NX-0, M0), locally advanced (T3-4, any N, M0) and metastatic (any T, any N, M1).

The early diagnosis of prostate cancer is of major importance because when PCa is detected at early stages it can be easily treated which results in a 5-year relative survival rate of 100%. On the other hand, when PCa is diagnosed at advanced stages, the 5-year relative survival rate drops to 30% (Jemal, Siegel et al. 2009). Prostate-specific antigen (PSA) serum levels, Digital rectal examination (DRE) and transrectal ultrasonography (TRUS) guided biopsy constitute the three major diagnostic means for the detection of prostate cancer (Bracarda *et al.* 2005).

Prostate cancer shows particular altered glycosylation patterns. By immunohistochemistry studies, carbohydrate antigens, SLe^x and Le^y, known to be minimal or absent in benign secretory epithelial cells, are highly expressed in tumour tissues (Martensson *et al.* 1995; Zhang *et al.* 1997). In addition, the up regulation of SLe^x correlates with poor prognosis of the tumour (metastatic prostate cancer) (Jorgensen *et al.* 1995; Jorgensen *et al.* 1997). Some glycosyltransferases that catalyze the formation of these carbohydrate antigens in the Golgi have been found to be altered in several metastatic prostate cancer cells which have E-selectin ligand activity. In particular, fucosyltransferases (FUT), FUT3, FUT6 and FUT7, are up-regulated on metastatic PCa cells and correlate with the level of expression of SLe^x bearing glycoproteins and E-selectin binding activity (Barthel *et al.* 2008).

2.2 Pancreatic cancer

Although pancreatic cancer represented only around 3% of all cancer cases in 2008 is the fourth leading overall cause of cancer death in Europe and the United States (Figure 9). In addition, it is the cancer with the lowest 5-Year Survival Rate (about 5%), which have not improved, contrarily to most of the other cancer types, since 1975 (Jemal *et al.* 2009; Ferlay *et al.* 2010b). This poor survival is because of its high aggressiveness and rapid progression (Bardeesy and DePinho 2002; Real 2003) together with the inadequateness of existing biomarkers (Goggins 2005), which leads to a late diagnosis, after metastasis in 78% of the diagnosed patients (Jemal *et al.* 2009).

The pancreas is a gland organ in the digestive and endocrine system, located across the back of the abdomen, behind the stomach and attached to duodenum. It is both an endocrine gland producing several important hormones, including insulin, glucagon, and somatostatin, as well

as an exocrine gland, secreting pancreatic juice containing digestive enzymes that pass to the small intestine. It is divided into four anatomical zones: head, neck, body and tail.

The majority of pancreatic cancers is classified as pancreatic ductal adenocarcinoma (PDAC) and affects the exocrine part of the gland. Sixty-five per cent are located within the head of the pancreas, 15% in the body of the gland, 10% in the tail and 10% are multifocal. PDAC has a particularly intense desmoplastic stroma, which can account for a large proportion of the pancreatic tumour volume. It comprises extracellular matrix, together with a number of different host cell types, including fibroblasts, small endothelial-lined vessels, residual normal epithelia and a variety of inflammatory cells, which are both locally derived and recruited from the circulation. Pancreatic intraepithelial neoplasias (PanINs) have been described as precursors lesions of this disease (Ghaneh *et al.* 2007) (Figure 12). Pancreatic adenocarcinoma may spread to the duodenum, bile duct or peripancreatic tissues and further into the stomach, spleen, colon or adjacent large blood vessels. Lymphatic invasion can be a feature in some cases. Distant metastasis may arise mainly in liver, peritoneum and lungs (Lack 2003).

The TNM system classifies pancreatic cancer according to its anatomic extent (Table 3). This classification is used to stage pancreatic cancer.

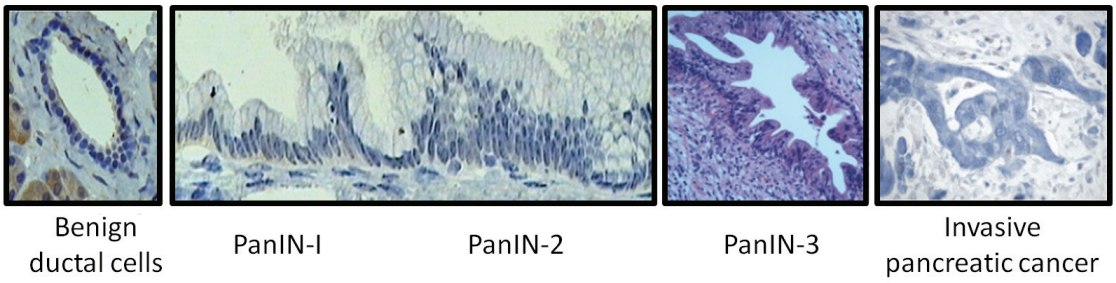


Figure 12. Histological images of benign pancreatic ductal epithelial cells, progressive pancreatic intraepithelial neoplasia (PanIN) and invasive pancreatic carcinoma. Extracted and modified from Ghaneh *et al.* 2007.

Pancreatic cancer cells show an overexpression of sialyl-Lewis antigens, sialyl-Lewis A (SLe^a) or sialyl-Lewis X (SLe^x), on cell surface glycoproteins or glycosphingolipids (Hosono *et al.* 1998; Peracaula *et al.* 2005). Lewis X and related antigens, such as SLe^x, have been found to be expressed in pancreatic cancer tissues at higher rates than inflamed pancreas (chronic pancreatitis) while there were hardly detected in healthy pancreatic tissues (Kim *et al.* 1988; Satomura *et al.* 1991; Sinn *et al.* 1992). The expression of these antigens inversely correlated with patients' survival (Nakamori *et al.* 1997; Amado *et al.* 1998).

Table 3. Classification of Pancreatic cancer*

TNM Clinical Classification

T - Primary tumour

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour limited to the pancreas, 2 cm or less in greatest dimension
T2	Tumour limited to the pancreas, more than 2 cm in greatest dimension
T3	Tumour extends directly into any of the following: duodenum, bile duct, peripancreatic tissues
T4	Tumour extends directly into any of the following: stomach, spleen, colon, adjacent large blood vessels

N - Regional Lymph Nods

Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node or nodes
N1a	Metastasis in a single regional lymph node
N1b	Metastasis in multiple regional lymph nodes

M - Distant Metastasis

MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

G Histopathological Grading

Gx	Grade cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3-4	Undifferentiated

Stage Grouping

Stage	**T**	**N**	**M**
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
	T2	N0	M0
Stage II	T3	N0	M0
Stage III	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
Stage IVA	T4	Any N	M0
Stage IVB	Any T	Any N	M1

* applies only to carcinomas of the exocrine pancreas. Extracted from Sobin and Wittekind 1997.

3 Tumour markers

A tumour marker is a substance that can be detected in higher than normal amounts in the blood, urine, or body tissues of some patients with certain types of cancers. Tumour markers are divided into two broad categories: (1) Tumour-derived markers, these are produced by the neoplastic cells; and (2) tumour-associated or host-response markers- these include products of normal tissue produced in response to the presence of neoplastic tissue. They may be indicators of tumour stage and grade and may be used in screening, diagnosis, prognosis of cancer and also to assess treatment response and monitor recurrence after treatment (Eissa and Sohair 1998). Table 4 shows the tumour markers approved by the FDA (Food and Drug Administration) for different cancers.

Different tumour markers are found in different types of cancer, and levels of the same tumour marker can be altered in more than one type of cancer. Some tumour marker levels can also be altered in patients with noncancerous conditions. In addition, tumour marker levels are not altered in all people with cancer, especially if the cancer is in its early stage. In this regard, the utility of a tumour marker in the clinical practice is determined by two factors: sensitivity (proportion of cancer patients which are correctly identified) and specificity (proportion of non-cancer patients which are correctly identified).

ROC (Receiver operating characteristic) curves are usually used to asses diagnosis accuracy of a given tumour marker and to compare it among other diagnosis tests/markers. These are graphical plots of the sensitivity *vs.* (1-specificity). The area under the ROC plot (ROC AUC) is a taken as a numerical measure of the diagnostic accuracy of the method. Values range between 1.0 (perfect separation of the test values of the two groups) and 0.5 (no apparent distributional difference between the two groups of test). It is possible to test whether the diagnostic test is at all effective in distinguishing the two populations as well as to estimate the ROC area by a confidence interval. In particular, the rejection of the hypothesis that the theoretical area is 0.5 provides evidence that the laboratory test does have the ability to distinguish between the two groups ($P<0.05$) (Zweig and Campbell 1993).

Table 4. Food and Drug Administration-approved cancer biomarkers*

Biomarker	Type	Source	Cancer type	Clinical use
AFP	Glycoprotein	Serum	Nonseminomatous testicular	Monitoring
Human chorionic gonadotropin-β	Glycoprotein	Serum	Testicular	Staging
AFP-L3	Glycoprotein	Serum	Hepatocellular	Risk assessment in patients with chronic liver diseases
CA19-9	Mucin	Serum	Pancreatic	Monitoring
CA125	Mucin	Serum	Ovarian	Monitoring
Pap smear	Cervical smear	Cervix	Cervical	Screening
CEA	Glycoprotein	Serum	Colon and others	Monitoring
Epidermal growth factor receptor	Glycoprotein	Colon	Colon	Selection of therapy
FDP	Glycoprotein	Serum	Colorectal	Monitoring
KIT (CD117)	Glycoprotein (IHC)	Gastrointestinal tumour	GIST	Diagnosis and selection of therapy
Thyroglobulin	Protein	Serum	Thyroid	Monitoring
PSA (total)	Glycoprotein	Serum	Prostate	Screening and monitoring
PSA (complex)	Glycoprotein	Serum	Prostate	Monitoring
PSA (free PSA %)	Glycoprotein	Serum	Prostate	Benign prostatic conditions versus Prostate cancer diagnosis
CA15-3	Mucin	Serum	Breast	Monitoring
CA27-29	Mucin	Serum	Breast	Monitoring
Cytokeratins	Protein (IHC)	Breast tumour	Breast	Prognosis
Oestrogen and progesterone receptor	Protein (IHC)	Breast tumour	Breast	Selection for hormonal therapy
HER2/NEU	Glycoprotein (IHC)	Breast tumour	Breast	Prognosis and selection of therapy
HER2/NEU	Glycoprotein	Serum	Breast	Monitoring
HER2/NEU	DNA (FISH)	Breast tumour	Breast	Prognosis and selection of therapy
Chromosomes 3, 7, 9 and 17	DNA (FISH)	Urine	Bladder	Screening and monitoring
NMP22 Protein	Protein	Urine	Bladder	Screening and monitoring
FDP	Glycoprotein	Urine	Bladder	Monitoring
BTA	Glycoprotein	Urine	Bladder	Monitoring

*Extracted and modified from Ludwig and Weinstein 2005.
Abbreviations: AFP, Alpha-fetoprotein; AFP-L3, Alpha-fetoprotein L3 subfraction; BTA, bladder tumour-associated antigen; CA, cancer antigen; CEA, carcinoembryonic antigen; FDP, fibrin and fibrinogen degradation products; FISH, fluorescent in-situ hybridization; GIST, gastrointestinal stromal tumour; IHC, immunohistochemistry; NMP22, nuclear matrix protein 22; PSA, prostate-specific antigen.

As can be seen in table 4 most tumour markers are either glycans or glycoproteins. Because oncogenesis leads to a remarkable alteration of cellular glycosylation, as described in section 1.5, tumour-secreted glycoproteins may reflect the altered glycosylation pattern of cancer cells and are likely to be good candidates for tumour markers (Orntoft and Vestergaard 1999).

3.1 Tumour markers in prostate cancer

Prostate-specific antigen (PSA) serum level is currently considered the best tumour marker available in clinical medicine for the purpose of monitoring and diagnosing prostate carcinoma, both uses having been approved by the FDA (Food and Drug Administration) (Table 4). The particularities of PSA will be discussed later in section 3.1.1.

Historically the earliest serum marker for prostate cancer was human prostatic acid phosphatase (PAP) (Gutman and Gutman 1938). However, with the introduction of prostate specific antigen (PSA), it was rendered almost obsolete as it was inferiorly effective (Lowe and Trauzzi 1993). Recently, early prostate cancer antigen-2 (EPCA-2) detection in serum, has shown some promising results as it was able to differentiate between men with and without PCa with 92% specificity and 94% sensitivity, whereas the specificity of PSA in the same population was 65% (Leman *et al.* 2007).

Other PCa markers are tissue markers such as Glutathione S-transferase π (GSTP1) hypermethylation and α-methylacyl coenzyme A racemase (AMARC) (Rubin *et al.* 2002) which have also been tested in urine (Rogers *et al.* 2004; Zielie *et al.* 2004). Other urine PCa markers are DD3 (PCA3) mRNA which has been standardized in an easy to use platform (APTIMA PCA3 Molecular Urine Test) that retains 69% sensitivity and 79% specificity in men undergoing prostate biopsy (Groskopf *et al.* 2006). This molecular assay has been further evaluated in a multicenter trial in Holland (van Gils *et al.* 2007) and in the United States (Sokoll *et al.* 2008a) where the test has performed similarly.

3.1.1 Prostate-Specific Antigen

Prostate-Specific Antigen (PSA) is a 28 kDa serine protease, member of the tissue kallikrein family. It has a single glycan chain linked to the Asn-45. It is produced primarily by the prostatic epithelium and it is secreted into the lumen of the gland to become a major component of the seminal plasma (0.5-3 mg/ml) (Diamandis 1998).

PSA is secreted as propeptide, which is inactive. The removal of the first seven aminoacids yields to the mature, active PSA which acts upon semenogelin I, semenogelin II and fibronectin

to cause liquefaction of the seminal plasma clot after ejaculation (Lilja *et al.* 1987). Other functions attributed to PSA include antiangiogenic properties (Mattsson *et al.* 2008; Mattsson *et al.* 2009) and as activator of different substrates such as: IGFBP proteins -by releasing IGF-1 (Cohen *et al.* 1994), TGF-beta (Killian *et al.* 1993) and Parathyroid hormone-related protein (Iwamura *et al.* 1996).

PSA is not found in the blood of healthy patients because of its specific location in the prostate. However, prostate cancer patients show a characteristic loss of the basal cell layer of prostate glands which allows PSA contained in the prostatic glands access to the peripheral circulation and be detected in blood (Balk *et al.* 2003).

The predominant molecular PSA form present in blood is the 80-90 kDa complex of PSA with α-1-antichymotrypsin (ACT), and minor fractions of PSA exist as complexes with other protease inhibitors, predominantly α-2-macroglobulin and α-1-antitrypsin (Balk *et al.* 2003). These fractions are called "complexed PSA". Free PSA (non-complexed or unbound PSA) represents a small but variable proportion of the total serum PSA concentration, containing also a mixture of different inactive PSA forms: nicked PSA (mature forms with different broken peptide bonds) and proPSA forms with the N-terminal propeptide truncated at different positions -2, -4, and -5 and -7 proPSAs (Mikolajczyk *et al.* 2002).

The main limitation of PSA is that it is not fully specific for prostate cancer as other prostatic pathologies -benign prostate hyperplasia (BPH) and prostatitis- can show serum PSA elevations (Catalona *et al.* 1994; Bracarda *et al.* 2005). The determination of tPSA level threshold to diagnose PCa is very difficult in terms of both specificity and sensitivity. Catalona et al., 1991 (Catalona *et al.* 1991) reported a 22% incidence rate of prostate cancer when serum PSA levels were between 4 and 10 ng/mL. This rate increased to 67% when serum PSA levels were higher than10 ng/mL. This study and subsequent ones lead to the establishment of the 4 ng/ml threshold. However, there is an appreciable risk of false positives as about 25-50% of BPH patients have tPSA levels higher than 4 ng/ml, which results in unnecessary biopsies (Laguna and Alivizatos 2000). On the other hand, recent studies show an important rate of PCa when tPSA levels are lower than 4 ng/ml (26.9 percent of prevalence among patients with values of PSA from 3.1 to 4.0 ng/mL) (Thompson *et al.* 2004). Thus, it has been suggested it is impossible to choose a tPSA threshold value below which the risk for PCa is zero (Damber and Aus 2008; Loeb and Catalona 2008).

There is an intense debate about the utility of PSA levels on prostate cancer screening (Lilja *et al.* 2008; Barry 2009). While a recent American study based on PSA levels and DRE did not

show any benefit in patients' survival (Andriole *et al.* 2009), another study in the European Union in 180,000 men randomized either to PSA screening every 4 years or to usual care, showed that prostate cancer mortality was reduced by 20%. However, it was associated with a high risk of overdiagnosis (Schroder *et al.* 2009). A subsequent analysis that corrected for contamination calculated the "true" benefit as a 31% reduction (Roobol *et al.* 2009).

In spite of its limitations, PSA is considered a useful marker of prostate pathologies, including prostate cancer, because PSA serum levels provide an early warning of the development and presence of prostate cancer. PSA is also very effective in monitoring after therapy, to follow PCa androgen-independent progression and recurrence after prostatectomy (Schroder *et al.* 2009).

Prostate-Specific Antigen improvement

Different approaches have been developed to improve PSA specificity, mainly in the PSA range 4-10 ng/mL, the traditional PSA "grey zone" where PCa diagnosis is especially controversial. These approaches have investigated different PSA features such as PSA density (Catalona *et al.* 2000), PSA velocity (Smith and Catalona 1994; Moul *et al.* 2007) and different PSA molecular forms such as complex PSA (Brawer *et al.* 1998), free PSA (Luderer *et al.* 1995; Prestigiacomo *et al.* 1996; Catalona *et al.* 2000), Benign Prostate-Specific Antigen (BPSA) (Linton *et al.* 2003) or ProPSAs (Mikolajczyk *et al.* 2004; Sokoll *et al.* 2008b). However, only PSA index (ratio free to total PSA) has been approved by the FDA to differentiate between PCa and BPH patients in the 4-10 ng/mL range (Table 4).

Prostate cancer cells have been described to possess altered glycosylation (see 2.1) which may affect nearly every glycoprotein produced in the malignant cell. Thus, PSA glycosylation has been studied with the aim of improve its specificity for the detection of PCa. The low concentration of PSA in the sera of patients (ng/mL) leaded researchers to use other physiological sources instead of serum in the initial structural studies of PSA glycosylation. Thus, PSA *N*-glycan chain was first characterized from seminal plasma of healthy donors. It was described as a sialylated complex biantennary structure, mostly core fucosylated and with a minor percentage of GalNAc using direct structural techniques such as 1H-Nuclear magnetic resonance (NMR) (Belanger *et al.* 1995) or *N*-glycan sequencing (Okada *et al.* 2001; Ohyama *et al.* 2004). In our group, these structures in seminal plasma PSA were confirmed using *N*-glycan sequencing and Mass spectrometry (MS) and, in addition, using these techniques, oligosaccharides linked to the PSA produced by the Prostate cancer cell line LNCaP were characterized for the first time (Peracaula *et al.* 2003b). This study showed a complete loss of

sialic acid in LNCaP derived PSA together with the presence of H2 structure (absent in the normal PSA) and an increase in the GalNAc content. These observations were consistent with previous chromatofocusing studies where higher pI in tumour-derived PSA (LNCaP or PCa serum PSA) than in healthy PSA (seminal plasma or BPH serum PSA) (Huber *et al.* 1995; Herrala *et al.* 1998; Wu *et al.* 1998)

Our group decided to further investigate PSA glycosylation in patients' sera as this is the source of major clinical significance. In the clinical field, PSA has mainly been analyzed in serum up to date. Recently the analysis of PSA glycosylation from other sources such as urine or seminal plasma has been performed (Jankovic and Kosanovic 2005, White *et al*. 2009) and some authors propose the use of seminal plasma and expressed-prostatic secretion (EPS) fluids (Drake *et al*. 2009) to analyze the different molecular forms of PSA. However, such fluids are not always obtained in the routine clinical practice. In addition, the procedures to get them could cause, in some cases, patients morbidity. For that, serum was considered the analytical PSA source of choice.

Tabares *et al.* 2006 compared by *N*-glycan sequencing PSA glycans from a metastatic PCa patient with a very high PSA and the ones from a healthy donor seminal plasma. The main differences were a decrease in the core fucosylation of PSA *N*-glycans from the PCa patient and also a different proportion of sialic acid α2-3 and α2-6 linked. PSA glycan differences between PCa and BPH patients' sera are very difficult to evaluate using structural techniques as PSA concentration in BPH sera is very low. Thus, other methodologies such as lectin affinity assays have been used (Barak *et al.* 1989; Basu *et al.* 2003; Ohyama *et al.* 2004). As discussed in section 1.6.4, sialylation may be evaluated using indirect techniques such as IEF or 2-DE. Different PSA spots are obtained when it is separated in a 2-DE gel (Charrier *et al.* 1999; Charrier *et al.* 2001; Isono *et al.* 2002; Jung *et al.* 2004). These studies have been mainly focused on fPSA 2-DE subforms, which in serum represent only 10-30% of the tPSA. But tPSA 2-DE subforms, which may be more representative of PSA modifications that occur during the PCa process, have not yet been fully evaluated in sera. Thus we decided to evaluate total PSA 2-DE subforms in the sera of PCa and BPH patients using a cohort of 40 samples (Chapter 3) and also to characterize which were the differences among these subforms that lead them to be segregated as different spots in a 2-DE gel (Chapter 4).

We chose to analyzed serum PSA due, on one hand, to the availability of the serum samples from the Hospital of Girona and, on the other hand, to the results of previous studies by us and others suggesting that glycosylation could be altered in serum PSA from prostate cancer patients.

3.2 Tumour markers in pancreatic cancer

The tumour marker currently used for pancreatic cancer is CA19-9. This marker is named after the monoclonal antibody used to detect it. CA19-9 antibody binds to the carbohydrate epitope SLe^a in sera where is mainly found on large mucin-like molecules (Magnani *et al.* 1983). However, its use has been only approved for monitoring the illness because its use in diagnosis is restricted by its false positive results (Grote and Logsdon 2007; Misek *et al.* 2007). This marker is unable to differentiate pancreatic cancer from benign pancreaticobiliary disorders such as chronic pancreatitis (CP).

Other markers for pancreatic cancer such as MUC1 (Gold *et al.* 2006), CEACAM1 (Simeone *et al.* 2007), or macrophage inhibitory cytokine 1 (MIC-1) (Koopmann *et al.* 2006) have been evaluated. Although some of them have shown better sensitivity than CA19-9, low specificities in distinguishing pancreatic cancers from pancreatitis are still a drawback. Thus, better pancreatic cancer markers still need to be identified as early diagnosis of pancreatic cancer is crucial to increase patients' survival.

As discussed on section 2.2, SLe^x and related Lewis antigens have been found to be overexpressed in PaC cell lines and tissues. Pancreatic cancer sera, but not sera from healthy patients, inhibit E-selectin binding of pancreatic tumour cells (Sawada *et al.* 1994), suggesting an over expression of the sialylated Lewis antigens in the PaC sera. Using glycoprotein microarray with multi-lectin detection, an increase in both fucosylation and sialylation of some serum glycoproteins has been described for PaC patients compared to healthy controls and pancreatitis patients (Zhao *et al.* 2007; Li *et al.* 2009). These data suggested that pancreatic cancer tumour may shed to the blood glycoproteins carrying SLe^x which could be used as PaC tumour markers. We therefore decided to use specific antibodies and *N*-glycan sequencing to investigate altered glycosylation of serum proteins from healthy controls, pancreatic cancer and pancreatitis patients (Chapters 5 and 6).

Chapter 2

Objectives

This work has been focused on the determination of glycan alterations of serum proteins in cancer and their use as tumour markers. For that, several methodologies including serum depletion by immunoaffinity methods, *N*-glycan sequencing, proteomics and specific glycan determination using monoclonal antibodies against carbohydrate antigens have been performed in order to accomplish the two main objectives of the present study:

1. Determine Prostate-specific antigen (PSA) glycoforms that could improve its specificity to distinguish between prostate cancer and benign prostatic hyperplasia patients.
   1.1. Evaluate PSA 2-DE subforms from a cohort of 20 prostate cancer patients and 20 benign hyperplasia patients
   1.2. Characterize at glycan level the different PSA 2-DE subforms.

2. Investigate glycoforms of serum proteins that could be used as markers of pancreatic cancer.
   2.1. Detect serum glycoproteins with increased Sialyl-Lewis X in pancreatic cancer patients.
   2.2. Describe other possible glycan changes in these glycoproteins that could also be used as tumour markers.

Chapter 3

Differential percentage of serum Prostate-specific antigen subforms suggests a new way to improve prostate cancer diagnosis

Differential Percentage of Serum Prostate-Specific Antigen Subforms Suggests a New Way to Improve Prostate Cancer Diagnosis

Ariadna Sarrats,[1] Josep Comet,[2] Glòria Tabarés,[3] Manel Ramírez,[4] R. Núria Aleixandre,[4] Rafael de Llorens,[1] and Rosa Peracaula[1]*

[1]*Unitat de Bioquímica i Biologia Molecular, Departament de Biologia, Universitat de Girona, Campus de Montilivi, Girona, Spain*
[2]*Unitat d'Urologia, Hospital Universitari Dr. Josep Trueta, Girona, Spain*
[3]*Protein Chemistry Department, Roche Diagnostics, Penzberg, Germany*
[4]*Laboratori ICS Girona, Hospital Universitari Dr. Josep Trueta, Girona, Spain*

BACKGROUND. Prostate-specific antigen (PSA) is the tumor marker currently used for prostate cancer (PCa) screening and diagnosis. However, its use is controversial as serum PSA levels are also increased in other non-malignant prostatic diseases such as benign prostatic hyperplasia (BPH). PSA sialic acid content is altered in tumor situation and modifies PSA's isoelectric point (p*I*). Our goal has been to evaluate serum PSA subforms from PCa and BPH patients by two-dimensional electrophoresis (2-DE) and to investigate whether they could be used to improve PCa diagnosis.

METHODS. PSA from 20 PCa and 20 BPH patients' sera was subjected to a four-step method to obtain serum PSA 2-DE subforms from free PSA (fPSA) plus PSA released from the complex with alpha-1-antichymotrypsin. Relative percentages of PSA spots were quantified and subjected to statistical analysis.

RESULTS. Five PSA subforms (F1, F2, F3, F4, and F5) of different p*I* were obtained. Relative percentages of F3 (%F3) and F4 (%F4) were different between PCa and BPH groups. %F3 decreased in cancers and this decrease correlated with the cancer stage, while F4 behaved oppositely. These observations were also found when only focusing on the patients within the low total PSA (tPSA) range 2–20 ng/ml.

CONCLUSIONS. %F3 showed a tendency of higher sensitivity and specificity than the currently used tPSA and %fPSA tests. Therefore, %F3 measurement should be investigated in a larger cohort of patients to study whether it could be introduced to improve PCa diagnosis. *Prostate 70: 1–9, 2010.* © 2009 Wiley-Liss, Inc.

KEY WORDS: **benign prostatic hyperplasia; prostate cancer; prostate-specific antigen; sialic acid; two-dimensional electrophoresis**

Abbreviations: ACT, alpha 1-antichymotrypsin; BPH, benign prostatic hyperplasia; BPSA, BPH associated PSA; CI, confidence interval; fPSA, free PSA; PBS, phosphate-buffered saline; PCa, prostate cancer; PSA, Prostate-specific antigen; ROC, receiver operating characteristics; RT, room temperature; TBST, 0.1% Tween in Tris-buffered saline; TNM, tumor-node-metastasis; tPSA, total PSA.

Grant sponsor: Ministerio de Ciencia e Innovación; Grant numbers: BIO 2004-0438, 2007-61323; Grant sponsor: La Marató de TV3 foundation; Grant number: 050932; Grant sponsor: Government of Catalonia; Grant number: 2005SGR00065; Grant sponsor: Roche Diagnostics (Barcelona, Spain).

*Correspondence to: Dr. Rosa Peracaula, Unitat de Bioquímica i Biologia Molecular, Departament de Biologia, Universitat de Girona, Campus de Montilivi, Girona 17071, Spain. E-mail: rosa.peracaula@udg.edu

Received 1 April 2009; Accepted 13 July 2009
DOI 10.1002/pros.21031
Published online 7 August 2009 in Wiley InterScience (www.interscience.wiley.com).

Ariadna Sarrats, Josep Comet, Glòria Tabarés, Manel Ramírez, R. Núria Aleixandre, Rafael de Llorens, Rosa Peracaula. “Differential percentage of serum prostate-specific antigen subforms suggests a new way to improve prostate cancer diagnosis”. *The prostate*. Vol. 70, Issue 1 (January 2010) : p. 1-9

Article first published online: 7 AUG 2009

http://dx.doi.org/ 10.1002/pros.21031

http://onlinelibrary.wiley.com/doi/10.1002/pros.21031/abstract

Chapter 4

Glycan characterization of total PSA 2-DE subforms from serum and seminal plasma

OMICS A Journal of Integrative Biology
Volume 14, Number 4, 2010

DOI: 10.1089/omi.2010.0050

Glycan Characterization of PSA 2-DE Subforms from Serum and Seminal Plasma

Ariadna Sarrats,[1] Radka Saldova,[2] Josep Comet,[3] Niaobh O'Donoghue,[2] Rafael de Llorens,[1] Pauline M. Rudd,[2] and Rosa Peracaula[1]

Abstract

Prostate-specific antigen (PSA) two-dimensional electrophoresis (2-DE) subforms (F1–F5) have been described to be altered in prostate cancer (PCa) compared to benign prostatic hyperplasia (BPH). To understand their molecular differences, characterization of these subforms from PCa serum and seminal plasma, namely, at the glycan level, was performed. PSA 2-DE subforms from two serum PCa samples and seminal plasma were analyzed by *N*-glycan sequencing using high-performance liquid chromatography (HPLC) combined with exoglycosidase array digestions and by mass spectrometry. F1, F2, and F3 subforms showed the same *N*-glycan pattern, which contained higher levels of sialic acid than the F4 subform, whereas the F5 subform was unglycosylated. When comparing PSA subforms from PCa with seminal plasma, a decrease in sialylation was observed. Furthermore, the analysis of F3, the more abundant PSA subform, showed a higher proportion of alpha 2–3 sialic acid and a decrease in core fucosylated glycans in the PCa sample. These *N*-glycan changes in PCa PSA subforms highlight the importance of glycosylation as an indicator of PCa disease.

Introduction

Prostate-specific antigen (PSA) is the tumor marker currently used in prostate cancer (PCa) screening and diagnosis. However, its use is controversial, as serum PSA levels are also increased in other nonmalignant prostatic diseases such as benign prostatic hyperplasia (BPH) (Bracarda et al., 2005). To address this issue, there have been several attempts to improve PSA specificity focusing on the measurement of different forms of PSA found in serum.

PSA is a glycoprotein produced by the prostatic epithelium and it is secreted as a proenzyme (proPSA) into the lumen of the prostate gland where it is activated. It is a normal component of seminal plasma where it is present at relatively high concentrations (0.5–3 mg/mL) (Diamandis, 1998). In prostate disease, the basement membrane can be disrupted, and PSA can access the peripheral circulation and be detected in the blood. The predominant molecular PSA form found in blood is complexed with protease inhibitors, mainly alpha-1-antichymotrypsin (ACT) (Balk et al., 2003). The remaining PSA forms are inactive and circulate as free PSA (fPSA). These include proPSA forms and internally cleaved PSA such as BPSA (BPH associated PSA) (Mikolajczyk et al., 2002).

Measurements of complex PSA (Brawer et al., 1998), fPSA (Catalona et al., 2000), specific free PSA forms such as proPSA (Mikolajczyk et al., 2004) and BPSA (Linton et al., 2003) have been studied to improve the specificity of PCa detection. However, only the percentage of PSA circulating in the free form (%fPSA) has been widely used by clinicians as a diagnostic tool in patients with low tPSA levels (Loeb and Catalona, 2008).

Other studies to improve the specificity of PSA in PCa diagnosis have focused on some of the biochemical characteristics of PSA. For example, glycosylation of PSA has been reported to be altered in the presence of tumors. In fact, secreted glycoproteins can reflect the changes in glycosylation pattern that usually take place on the tumor cells surface (Peracaula, 2007; Varki et al., 2008). Several authors have described modifications in the sialylation pattern of PSA derived from malignant origins compared with that from healthy individuals (Huber et al., 1995; Ohyama et al., 2004; Peracaula et al., 2003; Tabares et al., 2006). Sialic acid is a negatively charged carbohydrate. An increase or decrease in the content of this monosaccharide on a PSA *N*-glycan chain can modify the PSA's isoelectric point (pI). Two-dimensional gel electrophoresis has been used to evaluate sialic acid variations in PSA from different sources (Charrier et al., 1999; Isono et al., 2002; Jung et al., 2004; Tabares et al., 2007). Most of these studies have focused on fPSA 2-DE subforms, which represent only 10–30% of the total serum PSA. To enable the

[1]Unitat de Bioquímica i Biologia Molecular, Departament de Biologia, Universitat de Girona, Girona, Spain.
[2]Dublin–Oxford Glycobiology Laboratory, NIBRT, Conway Institute, University College Dublin, Belfield, Dublin, Ireland.
[3]Unitat d'Urologia, Hospital Universitari Dr. Josep Trueta, Girona, Spain.

analysis of all serum PSA subforms, we developed a sensitive and precise method to evaluate PSA 2-DE subforms from PCa and BPH sera, which included an initial ethanolamine treatment step to release PSA bound to ACT. Five individual PSA 2-DE subforms (called F1, F2, F3, F4, and F5) were detected and some were significantly altered between BPH and PCa patients. Compared to BPH, the relative percentage of F3 (%F3) negatively correlated with the stage of cancer, while the relative percentage of F4 (%F4) correlated positively with the stage of cancer. %F3 demonstrated a tendency for higher specificity and sensitivity than the currently used tPSA and % fPSA tests to distinguish between the two groups of patients (Sarrats et al., 2010).

The aim of the present study has been to unravel the biochemical differences among these PSA subforms (F1, F2, F3, F4, and F5) to develop future clinical assays to directly measure %F3. Thus, we have analyzed PSA 2-DE subforms from two prostate cancer patients' sera and also from seminal plasma from a healthy donor using *N*-glycan sequencing and mass spectrometry. Unfortunately, the biochemical analysis of PSA 2-DE subforms from a BPH patient was not possible due to the fact that BPH sera do not reach high enough PSA levels to allow *N*-glycan sequencing analysis. Seminal plasma was therefore taken as a source of healthy PSA.

These analyses have enabled us to characterize differences among the PSA 2-DE subforms (F1–F5) in each of the samples and to compare the glycosylation pattern between the samples originating from healthy and malignant origins.

Materials and Methods

PSA samples

Sera from two PCa patients (PCa A and PCa B) with high tPSA content (above 2.5 μg/mL) and seminal plasma from a healthy donor (~0.5 mg/mL fPSA) were obtained from Hospital Universitari, Dr. J. Trueta (Girona, Spain) following the standard operating procedures of its Ethics Committee. PCa patients were diagnosed by the Urology unit using transrectal ultrasound-guided biopsy and both showed multiple bone metastases detected by positron emission tomography and axial computed tomography.

PSA sample pretreatments and analyses are summarized in Figure 1.

Serum sample pretreatment

Serum PSA complexed to ACT was released using a previously published method with modifications (Peter et al.,

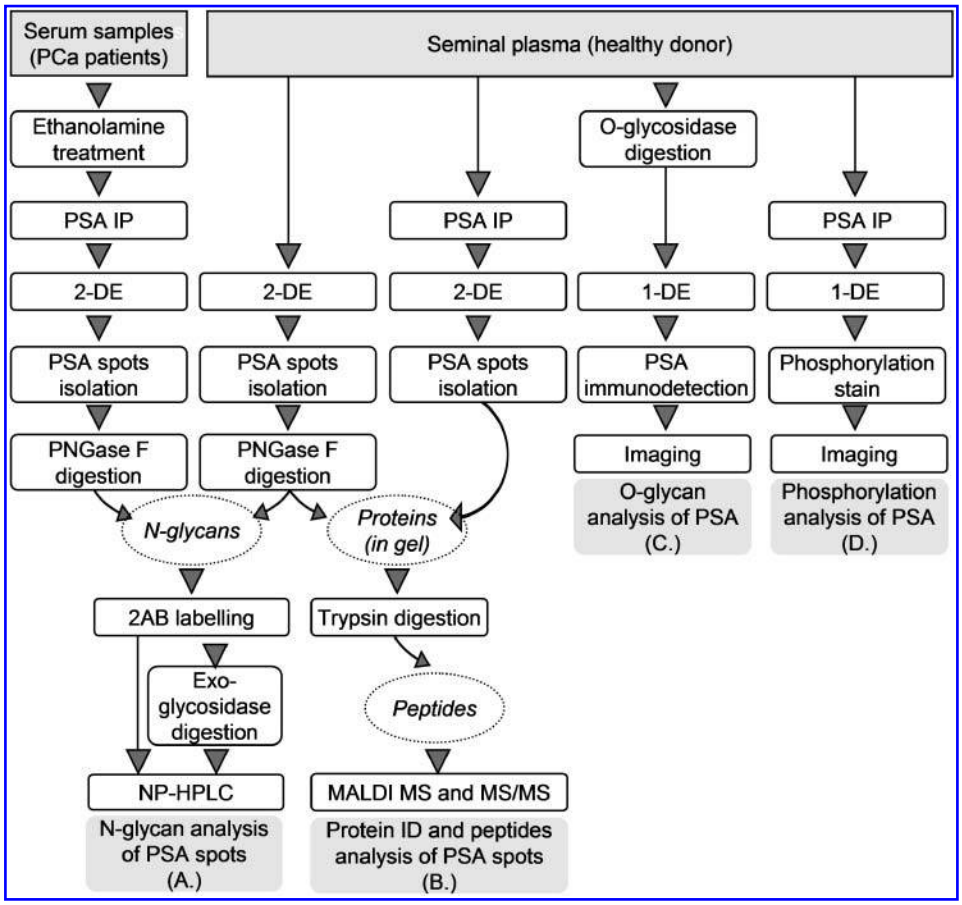


FIG. 1. Schematic illustration of workflow used in this work to obtain and characterize PSA 2-DE subforms (**A,B**) and to study PSA *O*-glycosylation (**C**) and phosphorylation (**D**). PSA IP, PSA immunopurification; 1-DE, one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; 2AB, 2-aminobenzamide; NP-HPLC, normal-phase HPLC; Protein ID, protein identification.

2000). A solution of 2,565 μL phosphate-buffered saline (PBS) and 265 μL 2 M ethanolamine (pH 12) was added to 2,850 μL of serum (up to a final pH of 10.3) and incubated at 25°C for 24 h. Before PSA isolation, the mixture was neutralized with 0.5 M HCl.

Total PSA was isolated by modification of a previously described direct immunoadsorption method (Peter et al., 1999). A suspension of 6 mL of 0.72 g/L streptavidin-coated magnetic beads was washed three times with 6 mL of washing buffer (50 mM Tris, 150 mM NaCl, pH 7.4, 1% Triton X-100) using magnetic separation. The beads were then incubated for 30 min at room temperature with slight shaking with 3 mL of the biotinylated mouse monoclonal antibody anti-tPSA M-36 (Roche Diagnostics, Germany), in a final concentration of 33.3 μg/mL dissolved in incubation buffer (50 mM Tris, 150 mM NaCl, pH 7.4, 0.1% Tween-20, 1% BSA). Then, the beads were washed as mentioned above and incubated for 1 h with 5,700 μL of neutralized treated serum (containing 2,850 μL of original serum). Afterward, the beads were washed as already described and the immunoadsorbed PSA was eluted with 360 μL of 2-DE rehydration buffer (8 M urea, 0.5% Triton X-100, 13 mM DTT, 1% Pharmalite 3-10 (Amersham Pharmacia Biotech, Germany), traces of Bromophenol blue) for 1 h.

Seminal plasma sample pretreatment

PSA from seminal plasma for MS analysis purposes was isolated using the procedure described above with some modifications: 4 mL of 0.72 g/L streptavidin-coated magnetic beads solution were washed three times with 4 mL of washing buffer and incubated with 2 mL of the biotinylated mouse monoclonal antibody anti-tPSA M-36 (Roche Diagnostics) (33.3 μg/mL in incubation buffer). Then, the beads were washed as mentioned above and incubated for 1 h with 4 mL of 1/20 diluted seminal plasma (in incubation buffer). Afterward, the beads were washed as already described and the immunoadsorbed PSA was eluted with 360 μL of 2-DE rehydration buffer for 1 h.

One milliliter of 1/10 diluted seminal plasma was also immunoadsorbed for phosphorylation analysis using the same protocol as above but the elution step was performed with 50 μL of 1×gel-loading buffer (30 mM Tris-HCl pH 6.8, 10% (v/v) glycerol, 2% SDS, 1.25% β-mercaptoethanol, 0.01% Bromophenol blue).

Two-dimensional electrophoresis (2-DE)

2-DE was performed according to the procedure we previously described (Sarrats et al., 2010). Active in-gel rehydration was effectuated with Immobiline dry strips pH 3–10, 18 cm (GE Healthcare, Sweden) using the 360 μL of the immunopurified PSA samples eluted in 2-DE rehydration buffer or 50 μL of seminal plasma diluted in 360 μL of 2-DE rehydration buffer. Following electrophoresis, 2-DE gels were stained by Coomassie blue and PSA spots were excised, cut into 1 mm^3 pieces and kept at −20°C until analysis (Royle et al., 2006).

The pI assignment to each PSA spot was calculated by measuring the spot distance to the anodic edge and transforming it into a pI value according to the relation between distance and pI supplied by the manufacturer.

N-glycans analysis

N-Glycans release, extraction and 2-aminobenzamide (2-AB) labeling. *N*-Glycans were released and extracted from the 1-mm^3 gel pieces of PSA spots according to the procedure described by Royle et al. (2006). Briefly, the gel pieces were washed and treated with PNGaseF to release the *N*-linked glycans. Afterward, *N*-glycans were fluorescently labeled with 2-aminobenzamide (2AB) by reductive amination (Bigge et al., 1995) (LudgerTag 2-AB labeling kit LudgerLtd, Abingdon, UK).

Simultaneous oligosaccharide sequencing by exoglycosidase digestions. The 2AB-labeled 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, USA) at the indicated concentrations: ABS—*Arthrobacter ureafaciens* sialidase (EC 3.2.1.18), 0.5 U/mL; NAN I— *Streptococcus pneumoniae* sialidase (EC 3.2.1.18), 1.7 U/mL; BTG—Bovine testes β-galactosidase (EC 3.2.1.23), 1 U/mL; SPG—*Streptococcus pneumoniae* β-galactosidase (EC 3.2.1.23), 0.1 U/mL; BKF—bovine kidney alpha-fucosidase (EC 3.2.1.51), 1 U/mL; GUH—*Streptococcus pneumoniae* β-N-acetylglucosaminidase, recombinant in *Escherichia coli* (EC 3.2.1.30), 8 U/mL. After incubation, enzymes were removed by filtration through a protein-binding EZ filters (Millipore Corporation, Bedford, MA, USA) (Royle et al., 2006).

Normal-phase high-performance liquid chromatography (NP-HPLC). Undigested and digested 2-AB-labeled *N*-glycans were subjected to NP-HPLC using a TSK-Gel Amide-80 4.6×250 mm column (Anachem, Luton, UK) on a 2695 Alliance separations module (Waters, Milford, MA, USA) equipped with a Waters temperature control module and a Waters 2475 fluorescence detector. 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. Gradient conditions were a linear gradient of 20–58% A, over 152 min at a flow rate of 0.4 mL/min. Samples were injected in 80% acetonitrile. Fluorescence was measured at 420 nm with excitation at 330 nm. The system was calibrated using an external standard of hydrolyzed and 2AB-labeled glucose oligomers to create a dextran ladder. Glycans were analyzed on the basis of their elution positions measured in glucose units (GU) (Royle et al., 2006).

Matrix-assisted laser desportion/ionization-time of flight (MALDI-TOF) mass spectrometry

Seminal plasma PSA 2-DE spots were in-gel digested with trypsin (sequencing grade modified, Promega Biotech Iberica, Spain) in the automatic Investigator ProGest robot (Genomic Solutions, Holliston, MA, USA). Briefly, excised gels spots were washed with ammonium bicarbonate buffer (50 mM NH_4HCO_3) and acetonitrile. Proteins were reduced with 10 mM DTT solution during 30 min, and alkylated with a 55 mM solution of iodine acetamide. After the washings with buffer and acetronitrile, proteins were digested overnight, at 37°C with 0.27 nmol of trypsin. Tryptic peptides were extracted from the gel matrix with 10% formic acid and acetonitrile; the extracts were pooled and dried in a vacuum centrifuge.

Proteins were analyzed in a MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems, IL, USA) mass spectrometer. The digests were redissolved in 0.1% trifluoroacetic acid in 50% acetonitrile. Typically 0.5 to 1.0 μL of sample was mixed with the same volume of a matrix solution (2–5 mg/mL α-cyano-4-hydroxycinnamic acid in 50% MeCN, 0.1–0.2% trifluoroacetic acid) and spotted to the MALDI plate. MS spectra were acquired in positive reflector mode (voltage of 20 kV in the source 1 and laser intensity ranged from 5,800–6,200). Typically, 500 shots per spectrum were accumulated. MS/MS spectra were acquired using collision-induced dissociation with atmospheric air as the collision gas. A MS-MS 1-kV positive mode was used.

MS and MS/MS spectra from the same spot were merged in a single mascot generic file (mgf) file prior to submission for database searching. Mgf files were submitted for database searching in a MASCOT search engine against nonredundant SwissProt database. The search parameters were: human taxonomy, one missed cleavage allowed, carbamidomethyl of cystein as fixed modification, oxidation of methionine and deamidation of asparagine or glutamine as variable modifications. Peptide tolerance was 100 ppm and 0.25 Da, respectively, for MS and MS/MS spectra. Only proteins with scores above significant Mascot level were considered as positive hits.

O-Glycosylation analysis

To investigate the presence of *O*-glycans attached to PSA, glycoproteins from seminal plasma (healthy donor) were digested with ABS plus *O*-glycosidase. ABS is used to release sialic acid residues which block *O*-glycosidase to access to the oligosaccharide chain. ABS digestion and a negative control without digestion (buffer without enzymes) were also performed. Both enzymes were purchased from Roche.

A total of 4.5 ng of PSA (9 μL of 1/1,000 diluted seminal plasma) were incubated in 10 μL of 0.1% SDS for 10 min at 70°C. Then, the solution was cooled down for 3 min at 4°C. After that, ABS (1 U/mL) or ABS (1 U/mL) with O-glycosidase (50 mU/mL) digestions were performed in 20 μL of 100 mM sodium acetate buffer, pH 5.5, 1% Triton X-100 for 18 h at 37°C. For the negative control of the digestion the volume of enzymes were substituted for water.

The products of the digestions were run on a 15% SDS-PAGE gel and then transferred to a PVDF membrane. PSA was immunodetected as previously described (Sarrats et al., 2010).

Phosphorylation analysis

Analysis of phosphorylation was performed using the ProQ-Diamond phosphoprotein gel stain (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA), which detects phosphate groups attached to serine, threonine, and tyrosine residues of proteins (Schulenberg et al., 2004). A total of 25 μg of PSA was immunopurified and electrophoresed together with 1 μL of PipermintStick phosphoprotein molecular weight standards (Molecular Probes, Invitrogen), which contains a mixture of two phosphorylated and four nonphosphorylated proteins. Thus, the standards serve both for as molecular weight markers and as positive and negative controls for the phosphoprotein gel stain. The gel was stained according to the standard protocol for minigels supplied by the manufacturer and visualized by a UV transilluminator. Then, the gel was Coomassie stained to detect all protein bands present.

Results

N-Glycan analysis of PSA 2-DE subforms

PSA from seminal plasma (healthy donor) and from two serum samples (PCa A and PCa B) was separated by 2-DE and stained with coomassie to obtain PSA 2-DE subforms. The serum samples were previously subjected to ethanolamine treatment to release PSA bound to alpha-1-antichymotrypsin (ACT), and then immunopurified. Five spots corresponding to F1, F2, F3, F4, and F5 subforms of PSA could be observed. Their experimental pIs were: 6.4, 6.6, 6.8, 7.0, and 7.2 respectively. However, F5 could not be detected in any of the serum samples analyzed (Fig. 2).

These spots were excised from the 2-DE gels and treated with PNGaseF. *N*-Glycans released were 2-AB labeled and subjected to NP-HPLC. Four main peaks (3–6) were present in the *N*-glycan profiles from the seminal plasma PSA and the PCa A serum PSA sample (Fig. 3). *N*-Glycan assignments of these peaks were performed by exoglycosidase digestions (data not shown). Relative areas and structures assigned to each peak are summarized in Table 1. Peak glucose units (GUs) corresponded to those previously described for the whole PSA *N*-glycans from seminal plasma and a PCa

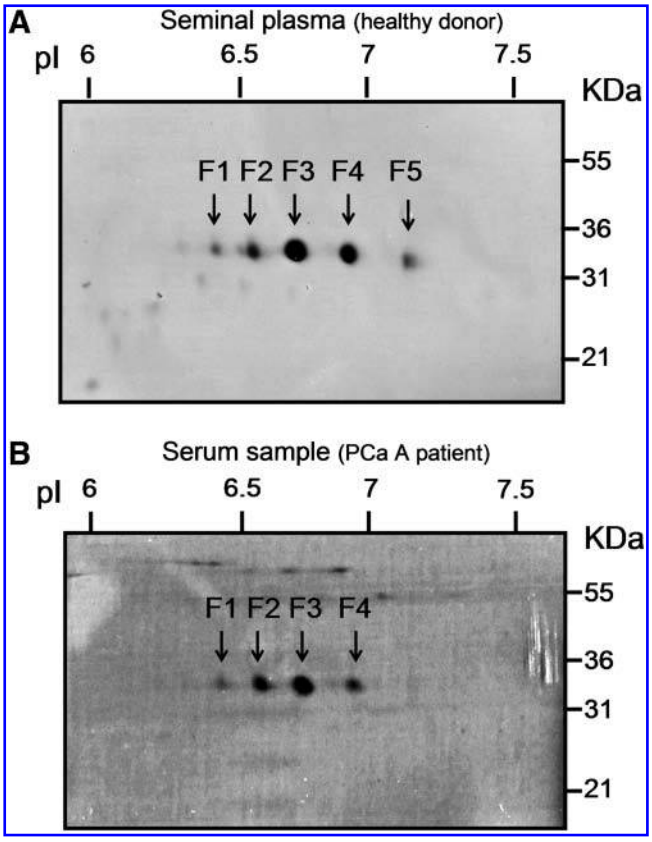


FIG. 2. PSA 2-DE subforms of seminal plasma from a healthy donor (**A**) and of a serum from a prostate cancer patient (PCa A) (**B**). Figures correspond to immunopurified samples.

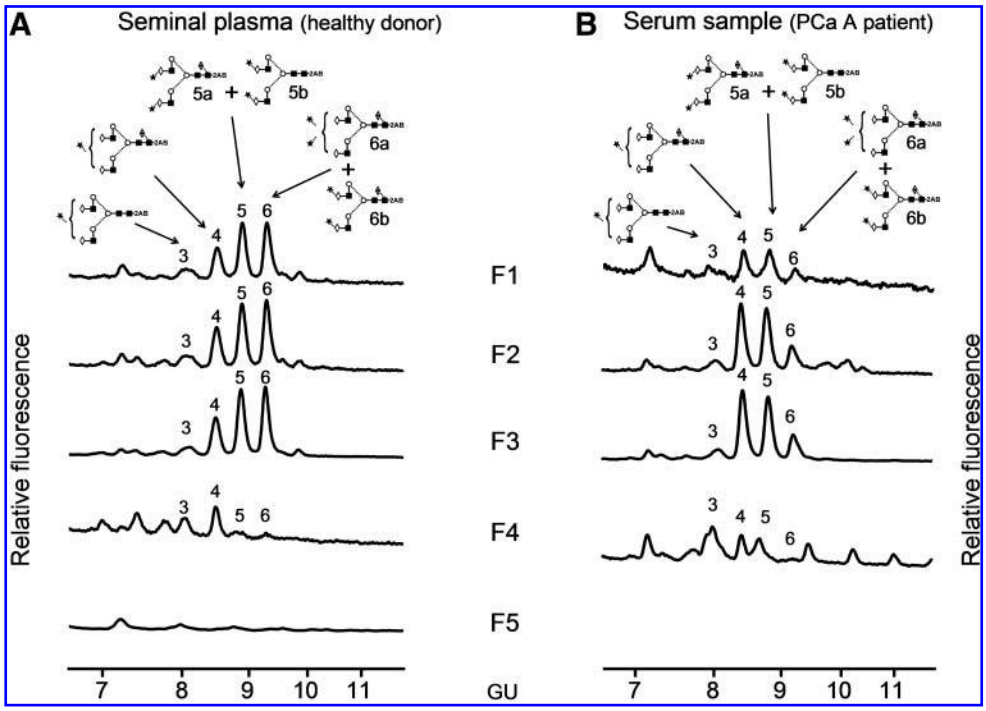


FIG. 3. NP-HLPC chromatograms of *N*-glycans released from each PSA subform (F1–F5) of (**A**) seminal plasma from a healthy donor and (**B**) serum from a prostate cancer patient (PCa A). See Table 1 for key legend of glycan symbols representation.

patient (Tabares et al., 2006). Spots F1, F2, and F3 contained mono- and disialylated complex biantennary structures, F4 contained mainly monosialylated complex biantennary structures and F5 was found not to be *N*-glycosylated (Fig. 3). This is in agreement with the lower molecular weight that F5 showed in the 2-DE gel (Fig. 2A) and with previous data from Isono et al. (2002). In this article, spots corresponding to F1 to F4 positively stained with Periodic acid-Schiff (PAS) carbohydrate stain, whereas spot F5 did not stain, consistent with its unglycosylated status.

F1, F2, and F3 had basically the same peak areas, and consequently, the same proportion of mono- and disialylated structures. Therefore, the sialic acid content in the *N*-glycan chains of F1, F2, and F3 cannot account for their different pI. The proportion of mono- and disialylated structures varied between the seminal plasma and the serum samples. F1, F2, and F3 seminal plasma subforms had a higher proportion of disialylated structures (~70%) compared to serum ones (~50%) (Table 1).

PCa B serum sample contained less amount of PSA and F2, F3, and F4 could only be analyzed by ABS digestion (data not shown). F2 and F3 had the same profile and contained a higher amount of sialylated glycan structures than F4 in agreement with the above results obtained for PCa A serum and seminal plasma subforms.

The more abundant subform F3 was studied in more detail to investigate the type of sialic acid linkage and its core fucose content, because these have been reported to differ in PSA from benign and malignant origins (Peracaula et al., 2003, Tabares et al., 2006). Thus, F3 from PCa A and from seminal plasma was digested with NANI and ABS (Fig. 4) to study sialic acid linkages and core fucosylation, respectively. The digestion with NANI showed a higher proportion of alpha 2–3 sialic acid in F3 from the prostate cancer sample compared to that from the seminal plasma one (53 vs. 22%).

ABS, at the working concentration, may partially digest *O*-acetylated sialic acids. In our analyses all the peaks in the NP-HPLC profile of *N*-glycans extracted from the PSA subfoms were digested after ABS treatment; therefore, we could say that is likely that PSA *N*-glycans do not contain *O*-acetylated sialic acids, although MS analysis would be necessary to confirm it.

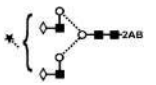
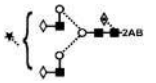
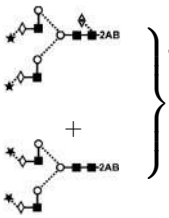
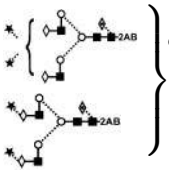
After ABS digestion, two peaks were observed and could be assigned to A2G(4)2 (peak 1) and to F(6)A2G(4)2 (peak 2) after a complete set of exoglycosidases (Fig. 4). The relative area of peak 2 (F(6)A2G(4)2) was taken as measure of core fucosylation content. In the seminal plasma sample, core fucosylated structures were more abundant than in the PCa sample A (66 vs. 46%).

MALDI-TOF MS analysis of PSA 2-DE subforms

Analysis of peptide mass fingerprints was performed on the protein spots of the 2-DE seminal plasma gel after *N*-glycans release. Data confirmed that PSA was the only glycoprotein significantly present in any of the spots and so it was assumed that all *N*-glycans released were exclusively from this glycoprotein.

Further MS analysis of the PSA spots was performed to investigate possible modifications in the primary PSA sequence that could explain the distinct pI of the different PSA subforms, namely, for F1, F2 and F3. For this purpose, PSA from seminal plasma was first immunopurified and then separated in a 2-DE gel. PSA spots were excised from the gel and analyzed by MALDI-TOF/TOF. PSA was the only protein identified in each spot and neither modifications of the

Table 1. *N*-Glycans in PSA Subforms from Seminal Plasma (Healthy Donor) and Serum (PCa A Patient)

			A. Seminal Plasma (healthy donor)					*B. Serum sample (PCa A patient)*				
Peak ID[a]	*Assigment*[b]	*Structure*[c]	*GU*[d]	*Peak area (%)*				*GU*[4]	*Peak area (%)*			
				F1	F2	F3	F4		F1	F2	F3	F4
3	A2G(4)2S(6)1		8.06 ± 0.03	10	9	7	31	8.03 ± 0.07	18	9	8	46
4	F(6)A2G(4)2S(6)1		8.49 ± 0.01	20	22	22	48	8.45 ± 0.02	31	40	40	21
5	F(6)A2G(4)2S(3)2 *(5a)* + A2G(4)2S(6)2 *(5b)*		8.86 ± 0.04	33	33	35	13	8.81 ± 0.06	39	38	37	30
6	F(6)A2G(4)2S(3,6)2 *(6a)* + F(6)A2G(4)2S(6)2*(6b)*		9.26 ± 0.01	36	37	37	7	9.23 ± 0.02	12	13	16	3
3 + 4	% Monosialylated glycans			30	31	28	80		49	49	47	67
5 + 6	% Disialylated glycans			70	69	72	20		51	51	53	33

[a]Peak ID relates to Figures 3 and 4.
[b]A2 biantenary; G(4), galactose (β1–4 linked); F(6) at the start of the abbreviation indicates fucose linked α1–6 to core GlcNAc; M, mannose; S, sialic acid; sialic acid linkage: (3), α2–3 and (6), α2-6.
[c]Symbol representation of glycans for this table and Figures 3 and 4: GlcNAc, filled square; mannose, open circle; galactose, open diamond; fucose, open diamond with a dot inside; sialic acid, filled star; beta linkage, solid line; alpha linkage, dotted line; unknown linkage, ~ ; the linkage itself is indicated by the angle linking adjacent residues, thus 1–4-linkage, horizontal line (-); 1–3-linkage, angled line (/); 1–6-linkage, angled line (\); 1–2-linkage, vertical line (|).
[d]GU values of are shown as means ± SD of all spots.

primary sequence nor deamidations of asparagines or glutamines were detected. PSA peptides identified in each spot are shown in Table 2. Peptides containing asparagines or glutamines did not show any shift of mass from the calculated one, and most of them were sequenced by MS/MS confirming that no deamidations had taken place.

O-*Glycan analysis of PSA*

PSA *N*-glycosylation has been investigated by different authors (Okada et al., 2001; Peracaula et al., 2003; Tabares et al., 2006; Tajiri et al., 2008). However, no evidence of O-linkages has been reported. To investigate whether *O*-glycans could be attached to PSA and thereby contribute to the observed differences in subforms pI, a Western blot of PSA from seminal plasma digested with a combination of sialidase (ABS) and *O*-glycosidase was performed and compared to that digested with sialidase only. The bands of PSA observed for the two digested samples showed the same molecular mass, which was lower than the undigested PSA sample (data not shown). The fact that *O*-glycosidase did not contribute to the decrease of PSA molecular mass suggests that *O*-glycans are most likely not present in PSA from seminal plasma.

Phosphorylation analysis of PSA

Analysis of phosphorylation was performed using the ProQ-Diamond phosphoprotein gel stain following electrophoresis of immunopurified PSA. Neither PSA nor negative controls stained positively for the presence of phospho-forms, whereas positive controls did, as expected. All control proteins and PSA bands were subsequently visualized using Coomassie staining. The intensity of Coomassie stained PSA was higher than any of the phosphoproteins controls, suggesting that the absence of signal for phosphorylated PSA was not due to limitations in the amount of protein sample (data not shown).

Discussion

Analysis of PSA glycosylation from sera is a challenging work as some of the differences between BPH and PCa serum PSA could be due to a different glycosylation pattern of this molecule and could therefore be exploited to improve specificity in PCa diagnosis. The low amount of PSA in sera is, however, the limiting step to fully characterize the glycan structures of PSA. So far, few groups have reported the detailed glycan structures of serum PSA from PCa patients (Tabares et al., 2006; Tajiri et al., 2008). Taking into account that the PSA 2-DE subforms (F1–F5) show different proportions between BPH and PCa sera (Sarrats et al., 2010), in this work we have characterized by *N*-glycan sequencing these 2-DE spots of PSA samples from PCa patients' sera. We also performed the *N*-glycan analysis of the corresponding F1–F5 PSA subforms from seminal plasma to compare them with those originating from PCa.

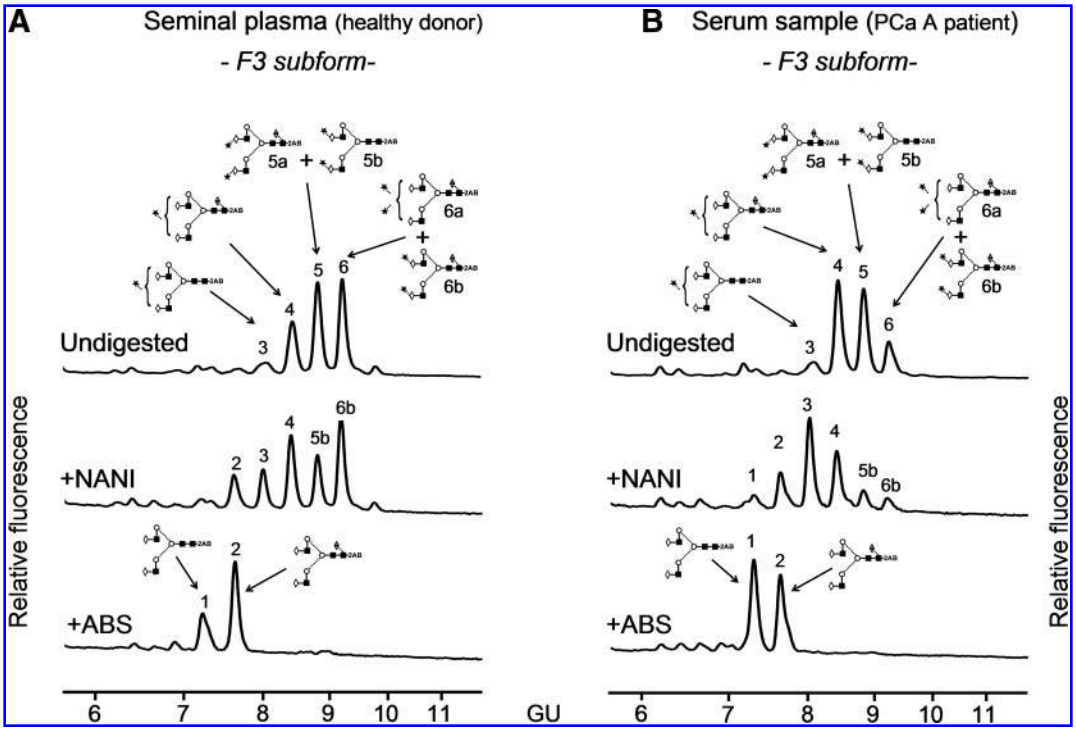


FIG. 4. NP-HPLC chromatograms of *N*-glycans after sialidase digestions with NANI and ABS from the F3 PSA subform of (**A**) seminal plasma from a healthy donor and (**B**) serum from a prostate cancer patient (PCa A). See Table 1 for key legend of glycan symbols representation.

Characterization of PSA 2-DE subforms

The *N*-glycan analysis of PSA subforms from both seminal plasma and PCa patients' sera showed that F1, F2, and F3 exhibited the same *N*-glycan pattern, which differed from F4 and F5. This may account for some of the differences in F1–F5 pI. The different content of sialic acid in the *N*-glycans from F3, F4, and F5 PSA subforms correlated with their different experimental pI detected by 2-DE. The subform F5 did not appear to be glycosylated and had a pI of 7.2, which corresponds to the theoretical one for PSA (7.26). F4 had a lower pI (7.0), which can be explained by the presence of mainly monosialylated *N*-glycans that contain negative charge. The F3 subform showed an even lower pI (6.8). It contains a much higher proportion of disialylated *N*-glycans, which would again contribute to this further decrease in pI. However, F1, F2, and F3 subforms all showed the same *N*-glycosylation pattern despite having different pI values (6.4, 6.6 and 6.8, respectively). Therefore, it seems likely that factors other than *N*-glycosylation may be modifying the theoretical pI of PSA (7.26), such as changes in the primary sequence or different posttranslational modifications (PTM). Deamidation of asparagines or glutamines have been suggested as modifications responsible for the spot trains of some serum proteins obtained in a 2-DE gel (Sarioglu et al., 2000). The deamidation process generates negatively charged aspartic and glutamic acid residues, which can modify a protein's pI. However, in the MS analysis of the seminal plasma PSA

Table 2. PSA Peptides Identified in Seminal Plasma PSA Spots

Peptide[a]	*Sequence*	*Cal. mass*[b]	*F1* *Obs. mass*[c]	*F2* *Obs. mass*[d]	*F3* *Obs. mass*[c]	*F4* *Obs. mass*[c]	*F5* *Obs. mass*[c]
25–33	IVGGWECEK	1,077.5033		1,077.4530		1,077.4723	1,077.4814 †
34–45	HS**Q**PW**Q**VLVASR	1,407.7491	1,407.8263 †	1,407.6949 †	1,407.7418 †	1,407.7126 †	1,407.7264 †
110–125	FLRPGDDSSHDLMLLR	1,887.9381	1,888.0737	1,887.8724 †	1,887.9420 †	1,887.9043 †	1,887.9192 †
48–68	AVCGGVLVHP**Q**WVLTAAHCIR	2,344.2165	2,344.4065	2,344.1399 †	2,344.2327 †	2,344.1682 †	2,344.1848 †
171–191	L**Q**CVDLHVIS**N**DVCA**Q**VHP**Q**K	2,460.2122					2,460.2205
25–45*	IVGGWECEKHS**Q**PW**Q**VLVASR	2,466.2346				2,466.1799	
170–191*	KL**Q**CVDLHVIS**N**DVCA**Q**VHP**Q**K	2,588.3071		2,588.2617 †	2,588.3479 †	2,588.3013 †	2,588.2996 †
138–169	VMDLPT**Q**EPALGTTCYASGW GSIEPEEFLTPK	3,540.6548					3,540.7874

[a]1 trypsin miss cleavage is indicated with *.
[b]Calculated mass of the peptides with carbamidomethylated cysteines and oxidated methionines.
[c]Observed mass of the peptides in the MALDI-TOF analysis. Peptides sequenced by MS/MS are marked with †.

subforms, neither modifications of the primary sequence nor deamidations were detected. *O*-glycosylation was investigated as a possible contributing factor to the differences in the subforms pI. However, in our hands, *O*-glycan modifications were not detected in PSA. Phosphorylation has also been described (along with glycosylation) as one of the most common PTM that can contribute to changes in a protein's pI (Gorg et al., 2004). The phosphorylation status of PSA was therefore analyzed, but no phosphorylated residues were detected. Other authors have suggested that the presence of proPSA forms are constituents of the total free PSA subforms detected in the serum by 2-DE. Tabares et al. (2007) reported the presence of proPSA forms in some of the free PSA serum 2-DE subforms. However, in our PSA 2-DE subforms from serum, the contribution from proPSA would be quite small as the majority of the PSA analyzed corresponded to active PSA previously bound to ACT. In addition, it is well known that PSA from seminal plasma does not contain proPSA forms (Mikolajczyk et al., 2002) and we detected the same five PSA subforms (F1–F5) that in serum. Taken together, the contribution of proPSA to the detected spot pattern can be ruled out.

In summary, differences in *N*-glycosylation partially explain the different isoelectric points observed for the PSA subforms, although the modifications that lead to different pIs between F1, F2, and F3 remain unknown.

N-*Glycosylation of PSA 2-DE subforms in prostate cancer: Changes in sialylation and fucosylation*

In the present study we describe a decrease of disialylated *N*-glycans in F4 compared to those in F3 for both seminal plasma and serum samples. Taking into account that the pIs of these subforms are the same for PSA samples from PCa serum, BPH serum, and seminal plasma (Sarrats et al., 2010) the decrease in sialylation described for the PSA F4 subform is likely to occur also in the corresponding F4 subform from BPH serum. When comparing the percentages of these subforms in BPH and PCa sera, a decrease in F3 and an increase in F4 in PCa patients sera compared to BPH patients sera has been reported (Sarrats et al., 2010). Taken together, a reduction in sialylation of serum PSA in PCa patients compared to that in BPH patients is suggested. In support of this hypothesis, PSA from BPH was described as possessing increased sialylation when compared with that from PCa and LNCaP in the work by Huber et al. (1995). The latter used chromatofocusing techniques combined with sialidase digestion. Recently, Meany et al. (2009) developed five lectin immunosorbant assays to analyze sialylation of total and free serum PSA. SNA and MAA lectin assays for total PSA showed higher electrochemiluminescence intensities in BPH patients than PCa patients, suggesting a higher sialylation of total serum PSA in BPH than in PCa patients.

Our previous results indicated a negative correlation between the percentage of F3 and a positive correlation between the percentage of F4 and the PCa stage (Sarrats et al., 2010). Thus, the potential decrease in PSA associated sialylation might be more marked as the cancer becomes more advanced. In fact, in the prostate cancer cell line LNCaP, which was derived from a node metastasis, a complete absence of sialylated structures was reported for PSA (Peracaula et al., 2003). A decrease in SNA reactivity on free PSA oligosaccharide chain has also been reported in PCa patients with metastatic tumors compared to those with localized tumors, suggesting a relationship between the progression of this cancer and the level of sialic acid (Kosanovic and Jankovic, 2005).

A general decrease in sialic acid content can be observed when comparing the percentage of mono/disialylated structures between PSA spots from PCa patient serum with those from healthy donor's seminal plasma. Seminal plasma PSA contains a greater proportion of disialylated structures in the F1, F2, and F3 subforms compared with those from PCa serum PSA (Table 1). We further examined the specific sialic acid linkage and observed an increase in alpha 2–3 linked sialic acid in the F3 PSA subform for the PCa patient (53%) compared with that from the healthy donor's seminal plasma (22%). These changes in this major subform may be considered representative of the whole PSA, as F3 accounts for 50–70% of all PSA subforms. The percentage of alpha 2–3 sialic acid in seminal plasma PSA was consistent with our previous analyses (25%) (Tabares et al., 2006) and with studies by Ohyama and coworkers ($\sim$20%) (Ohyama et al., 2004). In the same study, we observed a different proportion of alpha 2–3 linked sialic acid in the PSA present in patients, which was decreased in relation to that from healthy seminal plasma (15 vs. 25%) (Tabares et al., 2006). However, our present results are more consistent with the observations made by other authors (Ohyama et al., 2004; Tajiri et al., 2008).

The percentage of core fucosylation in the main PSA subform, F3, from a PCa patient's serum (46%) was lower than that present in the same subform from healthy donor's seminal plasma PSA (66%). This result is consistent with our previous work with serum PSA from a different PCa patient, although there, the decrease in core fucosylated structures in PCa compared to seminal plasma was more pronounced (16 vs. 80%) (Tabares et al., 2006). In agreement with these results, recently a decrease in the core fucosylated biantennary structure has also been reported in seminal plasma PSA from PCa patients compared to seminal plasma from healthy and BPH patients (White et al., 2009). These data suggest that this *N*-glycan modification might be used for detecting tumour related PSA.

These changes in PSA *N*-glycan chain are likely to occur within the prostate cell, throughout the secretory pathway, and these will probably include changes in alpha 2–3 and 2–6 sialyltransferases and in fucosyltransferases expression. Changes in the expression of some glycosyltransferases have been described in prostate tumor cells compared to normal prostate tissue (Barthel et al., 2008). The study of the molecular mechanisms involved in the glycosylation changes in prostate tumor tissues, such as alteration in the glycosyltransferase expression pattern, would be required to explain PSA *N*-glycan changes.

Conclusions

N-Glycan analysis of each PSA subform (F1–F4) from both prostate cancer sera and seminal plasma revealed differences in *N*-glycan sialylation for F3 and F4 subforms. F3 (which is decreased in PCa patient sera compared to BPH) showed both mono and disialylated *N*-glycans, whereas F4 (which is increased in PCa patient sera) showed mainly monosialylated glycans in all samples analyzed. Assuming that these *N*-glycan changes between F3 and F4 would also

be present in the PSA subforms from BPH serum, the degree of PSA sialylation might be exploited to distinguish PCa from BPH. However, the development of assays to directly measure sialylation and/or core fucosylation of PSA serum is required to analyze a large set of samples and to determine whether these PSA *N*-glycan changes could help to discriminate between benign and malignant conditions of the prostate.

Acknowledgments

A.S. acknowledges University of Girona for a pre-doctoral fellowship and for a short-term mobility fellowship. We thank Dr Marion Boland for careful reading of this manuscript, the UCD Conway Mass Spectrometry Resource (NIBRT, University College Dublin), and the Proteomics Platform of Barcelona Science Park (University of Barcelona) for the Mass Spectrometry analysis. This work was supported by EUROCarbDB (http://www.eurocarbdb.org) RIDS Contract No. 011952, the Spanish *Ministerio de Ciencia e Innovación* (grant BIO 2007-61323, awarded to R.P), *La Marató de TV3* foundation (grant 050932, awarded to R.P), and the Government of Catalonia (grant 2005SGR00065, awarded to R.L.).

Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

References

Balk, S.P., Ko, Y.J., and Bubley, G.J. (2003). Biology of prostate-specific antigen. J Clin Oncol 21, 383–391.

Barthel, S.R., Gabino, J.D., Wiese, J.K., Jaynes, J.M., Siddiqui, J., and Dimitroff. C.J. (2008). Analysis of glycosyltransferase expression in metastatic prostate cancer cells capable of rolling activity on microvascular endothelial (E)-selectin. Glycobiology 18, 806–817.

Bigge, J.C., Patel, T.P., Bruce, J.A., Goulding, P.N., Charles, S.M., and Parekh, R.B. (1995). Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. Anal Biochem 230, 229–238.

Bracarda, S., De Cobelli O., Greco C., Prayer-Galetti T., Valdagni R., Gatta G., et al. (2005). Cancer of the prostate. Crit Rev Oncol Hematol 56, 379–396.

Brawer, M.K., Meyer, G.E., Letran, J.L., Bankson, D.D., Morris, D.L., Yeung, K.K., et al. (1998). Measurement of complexed PSA improves specificity for early detection of prostate cancer. Urology 52, 372–378.

Catalona, W.J., Southwick, P.C., Slawin, K.M., Partin, A.W., Brawer, M.K., Flanigan, R.C., et al. (2000). Comparison of percent free PSA, PSA density, and age-specific PSA cutoffs for prostate cancer detection and staging. Urology 56, 255–260.

Charrier, J.P., Tournel, C., Michel, S., Dalbon, P., and Jolivet, M. (1999). Two-dimensional electrophoresis of prostate-specific antigen in sera of men with prostate cancer or benign prostate hyperplasia. Electrophoresis 20, 1075–1081.

Diamandis, E.P. (1998). Prostate-specific antigen: its usefulness in clinical medicine. Trends Endocrinol Metab 9, 310–316.

Gorg, A., Weiss, W., and Dunn, M.J. (2004). Current two-dimensional electrophoresis technology for proteomics. Proteomics 4, 3665–3685.

Huber, P.R., Schmid, H.P., Mattarelli, G., Strittmatter, B., Van Steenbrugge, G.J., and Maurer A. (1995). Serum free prostate specific antigen: isoenzymes in benign hyperplasia and cancer of the prostate. Prostate 27, 212–219.

Isono, T., Tanaka, T., Kageyama, S., and Yoshiki, T. (2002). Structural diversity of cancer-related and non-cancer-related prostate-specific antigen. Clin Chem 48, 2187–2194.

Jung, K., Reiche, J., Boehme, A., Stephan, C., Loening, S.A., Schnorr, D., et al. (2004). Analysis of subforms of free prostate-specific antigen in serum by two-dimensional gel electrophoresis: potential to improve diagnosis of prostate cancer. Clin Chem 50, 2292–2301.

Kosanovic, M.M., and Jankovic, M.M. (2005). Sialylation and fucosylation of cancer-associated prostate specific antigen. J Buon 10, 247–250.

Linton, H.J., Marks, L.S., Millar, L.S., Knott, C.L., Rittenhouse, H.G., and Mikolajczyk, S.D. (2003). Benign prostate-specific antigen (BPSA) in serum is increased in benign prostate disease. Clin Chem 49, 253–259.

Loeb, S., and Catalona, W.J. (2008). What to do with an abnormal PSA test. Oncologist 13, 299–305.

Meany, D.L., Zhang, Z., Sokoll, L.J., Zhang, H., and Chan, D.W. (2009). Glycoproteomics for prostate cancer detection: changes in serum PSA glycosylation patterns. J Proteome Res 8, 613–619.

Mikolajczyk, S.D., Marks, L.S., Partin, A.W. and Rittenhouse, H.G. (2002). Free prostate-specific antigen in serum is becoming more complex. Urology 59, 797–802.

Mikolajczyk, S.D., Catalona, W.J., Evans, C.L., Linton, H.J., Millar, L.S., Marker, K.M., et al. (2004). Proenzyme forms of prostate-specific antigen in serum improve the detection of prostate cancer. Clin Chem 50, 1017–1025.

Ohyama, C., Hosono, M., Nitta, K., Oh-Eda, M., Yoshikawa, K., Habuchi, T., et al. (2004). Carbohydrate structure and differential binding of prostate specific antigen to Maackia amurensis lectin between prostate cancer and benign prostate hypertrophy. Glycobiology 14, 671–679.

Okada, T., Sato, Y., Kobayashi, N., Sumida, K., Satomura, S., Matsuura, S., et al. (2001). Structural characteristics of the N-glycans of two isoforms of prostate-specific antigens purified from human seminal fluid. Biochim Biophys Acta 1525, 149–160.

Peracaula, R. (2007). Altered glysosylation in tumour proteins: biological implications. Afinidad 64, 346–355.

Peracaula, R., Tabares, G., Royle, L., Harvey, D.J., Dwek, R.A., Rudd, P.M., et al. (2003). Altered glycosylation pattern allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. Glycobiology 13, 457–470.

Peter, J., Unverzagt, C., Lenz, H., and Hoesel, W. (1999). Purification of prostate-specific antigen from human serum by indirect immunosorption and elution with a hapten. Anal Biochem 273, 98–104.

Peter, J., Unverzagt, C., and Hoesel, W. (2000). Analysis of free prostate-specific antigen (PSA) after chemical release from the complex with alpha(1)-antichymotrypsin (PSA-ACT). Clin Chem 46, 474–482.

Royle, L., Radcliffe, C.M., Dwek, R.A., and Rudd, P.M. (2006). Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions. Methods Mol Biol 347, 125–143.

Sarioglu, H., Lottspeich, F., Walk, T., Jung, G., and Eckerskorn, C. (2000). Deamidation as a widespread phenomenon in two-dimensional polyacrylamide gel electrophoresis of human blood plasma proteins. Electrophoresis 21, 2209–2218.

Sarrats, A., Comet, J., Tabares, G., Ramirez, M., Aleixandre, R.N., De Llorens, R., et al. (2010). Differential percentage of

serum prostate-specific antigen subforms suggests a new way to improve prostate cancer diagnosis. Prostate 70, 1–9.

Schulenberg, B., Goodman, T.N., Aggeler, R., Capaldi, R.A. and Patton, W.F. (2004). Characterization of dynamic and steady-state protein phosphorylation using a fluorescent phosphoprotein gel stain and mass spectrometry. Electrophoresis 25, 2526–2532.

Tabares, G., Radcliffe, C.M., Barrabes, S., Ramirez, M., Aleixandre, R.N., Hoesel, W., et al. (2006). Different glycan structures in prostate-specific antigen from prostate cancer sera in relation to seminal plasma PSA. Glycobiology 16, 132–145.

Tabares, G., Jung, K., Reiche, J., Stephan, C., Lein, M., Peracaula, R., et al. (2007). Free PSA forms in prostatic tissue and sera of prostate cancer patients: analysis by 2-DE and western blotting of immunopurified samples. Clin Biochem 40, 343–350.

Tajiri, M., Ohyama, C., and Wada, Y. (2008). Oligosaccharide profiles of the prostate specific antigen in free and complexed forms from the prostate cancer patient serum and in seminal plasma: a glycopeptide approach. Glycobiology 18, 2–8.

Varki, A., Kannagi, R., and Toole, B. (2008). Glycosylation changes in cancer. In *Essentials of Glycobiology,* 2nd ed. A. Varki, R. Cummings, J. Esko, H. Freeze, P. Stanley, C. Bertozzi, et al., editors. (Cold Spring Harbour Laboratory Press, New York), pp. 617–632.

White, K.Y., Rodemich, L., Nyalwidhe, J.O., Comunale, M.A., Clements, M.A., Lance, R.S., et al. (2009). Glycomic characterization of prostate-specific antigen and prostatic acid phosphatase in prostate cancer and benign disease seminal plasma fluids. J Proteome Res 8, 620–630.

Address correspondence to:
Dr. Pauline M. Rudd
Dublin–Oxford Glycobiology Laboratory
NIBRT, Conway Institute
University College Dublin
Belfield, Dublin 4, Ireland

E-mail: pauline.rudd@nibrt.ie
OR
Dr. Rosa Peracaula
Unitat de Bioquímica i Biologia Molecular
Departament de Biologia
Universitat de Girona
Campus de Montilivi
Girona 17071, Spain

E-mail: rosa.peracaula@udg.edu

Chapter 5

Glycosylation of liver acute-phase proteins in pancreatic cancer and chronic pancreatitis

Ariadna Sarrats, Radka Saldova, Eva Pla, Esther Fort, David J. Harvey, Weston B. Struwe, Rafael de Llorens, Pauline M. Rudd, Rosa Peracaula. “Glycosylation of liver acute-phase proteins in pancreatic cancer and chronic pancreatitis”. Proteomics Clinical Applications. Vol. 4, issue 4 (April 2010) : 432-448. DOI: 10.1002/prca.200900150

http://dx.doi.org/10.1002/prca.200900150

http://onlinelibrary.wiley.com/doi/10.1002/prca.200900150/full

Received: July 10, 2009

Revised: October 6, 2009

Accepted: December 18, 2009

Abstract

Purpose: Glycosylation of acute-phase proteins (APP), which is partially regulated by cytokines, may be distinct in disease and provide useful tumour markers. Thus, we have examined the glycosylation of major serum APP in pancreatic cancer (PaC), chronic pancreatitis (CP) and control patients.

Experimental design: Using a specific anti-sialyl Lewis X antibody and N-glycan sequencing, we have determined glycosylation changes on α-1-acid glycoprotein (AGP), haptoglobin (HPT), fetuin (FET), α-1-antitrypsin (AT) and transferrin (TRF).

Results: Increased levels of sialyl Lewis X (SLex) were detected on AGP in advanced PaC and CP and on HPT, FET, AT and TRF in CP. An increase in N-glycan branching was detected on AGP and HPT in the advanced stage of PaC and CP and on FET and TRF in the CP. A core fucosylated structure was increased on AGP and HPT only in the advanced PaC patients.

Conclusions and clinical relevance: Changes in APP SLex and branching are probably associated with an inflammatory response because they were detected in both advanced PaC and CP patients and these conditions give rise to inflammation. On the contrary, the increase in APP core fucosylation could be cancer associated and the presence of this glycoform may give an advantage to the tumour.

Keywords

Acute-phase proteins; Core fucose; Liver; Pancreatic cancer; Sialyl Lewis X

Chapter 6

Identification of potential pancreatic cancer serum markers: increased Sialyl-Lewis X glycoproteins.

Identification of potential pancreatic cancer serum markers: increased Sialyl-Lewis X glycoproteins

Ariadna Sarrats[1], Maria-José Ferri[2], Joan Figueras[3],
Esther Fort[4], Rafael de Llorens[1], Rosa Peracaula[1]*

[1]Unitat de Bioquímica i Biologia Molecular, Departament de Biologia, Universitat de Girona, Campus de Montilivi, Girona, Spain.

[2]Laboratori ICS Girona, Hospital Josep Trueta, Girona, Spain.

[3]Unitat de Cirugia Hepato-Biliar i Pancreàtica, Servei de Ciriurgia, Hospital Josep Trueta, IDiBGi, Girona, Spain

[4]Unitat de Digestiu, Hospital Josep Trueta, Girona, Spain.

*To whom correspondence should be addressed: Rosa Peracaula: e-mail: rosa.peracaula@udg.edu, Tel:+34 972418370; Fax: +34 972418370

Abstract

Pancreatic cancer cells and tissues usually show an enhanced expression of the Sialyl-Lewis X (SLe^x) and related epitopes. Pancreatic tumours may secrete some of the proteins carrying such increased SLe^x determinant into serum and these could be used as PaC markers. In a previous study we identified serum glycoproteins with increased SLe^x in pancreatic cancer (PaC) and also in chronic pancreatitis patients (CP), in particular several acute-phase proteins, mainly secreted by the liver. Our purpose has been to identify other serum glycoproteins with increased SLe^x only in PaC patients.

Therefore, serum samples from three healthy controls, eight PaC and five CP patients were depleted of the twelve most abundant serum proteins, including eight of the more abundant acute-phase proteins, electrophoresed and subjected to SLe^x immunodetection. Proteins that differentially expressed SLe^x in PaC with respect CP and controls were found in 5 bands and were trypsin digested and identified using a LC-ESI-QTOF mass spectrometry. They corresponded to alpha-2-macroglobulin (band 1 and 2), ceruloplasmin (band 2), inter-alpha-trypsin inhibitor heavy chain H4 (band 3 and 4), complement C3 (band 4), complement C6 (band 4) and complement C4 (band 5). All these proteins were again mainly liver derived APP, not representing specific proteins from pancreas. However, their increase in SLe^X was only detected in PaC patients; therefore the evaluation of the SLe^X content of these APP in a larger group of patients would determine their usefulness as PaC biomarkers.

Introduction

Pancreatic cancer (PaC) 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 in 2008. 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% (Jemal *et al.* 2009; Ferlay *et al.* 2010a).

CA19-9 serum detections is currently used in the monitoring of PaC 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 (CP) (Grote and Logsdon 2007; Misek *et al.* 2007). Thus, the availability of adequate biomarkers for pancreatic cancer detection is of major interest. The ideal pancreatic serum marker would be a specific substance produced by the tumour that could reach the bloodstream and be detected in the sera of PaC patients.

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 (Varki *et al.* 2008). In particular, SLe^x and related Lewis antigens have been found to be overexpressed PaC cell lines (Hosono *et al.* 1998; Peracaula *et al.* 2005) and tissues (Kim *et al.* 1988; Satomura *et al.* 1991; Sinn *et al.* 1992). An increase of sialylated Lewis antigens and both fucosylation and sialylation of certain glycoproteins have been detected in the sera of PaC patients compared to healthy individuals and pancreatitis patients (Sawada *et al.* 1994; Zhao *et al.* 2007; Li *et al.* 2009). These data suggests that pancreatic tumour may shed to the blood glycoproteins carrying SLe^x which could be used as PaC tumour markers.

In a previous work we identified serum glycoproteins carrying increased SLe^x in both advanced PaC and CP patients (Sarrats *et al.* 2010b). 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 APP were also found to bear increased SLe^x levels only in CP patients (alpha-1-antitrypsin and fetuin). Although SLe^x on these APP may be used as cancer prognostic factors these modifications are not specific enough to be used as pancreatic cancer markers.

In the present work, we have used IgY-12 High Capacity Spin columns to deplete the twelve most abundant serum proteins, which include all APP previously found to bear increased SLe^x in both PaC and CP patients (Sarrats *et al.* 2010b) in order to identify other glycoproteins with enhanced SLe^x only in PaC patients.

Methods

Serum samples were obtained from 3 healthy controls (1 female and 2 males; age range 57-60), 8 pancreatic cancer patients (3 females and 6 males; age range 53-68, 4 stage III (T3 N1 M0) and 4 stage IVb (T3 N1 M1)) and 5 chronic pancreatitis patients (5 males, age range 47-72) 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 Digestive and Pathology Units.

Serum samples (20μL of each) were depleted using the ProteomeLab IgY-12 High Capacity Spin column (Proteome Partitioning Kit, Beckman Coulter, Fullerton, CA, USA), following centrifugation using a 0.22 μm Spin-X Centrifuge Tube Filter (Costar, Corning, NY, USA) for 10 min at 2000 rpm according to manufacturer's protocols. This column facilitates 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 immunedepletion was concentrated to ~100 μL using Microcon YM-3 Centrifugal Filter Device (Millipore, Billerica, MA, USA).

Protein concentration was determined by the Bradford protein assay using bovine serum albumin as standard (Quick Start Bradford Protein Assay, BioRad, Hercules, CA, USA). After Immunodepletion and concentration of serum samples, 25.24 μg of total protein was electrophoresed under reducing conditions on a 12% or 8% polyacrylamide gels which were either Coomassie stained or transferred onto a PVDF membrane (Millipore Corporation, Bedford, MA, USA). Transferred proteins were Ponceau stained (Ponceau S solution, DIG Glycan Differentiation Kit, Roche Diagnostics, Germany) and after that, SLe^x was immunodetected as previously described (Sarrats *et al.* 2010b) (Proteins contained in the bands with specific SLe^x immunodetection for the pancreatic cancer patients group were in-gel digested with trypsin, extracted and analyzed in a LC-ESI-QTOF mass spectrometer as described in (Sarrats *et al.* 2010b). 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, 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 $P < 0.05$. Peptide summary was selected as the report format. Only proteins with at least 2 peptides identified were accepted as positive hits.

Results and discussion

Depletion of serum samples with ProteomeLab IgY-12 High Capacity Spin column reduced total protein amount by about 90% (range 85.1%-93.9%). Depleted serum samples from healthy controls (HC 1-3), pancreatic cancer patients (PaC 1-8) and chronic pancreatitis patients (CP 1-5) patients were electrophoresed in a 12% polyacrylamide gel, transferred onto a PVDF membrane and subjected to SLe^x immunodetection (Fig. 1). Different immunoreactive bands were observed in all the samples. Most of the PaC samples showed stronger SLe^x signal in the molecular weight region higher than 64 kDa (Fig 1A and 1C). Some of these bands were neither observed in any of the HC patients nor in any of the CP patients, which prompt for the identification of the serum glycoproteins bearing this PaC specific increase of SLe^x. For that, representative samples of each group of patients that show higher signal in the region above 64kDa were selected (HC1 for the healthy patients group; CP3 and CP5 for the pancreatitis group and PaC1, PaC4, PaC6 and PaC7 for the cancer group) and electrophoresed in an 8% polyacrylamide gel to improve band separation in the high molecular weight range. 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 (1-5) which showed stronger SLe^x immunoreactivity in the PaC patients than in the CP or HC were selected (Fig. 2). These bands were cut from the Coomassie-stained gel and subjected to protein identification (Table 1).

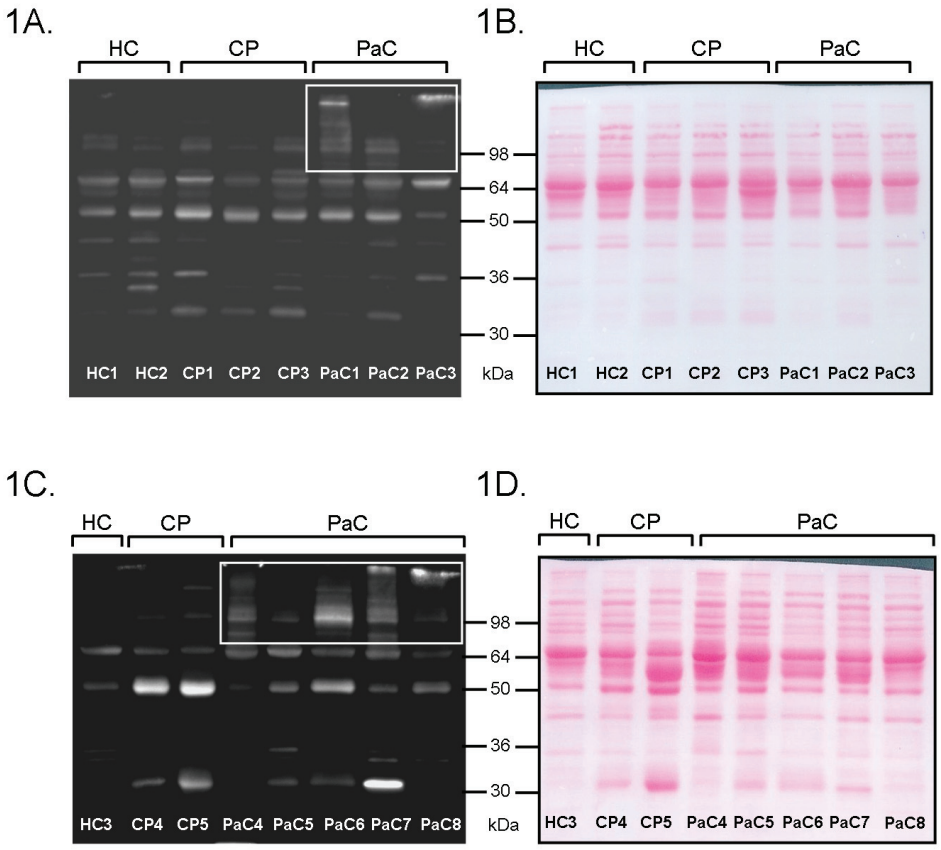


Figure 1. SLe^x immunodetection (1A and 1C) and corresponding Ponceau stain (1B and 1C) of the immunodepleted serum samples blots. Proteins were electrophoresed in a 12% polyacrylamide gel.

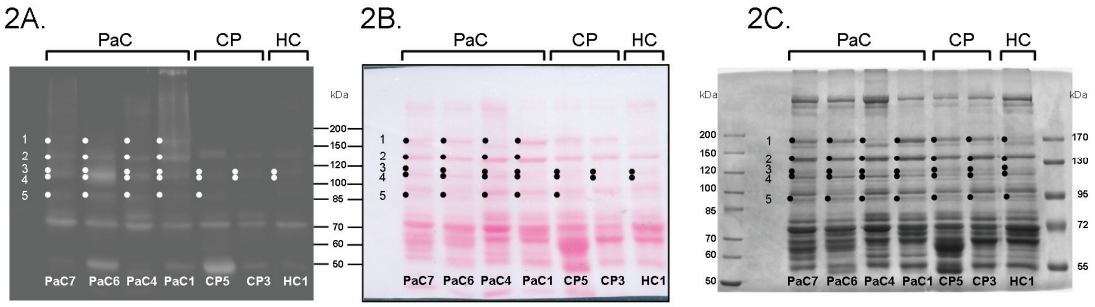


Figure 2. SLe^x immunodetection (2A) and corresponding Ponceau stain (2B) of depleted serum samples blots from gel 1. Coomassie stained of the same depleted serum samples gel 2 (2C). Proteins were electrophoresed in an 8% polyacrylamide gel.

Band 1 was found to contain alpha-2-macroglobulin (A2M). The experimental molecular weight of the band (~180 kDa) was in agreement with the classically described (Sottrup-Jensen *et al.* 1984; Arnold *et al.* 2006), which is higher than the theoretical one because of the glycans presence. A2M corresponds to one of the proteins depleted by the ProteomeLab IgY-12

column. Thus, this protein is not 100% removed from the sera after depletion protocol. It is difficult to give a clear interpretation of this event as the depletion process may have removed A2M at different yields in the different samples. However, except for serums PaC4 and HC1, similar intensities of the bands are observed in the Coomassie stained gels, which suggest that A2M may have increased SLe^x in the PaC patients. A2M functions as a non-specific protease inhibitor in mammals which is predominantly produced by the liver, and it is one of the major acute-phase serum proteins associated with inflammatory response (Kushner 1982). It has been described to posses 8 possible *N*-glycosylation sites (Sottrup-Jensen *et al.* 1984). Glycans in A2M from normal sera showed mainly complex, biantennary structures with and without core fucosylation. High-mannose structures (~8%) were also found in a minor proportion (Arnold *et al.* 2006). No glycan structures containing SLe^x were detected. In fact, 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 PaC patients, suggesting an interesting specific glycan alteration in PaC.

Band 2 was found to contain ceruloplasmin (CERU). A2M was also found in this band but at a much lower score and number of identified 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 assume CERU is the main protein present in this band. Band 2 intensities in the Coomassie stained gel were similar in all the patients, which means the enhanced SLe^x detection may be due preferentially to an increase of this epitope in the protein rather than a rise of the CERU serum levels. CERU is an acute-phase protein produced by the liver and secreted in plasma. It has 6 *N*-glycosylation sites with complex type, bi, tri and tetrantennary structures which have been described to be both sialylated and fucosylated and to contain SLe^x epitope in tri and biantennary structures (Harazono *et al.* 2006). Serum levels of CERU have been proposed as markers of different solid cancers, as they were increased in different cancer patients compared to healthy controls (Senra Varela *et al.* 1997; Ros-Bullon *et al.* 2001). They were not significantly different between lung cancer patients and patients with benign lung diseases. Interestingly, lung cancer patients showed different CERU heterogeneity in immunoelectrophoresis attributed to changes in its charge/size. However, CERU glycan microheterogeneity analyzed by crossed affinoimmunoelectrophoresis with WGA (*Wheat germ* agglutinin, a lectin that recognizes mainly *N*-acetyl-glucosamine and that has also found to have affinity to sialic acid) was not useful as an indicator of malignancy (Hansen *et al.* 1987). This lectin study could not give information about the detailed glycan structures of CERU separated by affinoimmunoelectrophoresis with WGA. In our study, the glycan differences

attributed to CERU in PaC and benign and CP patients are due to different content of SLe^x, which could be present in any type of their complex glycan structures.

Band 3 was found to contain Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), which is also an acute-phase response plasma glycoprotein synthesized mainly by the liver (Saguchi *et al.* 1995; Choi-Miura 2001). Although the theoretical molecular weight of this protein is 103 kDa, it shows an experimental molecular weight of 120 kDa (Pu *et al.* 1994). Two fragments may be derived from this intact form of the protein, the C-terminal 35 kDa and the N-terminal 85 kDa fragments. The 85 kDa ITIH4 fragment is further cleaved to produce an N-terminal 57 kDa fragment and a putative 28 kDa fragment. The latter is believed to be further processed by protease(s) to generate smaller fragments (Saguchi *et al.* 1995; Song *et al.* 2006). The 85 kDa fragment possesses four *N*-glycosylation sites (Liu *et al.* 2005; Chen *et al.* 2009) while the 35 kDa fragment may be *O*-glycosylated in three different residues (Pu *et al.* 1994; Nishimura *et al.* 1995). Band 3 may contain the intact form of ITIH4 because of its experimental molecular weight of ~120 kDa and the fact that the MS analysis identified peptides from both 35 kDa and 85 kDa fragments. Different ITIH4 fragmentation patterns are generated in different types of cancer and diabetes (Song *et al.* 2006). After serum enrichment of glycoproteins with a Multilectin affinity column, Heo and co-workers (Heo *et al.* 2007) identified increased ITIH4 in lung cancer patients compared to healthy individuals. The increase of this protein in lung cancer patients' sera had been previously described by proteome analysis (Fujii *et al.* 2005; Okano *et al.* 2006). An ITIH4 fragment was also identified as an early stage ovarian cancer marker (Zhang *et al.* 2004) and after isolation of *O*-glycosylated serum proteins with CGB lectin affinity chromatography, the 35 kDa ITIH4 fragment was found to be increased in ovarian carcinomas compared to other carcinomas and healthy controls (Mohamed *et al.* 2008). To our knowledge, there are no previous reports regarding ITIH4 glycosylation studies in illnesses, however, the increased levels of ITIH4 in (Heo *et al.* 2007; Mohamed *et al.* 2008) may be due to increased glycosylation (increased protein affinity to multilectin columns used to enrich the samples in glycoproteins). Somehow, this would be in agreement with our results were increased SLe^x is observed in the PaC patients.

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).

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. In fact, in the SLe^x western blot, only one immunoreactive band can be noticed in the molecular weight range of bands 3-4 from. In addition, band 4 is slightly more intense in the PaC patients. Thus, the increased SLe^x may be also due to an increase of all these proteins levels. Complement C3 and Complement component C6 are constituents of the complement system, which are produced mainly by the liver and circulate in the blood and extracellular fluid (Alberts *et al.* 2002). Complement proteins are considered positive acute-phase reactants (Ceciliani *et al.* 2002). C6 is a late complement component, member of the Membrane attack complex (MAC). Two *N*-glycosylation sites have been found in this protein (Liu *et al.* 2005). C3 is an early, pivotal component of complement. Its processing generates different fragments of different molecular weight (Alberts *et al.* 2002). Thus, band 4 may contain one of these fragments. It has been described to have three possible *N*-glycosylation sites (Liu *et al.* 2005).

In the work by Heo and co-workers (Heo *et al.* 2007) commented above, a C3 fragment was also found to be increased in lung cancer patients compared to healthy controls after serum enrichment of glycoproteins with a Multilectin affinity column, which may be also due to an increased of C3 fragment glycosylation in lung cancer patients. Recently, C3 have shown an increase of Sambucus nigra Agglutinin reactivity, which recognizes alpha-2,6 sialic acid, and also in fucosylation (Aleuria aurentia lectin reactivity) in plasma from colorectal cancer patients in comparison to adenoma and healthy individuals (Qiu *et al.* 2008). The increase in C3 fucosylation is consistent with the increased SLe^x found in our set of pancreatic cancer patients.

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 (Blanchong *et al.* 2001). In fact, we cannot know unambiguously which of the isotypes is present in band 5 as both C4-A and C4-B were matched to the same set of peptides. Any of them or both of them may be present. C4 plays a central role in the activation of the classical pathway of complement system. Likewise its other complement system counterparts, is produced mainly by the liver and secreted into the blood being considered a positive acute-phase protein (Alberts *et al.* 2002; Ceciliani *et al.* 2002). It is a ~200 kDa glycoprotein consisting of three polypeptides designated alpha, beta and gamma linked together by disulfide bonds. It is synthesized as single chain polypeptide, which is processed by intracellular cleavage to yield the secreted three-chain C4 molecule. Consequently, in an SDS-PAGE gel under reducing conditions, purified serum C4 is separated in three different bands of 93 kDa -alpha chain-, 78 kDa -beta chain- and 33 kDa -gamma chain- (Schreiber and Muller-Eberhard 1974; Morris *et al.* 1982; Chan and Atkinson 1983). Therefore, and due to its experimental molecular weight of ~90 kDa, band 5 corresponds to C4 alpha chain. In addition, identified peptides corresponded exclusively to this region of the protein sequence (data not shown). C4 may be *N*-glycosylated and four different sites, one in the beta chain and three in the alpha chain (Belt *et al.* 1984). Oligosaccharides in C4 alpha chain have been documented as complex, biantennary and fucosylated structures, which may include SLe^x epitope (Chan and Atkinson 1985). Increased SLe^x in band 5 in all PaC patients and in one CP patient may be due solely to glycosylation as similar levels of proteins are observed in all the samples (Fig. 2).

In agreement with our results, using glycoprotein microarrays with multi-lectin detection techniques, an increase in both fucosylation and sialylation of serum glycoproteins, such as serum amyloid P-component, Beta-2-glycoprotein 1 and alpha-1-□ glycoprotein, which are also mainly liver-derived, has been described for PaC patients compared to healthy controls and

pancreatitis patients (Zhao *et al.* 2007; Li *et al.* 2009). In the present study we have identified some serum glycoproteins with potentially increased SLe^x in PaC patients different from the high abundant APP that we had previously described (Sarrats *et al.* 2010b). Although these proteins turned out to be again APP, which are produced mainly by the liver, their microheterogeneity (increased SLe^x) may be used in the differentiation of PaC and CP. However, these modifications may not be specific for this type of cancer since changes in the glycan structures of these APP have been described in other cancer types. To better elucidate this issue, glycan characterization of this proteins should be performed in a larger study with more PaC and CP patients and also including other types of tumours, especially the ones with signs and symptoms similar to the ones in PaC patients.

The serum levels of these increased SLe^x proteins range between 0.1-2 mg/mL, which precludes the detection of potential minor proteins with increased SLe^x derived from the pancreatic tumour. Thus, the discovery of PaC specific serum markers should be oriented either on the serum depletion of more liver APP in order to search for differential glycosylation of minor serum pancreas-specific proteins or in the evaluation of proteins glycoforms specifically expressed by the pancreatic tumour tissues that may reach the bloodstream.

Chapter 7

General Discussion

Glycan alteration of serum proteins as tumor markers

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 alteration in malignant cells take a variety of forms and include changes in branching, fucosylation, sialylation, and in the expression of terminal glycan structures such as Lewis antigens (Varki *et al.* 2008).

The availability of accurate tumour markers for the diagnosis of cancer is of major importance because the identification of patients at early stages of cancer improves the rate of survival (Jemal *et al.* 2009). In particular, serum and urine tumour markers are of special interest because their evaluation does not require invasive procedures for the patients. Recently, the deficiency of proteomics to deliver reliable cancer biomarkers has renewed interest in glycomic profiling of serum and other body fluid glycoproteins. In this regard, efforts have been taken to (1) improve the specificity of known cancer biomarkers by defining glycoforms of proteins uniquely expressed by cancer cells and (2) reinvestigate the classical findings of glycan-specific antibodies associated with cancer, which are formed due to the presence of tumour aberrant glycan structures (Varki *et al.* 2008).

In this work, approach (1) has been used on the investigations of Prostate-Specific antigen, the currently FDA approved prostate cancer marker, to improve differential diagnosis between prostate cancer and benign prostatic hyperplasia patients. A consequence of approach (2) has been used in the discovery of new pancreatic cancer serum biomarkers based on the search of serum glycoproteins bearing increased Sialyl-Lewis X, as this glycan structure is aberrantly expressed in pancreatic cancer tissues.

Changes in Prostate-Specific Antigen glycosylation

Prostate-Specific Antigen (PSA) serum concentration is currently considered the best tumour marker available in clinical medicine for the purpose of monitoring and diagnosing prostate cancer (PCa), both uses having been approved by the FDA (Food and Drug Administration). However, PSA is not fully specific for PCa as other prostatic pathologies such as benign prostate hyperplasia (BPH) may show serum PSA elevations (Catalona *et al.* 1994; Bracarda *et al.* 2005). Prostate cancer cells show altered glycosylation (Martensson *et al.* 1995; Zhang *et al.* 1997; Barthel *et al.* 2008). Thus, glycosylation of PSA had been studied to differentiate PCa from BPH patients. In a previous work, our group described by *N*-glycan sequencing differences in sialylation when comparing PSA from a healthy origin (healthy donor seminal plasma) and from a malignant origin (prostate cancer cell line, LNCaP). In contrast to normal PSA glycans,

which were sialylated, LNCaP PSA oligosaccharides were all neutral (Peracaula *et al.* 2003b). Later, the group was able to analyze, using the same technique, PSA from sera of an advanced PCa patient (Tabares *et al.* 2006) and changes in glycosylation, namely sialic acid linkage and core fucosylation were found between the serum PSA of a PCa patient and the PSA from a healthy donor seminal plasma. However, the analysis of serum PSA from a BPH patient was not possible due to the fact that these patients do not reach high enough PSA levels to allow *N*-glycan sequencing analysis.

Other studies have also shown changes of sialic acid on PSA in tumour situation. Serum PSA from BPH patients was described as possessing increased sialylation when compared with that from PCa patients and the PSA produced by the prostate cancer cell line, LNCaP in the work by Huber *et al.* 1995, who used chromatofocusing techniques combined with sialidase digestions.

These previous studies pointed out that the content of sialic acid decreased in PSA from prostate cancer patients compared to PSA from BPH patients. Sialic acid is a negatively charged carbohydrate and can alter PSA theoretical isoelectric point (pI). Two-dimensional electrophoresis (2-DE) separates proteins according to their pI and molecular weight. Therefore, in the present work, 2-DE was used to indirectly evaluate serum PSA sialylation in PCa and BPH patients. Later, PSA 2-DE subforms from sera of a PCa patient and from seminal plasma of a healthy donor were also characterized by *N*-glycan sequencing, which confirmed PSA altered sialylation and revealed (confirmed) other PSA *N*-glycan changes in prostate cancer, such as core fucosylation (Figure 1).

Changes in Prostate-Specific Antigen sialylation

The separation of serum PSA from PCa and BPH patients, including free PSA and PSA released from ACT complex, by 2-DE resulted in 5 main subforms of the same molecular weight that we named F1 to F5 from the more acidic to the less acidic form (Sarrats *et al.* 2010a). Similar pattern of 2-DE subforms of free PSA from seminal plasma and from serum had been previously observed by different authors (Charrier *et al.* 1999; Isono *et al.* 2002; Jung *et al.* 2004; Tabares *et al.* 2007). The relative percentage of F3 subform (%F3) was significantly decreased in the PCa group of patients and correlate negatively with the stage of the disease. %F4, was increased in the PCa group, although not significantly, and correlate positively with the stage of the cancer (Sarrats *et al.* 2010a). This increase in the high pI PSA subform F4 in PCa patients was in agreement with our preliminary results where four metastatic PCa patients showed a greater proportion of high pI subforms compared to seminal plasma PSA from a healthy donor (Tabares *et al.* 2006).

ROC analysis of total 2-DE PSA subforms (especially %F3) showed a tendency of better discriminatory power between PCa and BPH patients than the currently clinical tests of tPSA and %fPSA, even when considering only the patients within the total PSA rage from 2-20 ng/mL (Sarrats *et al.* 2010a).

These results prompted for the investigation of the biochemical differences among PSA 2-DE subforms F1 to F5 that could lead to different pI. In particular, sialylation degree was the main candidate to be blamed for the change in the isoelectric point of these PSA 2-DE subforms. In a previous work, we had analyzed the glycosylation of both low pI PSA and high pI PSA from seminal plasma by *N*-glycan sequencing and reported that the high pI PSA was mostly monosialylated while low pI PSA contained both mono and disialylated *N*-glycans (Peracaula *et al.* 2003b). We subsequently performed *N*-glycan analysis of PSA subforms from sera of a PCa patient and from seminal plasma of a healthy donor. F1, F2 and F3 exhibited the same *N*-glycan HPLC profile, which differed from F4 and F5. The different content of sialic acid in the *N*-glycans from F3, F4 and F5 PSA subforms correlated with their different experimental pI detected by 2-DE. The subform F5 did not appear to be glycosylated and had a pI of 7.2, which corresponds to the theoretical one for PSA (7.26). F4 had a lower pI (7.0), which can be explained by the presence of mainly monosialylated *N*-glycans that contain negative charge. The F3 subform showed an even lower pI (6.8). It contained a much higher proportion of disialylated *N*-glycans, which would again contribute to this further decrease in pI. However, F1, F2 and F3 subforms all showed the same *N*-glycosylation pattern despite having different pI values (6.4, 6.6 and 6.8, respectively).

With the aim to explain the pI differences among these subforms, other modification of the PSA molecule that could lead to pI modification such as deamidations, phosphorylations or *O*-glycosylation were investigated. However, none of them turn out to be detected (Sarrats *et al.* 2010c). The presence of proPSA forms reported by other authors (Tabares *et al.* 2007) in serum free PSA 2-DE subforms was rule out due to the fact the majority of the PSA analyzed corresponded to active PSA previously bound to alpha-1-antichymotrypsin and therefore the contribution from proPSA would be quite small. In addition, it is well known that PSA from seminal plasma does not contain proPSA forms (Mikolajczyk *et al.* 2002) and the same five PSA subforms (F1-F5) that in serum were detected in this fluid. Other possible modifications of PSA that could lead to different pIs between F1, F2 and F3 may be sulphation of tyrosine residues (Moore 2003), carboxylation of aspartic or glutamic acid residues -giving an extra negative charge-, methylation of glutamic acid residues -eliminating a negative charge- (Nelson and Cox

2008), or acetylation either on the amino-terminal residue or on the ε-amino groups of lysine residues -eliminating a positive charge- (Polevoda and Sherman 2002), among others.

Although these modifications remain unrevealed, differences in PSA sialylation can explain the different isoelectric points observed between F3 and F4 and therefore the decrease in % F3 and the increase % F4 reported for the PCa patients compared to the BPH ones (Sarrats *et al.* 2010a). We observed a decrease of disialylated *N*-glycans in F4 compared to those in F3 for both seminal plasma and serum samples. Taking into account that the pIs of these subforms are the same for PSA samples from PCa serum, BPH serum and seminal plasma (Sarrats *et al.* 2010a), the decrease in sialylation described for the PSA F4 subform (Sarrats *et al.* 2010c), is likely to occur also in the corresponding F4 subform from BPH serum. We therefore suggest a reduction in sialylation of serum PSA in PCa patients compared to that in BPH patients, consistently with the previous work of Huber *et al.*, 1995 and the recently published by Meany *et al.* 2009, who reported a higher sialylation of total serum PSA in BPH than in PCa patients using SNA and MAA lectin immunosorbent assays.

We observed a negative correlation between the percentage of F3 and a positive correlation between the percentage of F4 and the PCa stage (Sarrats *et al.* 2010a). Thus, the potential decrease in PSA sialylation might be more marked as the cancer becomes more advanced. In fact, in the prostate cancer cell line LNCaP, which was derived from a node metastasis, a complete absence of sialylated structures was reported for PSA (Peracaula *et al.* 2003b). A decrease in SNA reactivity on free PSA oligosaccharide chain has also been reported in PCa patients with metastatic tumours compared to those with localized tumours, suggesting a relationship between the progression of this cancer and the level of sialic acid (Kosanovic and Jankovic 2005).

We observed an increase in alpha 2-3 linked sialic acid in the F3 PSA subform for the PCa patient (53%) compared with that from the healthy donor's seminal plasma (22%). These changes in this major subform may be considered representative of the whole PSA, as F3 accounts for 50-70% of all PSA subforms. The percentage of alpha 2-3 sialic acid in seminal plasma PSA was consistent with the study by Ohyama and coworkers (Ohyama *et al.* 2004) and with our previous analyses (Tabares *et al.* 2006). In the same study, we observed a different proportion of alpha 2-3 linked sialic acid in the PSA present in patients, which was decreased in relation to that from healthy seminal plasma (15% vs. 25%). However, our present results are more consistent with the observations made by other authors (Ohyama *et al.* 2004; Tajiri *et al.* 2008).

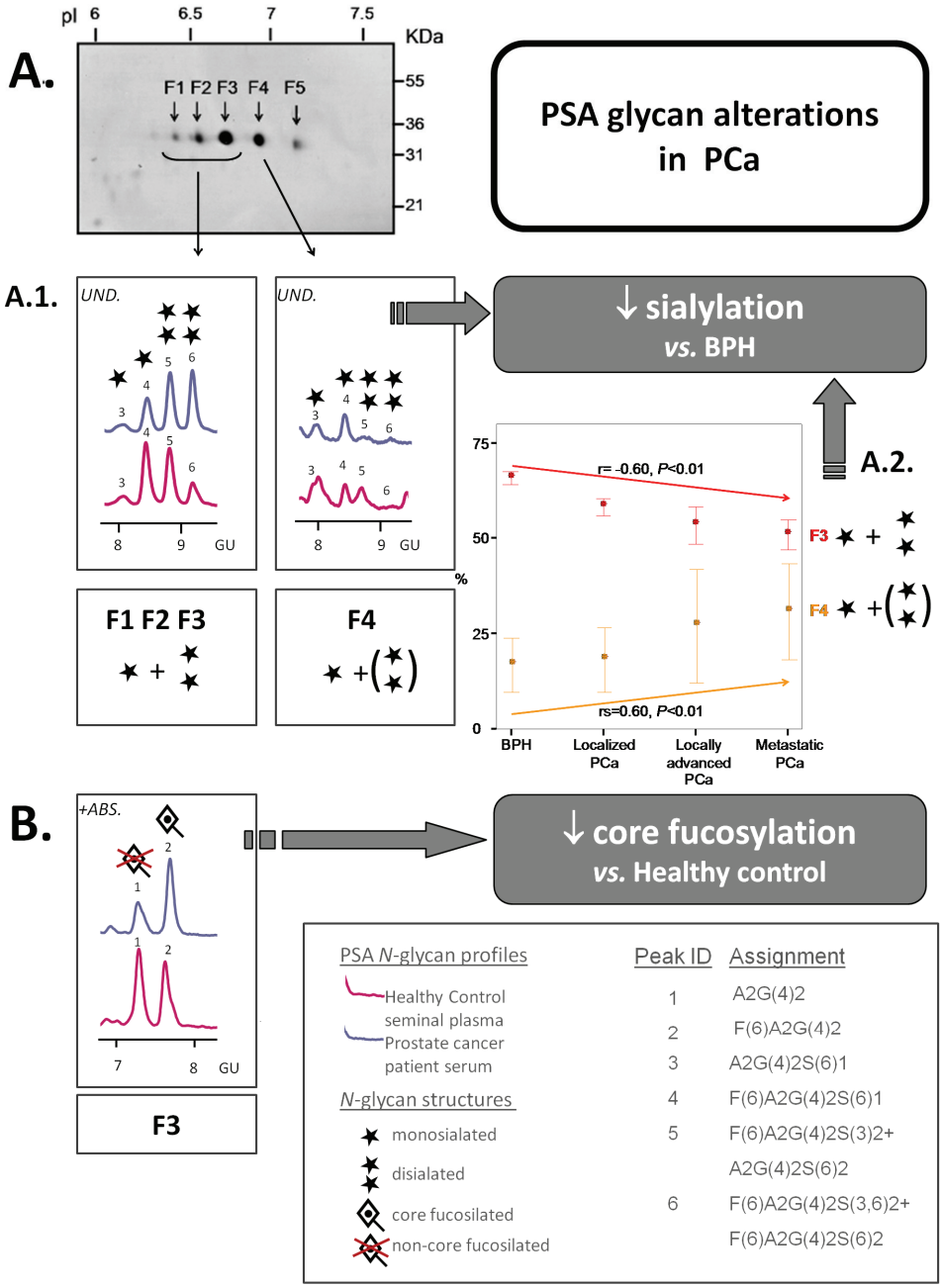


Figure 1. PSA glycan alteration in prostate cancer (PCa) compared to benign prostatic hyperplasia (BPH) patients and healthy controls (HC) (A.) Decreased sialylation of serum PSA from PCa compared to BPH was observed evaluation of PSA 2-DE subforms. (A.1.) Undigested *N*-glycan profiles of PSA 2-DE subforms from a HC seminal plasma and a PCa serum. F1, F2 and F3 showed a very similar profile containing mono and disialylated complex biantennary structures. Subform F4 contained mainly monosialylated complex biantennary structures. No glycans were detected in F5. (A.2.) Relative percentage of F3 (%F3) was decreased in PaC compared to BPH and this decrease correlate with the stage of the cancer. %F4 behaved oppositely. This changes were associated with a decrease of PSA sialic acid content in PCa patients compared to BPH ones. (B). *N*-glycan profiles of F3 PSA 2-DE subform after digestion with *Arthrobacter ureafaciens* sialidase. The percentage of core fucosylation is reduced in the PaC patient sample compared to the healthy control. Key to abbreviations: +ABS, digested with *Arthrobacter ureafaciens* sialidase; BPH; benign prostatic hyperplasia, GU, glucose units; HC; healthy control; PCa, prostate cancer; UND, undigested.

Changes in Prostate-Specific Antigen fucosylation

The percentage of a biantennary core fucosylated structure in the main PSA subform, F3, from a PCa patient's serum (46%) was lower than that present in the same subform from healthy donor's seminal plasma PSA (66%). This result is consistent with our previous work with serum PSA from a different PCa patient, although there, the decrease these core fucosylated structure was more pronounced (16% vs. 80%) (Tabares *et al.* 2006). Recently, a decrease in the same core fucosylated biantennary structure has been reported in seminal plasma PSA from PCa patients compared to seminal plasma from healthy and BPH patients (White *et al.* 2009). However, the work of Tajiri (Tajiri *et al.* 2008) showed by MS analysis of PSA glycopeptides that the relative abundances of the ions for fucosylated species among the total glycopeptide ions of two PCa patients analyzed (100% and 64%) were higher than the seminal plasma one (52%). These different results might be explained by the fact that only a small number of patients is usually analyzed in each study, together with the intrinsic heterogeneity of the PCa serum samples. These data suggest that this *N*-glycan modification might be used for detecting tumour related PSA.

Diagnostic relevance of changes in Prostate-Specific Antigen glycosylation

Assuming that *N*-glycan changes between F3 and F4 would also be present in the PSA subforms from BPH serum, the degree of PSA sialylation might be exploited to distinguish PCa from BPH. In addition, the proportion of core fucosylation structures might be used for detecting tumour related PSA. The development of assays to directly measure sialylation and/or core fucosylation of serum PSA is required to analyze a large set of samples and to determine whether these PSA *N*-glycan changes could help to discriminate between benign and malignant conditions of the prostate. In this regard we are currently setting an ELLA (Enzyme Linked Lectin Assay) methodology to evaluate PSA core fucosylation in a larger cohort of patients.

Biological implication of changes in Prostate-Specific Antigen glycosylation

The influence of the glycosylation on PSA enzymatic activity and on the other PSA functions has been barely analyzed. Mattson *et al*. (Mattsson *et al.* 2008) separated two main glycosylated PSA from plasma seminal, monosialylated and disialylated, and analyzed their enzymatic activity linked to the anti-angiogenic activity. No significant differences were described suggesting that the difference in sialic content would not influence in the biological activity of PSA. Similar results were obtained by Zhang et al. (Zhang *et al.* 1995) when analyzing the enzymatic activity of PSA subforms from seminal plasma separated by anion exchange

chromatography. The two active subforms that contain an intact PSA chain that differ in the content of sialic acid present the same enzymatic activity tested by the ability to form a complex with ACT, indicating again that changes in PSA sialylation are not influencing PSA enzymatic activity .

Samadi *et al.* 1999 suggested that PSA glycosylation could regulate the release of PSA from the tumour tissue into blood. They reported that at higher glycosylation (Con A reactivity) of cellular PSA, lower levels of serum PSA.

The changes in PSA *N*-glycan chain that we have reported are likely to occur within the prostate cell, throughout the secretory pathway, and these will probably include changes in alpha 2-3 and 2-6 sialyltransferases and in alpha 1-6 fucosyltransferase expression. Changes in the expression of some glycosyltransferases have been described in prostate tumour cells compared to normal prostate tissue (Barthel et al., 2008). The study of the molecular mechanisms involved in the glycosylation changes in prostate tumour tissues, such as alteration in the glycosyltransferase expression pattern or modifications of the sugar nucleotide donors or transporters levels would be required to explain PSA *N*-glycan changes. Further studies need to be performed to better understand the influence of glycosylation in PSA biology, especially regarding differently fucosylated glycoforms.

Changes in Acute-phase proteins glycosylation as pancreatic cancer markers

Sialyl-Lewis X (SLe^x) and related Lewis antigens have been found to be overexpressed in several tumours, including PaC cell lines and tissues (Kim *et al.* 1988; Satomura *et al.* 1991; Sinn *et al.* 1992; Hosono *et al.* 1998; Peracaula *et al.* 2005). The degree of expression of Sialyl-Lewis X/A determinants on cancer tissues has been reported to inversely correlate with the prognosis of several cancers such as colon, lung, breast, stomach, prostate, and urinary bladder cancer (Kannagi *et al.* 2004). Sialylated Lewis antigens and glycoproteins with increased fucosylation and sialylation have been also found in the sera of pancreatic cancer patients (Sawada *et al.* 1994; Zhao *et al.* 2007; Li *et al.* 2009) which suggested that pancreatic cancer tumour may shed to the blood glycoproteins carrying SLe^x.

In the present study, Western blot with an anti Sialyl-Lewis X antibody was used to identify serum glycoproteins bearing increased Sialyl-Lewis (SLe^x) that could be used as pancreatic cancer markers. *N*-glycan sequencing was performed in some of the identified glycoproteins to further investigate their altered glycosylation. The identified glycoproteins with altered glycosylation turn out to be Acute-phase proteins (Figure 2).

Acute-phase proteins (APP) are proteins synthesized by the liver hepatocytes that show changes in their plasma concentration as part of the acute phase response (APR). The latter is defined as a prominent systemic reaction of the organism to local or systemic disturbances in its homeostasis caused by infection, tissue injury, trauma or surgery, neoplastic growth or immunological disorders (Gruys *et al.* 2005). APP are classified in positive APP, whose serum concentration is increased from about 50% to as much as 1000-fold and in negative APP, which show a decrease in their serum concentration. Positive APP play different functional roles and contribute at different points of the inflammation process. They include complement system proteins, C-reactive protein, serum amyloid A, alpha-1-acid glycoprotein, haptoglobin and protease inhibitors such as α-1-antichymotrypsin, alpha-1-antitrypsin (AT) and inter-alpha-trypsin inhibitors. The decrease in serum concentration of negative APP is not considered essential for host defense and has been proposed to be a consequence of the cellular need to divert available amino acids for the production of positive APP. Negative APP include albumin, transferrin, fetuin and alpha-fetoprotein (Gabay and Kushner 1999; Ceciliani *et al.* 2002).

Changes in Acute-phase proteins Sialyl-Lewis X

After serum depletion of albumin and IgG, the acute-phase protein alpha-1-acid-glycoprotein was identified to have increased SLe^x in the sera of pancreatic cancer (PaC) and some chronic pancreatitis (CP) patients compared to healthy controls.

We subsequently performed *N*-glycan sequencing to investigate glycosylation of other major APP including alpha-1-acid-glycoprotein (AGP), haptoglobin (HPT), fetuin (FET), alpha-1-antitrypsin (AT) and transferrin (TRF) in PaC and CP patients and healthy controls, which were isolated from the sera of the patients by 2-DE. The *N*-glycans of these proteins appeared to be all complex type, and presented bi, tri and tetranntenary structures. TRF contained only bi and triantennary glycans and also bisected structures, which were not detected in the other APP. Several *N*-glycans were outer arm fucosylated and only one minor structure F(6)A2G(4)2S2, present in all APP, was core fucosylated. TRF contained in addition other core fucosylated structures. No GalNAc was detected. All galactoses were beta 1-4 linked to GlcNAc and all outer arm fucoses were alpha 1-3 linked. Thus, the summation of the relative peak areas containing sialylated structures with outer arm fucose linked to GlcNAc which was simultaneously attached to galactose was taken as a measure of the SLe^x antigen.

An increased SLe^x was observed in AGP, HPT and TRF in advanced PaC patients but also in the CP patients. This last group of patients showed in addition increased SLe^x in FET and AT (Sarrats *et al.* 2010b). AGP has been described to carry an increased SLe^x type fucosylation in patients

with acute inflammation (De Graaf *et al.* 1993; Brinkman-van der Linden *et al.* 1998; Higai *et al.* 2005) and chronic inflammation (rheumatoid arthritis and diabetes) (Brinkman-van der Linden *et al.* 1998; Poland *et al.* 2001; Higai *et al.* 2003; Higai *et al.* 2005), ovarian cancer (Saldova *et al.* 2007) and breast cancer (Abd Hamid *et al.* 2008). Overall fucosylation on AGP measured with crossed affinoimmunoelectrophoresis with *Aleuria aurantia* lectin (AAL) and anti-AGP antibody was proposed as a marker of progression and prognosis in different types of malignancies (Hashimoto *et al.* 2004). This increased fucosylation could be linked to an increased SLe^x expression as AAL has been described to have affinity for core fucose (Debray and Montreuil 1989), terminal fucose with any type of linkage (Kochibe and Furukawa 1980) including SLe^x determinant, which contains a terminal alpha 1-3 linked fucose (Haselhorst *et al.* 2001).

Using the same method, increased fucosylation on HPT have been observed in both acute (severe trauma) and chronic inflammation (rheumatic arthritis),These changes were linked to increased SLe^x on HPT, confirmed by Western-blot with a monoclonal anti-SLe^x antibody (Brinkman-van der Linden *et al.* 1998). Enhanced SLe^x type fucosylation have been described in different types of cancers including advanced ovarian cancer (Saldova *et al.* 2007) and prostate cancer (Fujimura *et al.* 2008). Lung cancer (Kossowska *et al.* 2005), pancreatic cancer (Okuyama *et al.* 2006) and pancreatitis patients (Nakano *et al.* 2008) showed increased AAL reactivity in HPT blots, which may be related, as commented above, to a rise of HPT glycoforms containing SLe^x. This last work with pancreatic cancer and pancreatitis patients would be in agreement with our results. Increased alpha 1-3 fucosylation has been described in transferrin in hepatocellular carcinoma patients (Yamashita *et al.* 1989).

Thus, we first proposed that increased SLe^x in APP were rather related to the inflammation than cancer itself and, therefore, did not have tumour diagnostic relevance. Cytokines involved in the induction of the inflammatory reaction have been described to regulate both APP synthesis and glycosylation (Gabay and Kushner 1999). Serum cytokines commonly increased in the sera of CP and PaC patients (Zeh *et al.* 2005) could regulate in a similar way liver glycosyltransferases, which would result in increased SLe^x on most the proteins synthesized by the hepatocytes, including the APP. In support to this hypothesis, stimulation of the hepatoma cell line HUH-7 with IL-1β showed an increase SLe^x on secreted AGP by enhancing the expression of the beta-galactoside alpha 2-3 sialyltransferase IV (ST3 Gal IV) and fucosyltransferase VI (FUT VI) (Azuma *et al.* 2000; Higai *et al.* 2006). Stimulation of hepatoma cells with IL-6 increased HPT AAL detected fucosylation, which was linked to an enhanced

expression of fucosylation-related genes such FUT6 and FUT8, GDP-fucose synthase (FX) and GDP-mannose-4,6-dehydratase (GMD) (Narisada *et al.* 2008).

However, after serum depletion of the twelve most abundant serum proteins, including the previously studied APP, we identified other protein bands with specific SLe^x increased immunoreaction only in the PaC patients. The glycoproteins identified in these bands corresponded to alpha-2-macroglobulin (A2M), ceruloplasmin (CERU), inter-alpha-trypsin inhibitor heavy chain H4 (ITH4), complement C3 (C3), complement component C6 (C6), complement C4-A (C4A), complement C4-B (C4B). C3 have been described to show an increased AAL reactivity in the plasma from colorectal cancer patients in comparison to adenoma and healthy individuals (Qiu *et al.* 2008). The increase of SLe^x in these other APP only in the PaC group of patients could be due to the fact that we were studying a different set of patients or to that we are considering different proteins whose glycosylation may be actually differently regulated. Therefore, extension of these studies to a larger cohort of patients is required to clearly state the significance of SLe^x increase in A2M, CERU, ITH4, C3, C6, and C4.

Changes in Acute-phase proteins branching

Using *N*-glycan sequencing we observed an increase in branching in the APP isolated by 2-DE. In particular, an increase in the amount of tetra-antennary structures on AGP and HPT was observed in CP and in advanced PaC, which may be linked to an up-regulation of *N*-acetylglucosaminyltransferase V (GnT-V), the enzyme responsible for the beta 1-6 branching (Taniguchi *et al.* 1999). A concomitant decrease in tri-antennary structures was observed in advanced patients, while in CP patients, the percentage of biantennary glycans was decreased. FET showed the same changes in CP patients but more significantly, and in addition, an increase of triantennary structures was also observed. These results suggest that N-acetylglucosaminyltransferase IV (GnT-IV), the enzyme responsible for the addition of the beta 1-4 branching (third antennae), may also be up-regulated in CP.

TRF showed a different repertoire of *N*-glycan structures but a change in branching pattern was also detected. TRF showed a decrease in biantennary structures in both advanced PaC and CP patients concomitant with an increase in bisected and tri-antennary glycans (Sarrats *et al.* 2010b).

Substantial increases in AGP glycoforms expressing biantennary glycans are apparent in the early phase of an acute-phase reaction. However, these are decreased to control levels after the second day after surgical trauma (De Graaf *et al.* 1993). There is no agreement regarding

branching changes observed in chronic inflammation. Some publications described an increase of biantennary glycans on AGP (Higai *et al.* 2005). Others reported that AGP Concanavalin A (ConA) reactivity showed a transition from initially elevated to decreased as disease became chronic, which indicates an increase in branching in chronic inflammation (Fassbender *et al.* 1991). Haptoglobin showed increased branching in ovarian cancer patients compared to controls (Turner 1995) and in prostate cancer compared to benign prostatic hyperplasia (Fujimura *et al.* 2008). Increase in TRF branching has been reported for hepatocellular carcinoma compared to healthy controls (Yamashita *et al.* 1989) and for chronic inflammation, in particular in different types of rheumatoid arthritis (Feelders *et al.* 1992).

Likewise SLe^x, in the APP whose *N*-glycans have been characterized (AGP, HPT, TRF and FET), we propose that increased branching in APP is rather related to the inflammation than cancer itself. Stimulation of the hepatic carcinoma cells HUH-7 with proinflammatory cytokines such as IL-1beta and/or IL-6 for 2 days increased AGP production and ConA reactivity (biantennary glycoforms). However, there was a decrease on AGP ConA reactivity after 5 days of stimulation, probably linked to a decrease of biantennary structures (increase of branching) (Azuma *et al.* 2000), consistently with our results, where an increase of branching is observed in chronic inflammation (pancreatitis). However, the mechanism by which these cytokines cause the branching modifications of APP has not yet been studied.

Changes in Acute-phase proteins core fucosylation

Core fucosylation of some APP was also determined using *N*-glycan sequencing experiments. Interestingly, core fucosylation of AGP and HPT was specifically increased in advanced pancreatic cancer patients but this increase was not significant for other APP in the same study with the same groups of patients, which supports the idea that glycosylation of APP may be differently regulated. CP patients also showed this modification although at a lower extent (Sarrats *et al.* 2010b). Consistently, increase in *Aspergillus oryzae* lectin (AOL) affinity of HPT has been previously described in PaC patients compared to controls (Okuyama *et al.* 2006). This lectin has strongest preference for the alpha1-6 fucose determinants rather that external ones (Matsumura *et al.* 2007). In the present work we have performed detailed *N*-glycan sequencing, which can discriminate between core and outer arm fucosylation and give more precise information than lectin binding studies. In addition, we have also included the pancreatitis patients group and have shown that increased core fucosylation is a cancer-specific modification of AGP and HPT. Lung cancer patients showed an increased core fucosylation in AGP and HPT compared to healthy controls (Ueda *et al.* 2007) and this *N*-glycan

modification was also observed in hepatoma patients compared to controls and chronic liver diseases in AGP, AT, FET and TRF (Matsumoto *et al.* 1994; Naitoh *et al.* 1999; Comunale *et al.* 2006), using different techniques such as MS, glycan sequencing or *Lens culinaris agglutinin* (LCA) which binds core fucose of bi and triantennary complex type *N*-glycans (Cummings and Etzler 2008).

Thus, core fucosylation of APP seems to be a modification more related to cancer than to inflammation. In particular the increased core fucosylation in APP may be linked to an increase of liver FUT VIII expression (Noda *et al.* 1998) which is the only known fucosyltransferase involved in the addition of core fucose (Miyoshi and Nakano 2008) and which is very low expressed in normal liver (Miyoshi *et al.* 1997). This increase in FUT VIII may be more marked in the PaC patients than in CP patients. Other mechanisms such as the enhancement in the levels of GDP-fucose synthase (FX) or GMD protein, which both contribute to the synthesis of GDP-fucose, or even in the levels of GDP-fucose transporter, could also occur and would contribute to the increase of the core fucosylated glycan structures.

Diagnostic relevance of Changes in Acute-phase proteins glycosylation

Increased core fucosylation on AGP and HPT seems to be a cancer-specific modification not related to inflammation, as described in this work (Sarrats *et al.* 2010b) and others (Okuyama *et al.* 2006) in PaC patients compared to the CP and HC groups. In a previous study of our group, serum Ribonuclease 1 was found to be much more core fucosylated in PaC sera than in control patients (Barrabes *et al.* 2007), which suggests that the quantification of core fucosylation in some PaC serum glycoproteins might be useful for PaC diagnostic or prognosis purposes. Core fucosylated alpha-fetoprotein, called AFP-L3, is very specifically increased in hepatocellular carcinoma and was approved as a tumour marker by the FDA in 2005 (Miyoshi *et al.* 2008).

Increases of SLe^x and branching and on certain APP such as AGP, HPT might reflect the inflammatory status of the patient, and could be used as markers of progression and prognosis of pancreatic and other cancers (Hashimoto *et al.* 2004; Abd Hamid *et al.* 2008). However, other APP such A2M, CERU, ITH4, C3, C6, C4-A and C4-B showed an specific SLe^x increase in PaC patients. The evaluation of SLe^x content of these proteins in a larger cohort of patients, together with the measurement of core fucosylation of AGP and HPT would determine their potential use as tumour markers. Since changes in SLe^x and core fucosylation of the studied APP have also been described in other cancer types, an overall study that could analyze APP *N*-glycan modifications in a larger number of samples including different types of malignancies

would be required to establish the clinical utility of APP core fucosylation or SLe^x changes in pancreatic adenocarcinoma.

The serum levels of these increased SLe^x APP range between 0.1-2 mg/mL, which precludes the detection of potential minor proteins with increased SLe^x derived from the pancreatic tumour. Thus, the discovery of PaC specific serum markers should be oriented either on the serum depletion of more liver APP in order to search for differential glycosylation of minor serum pancreas-specific proteins or in the evaluation of proteins glycoforms specifically expressed by the pancreatic tumour tissues that may reach the bloodstream.

Biological implication of Changes in Acute-phase proteins glycosylation

It is not clear whether APP biological functions could be affected by APP altered glycosylation in cancer and in inflammation. It has been proposed that the increase in SLe^x-substituted glycans on AGP might represent a mechanism for feedback inhibition of leukocyte extravasation into inflamed tissues. AGP expressing SLe^x may interact with E-selectin expressed at the surface of endothelial cells and compete with leukocytes also expressing SLe^x (Fournier *et al.* 2000). In addition, it may be stated that the terminal sialic acid alpha 2-3 on SLe^x determinant reduces the amount of free galactose accessible to the asialoglycoprotein cell receptor in the liver, which was initially described to clear-up glycoproteins with terminal galactose or GalNAc from circulation (Ashwell and Harford 1982). Recently, it has been demonstrated that this receptor can also mediate the clearance of glycoproteins with terminal sialic acid linked alpha 2-6 to galactose or GalNAc (Park *et al.* 2005; Steirer *et al.* 2009) .Thus, these glycoproteins would be cleared from blood at a more rapid rate than those terminating with sialic acid alpha 2-3 linked, including the ones bearing the SLe^x epitope. The presence of SLe^x glycans in APP in cancer and also in inflammation could therefore increase the serum concentrations of these APP. Certain APP such as AGP and AT have shown anti-apoptotic properties in animal models (Daemen *et al.* 2000), thus their increased serum levels in cancer patients may aid tumour progression.

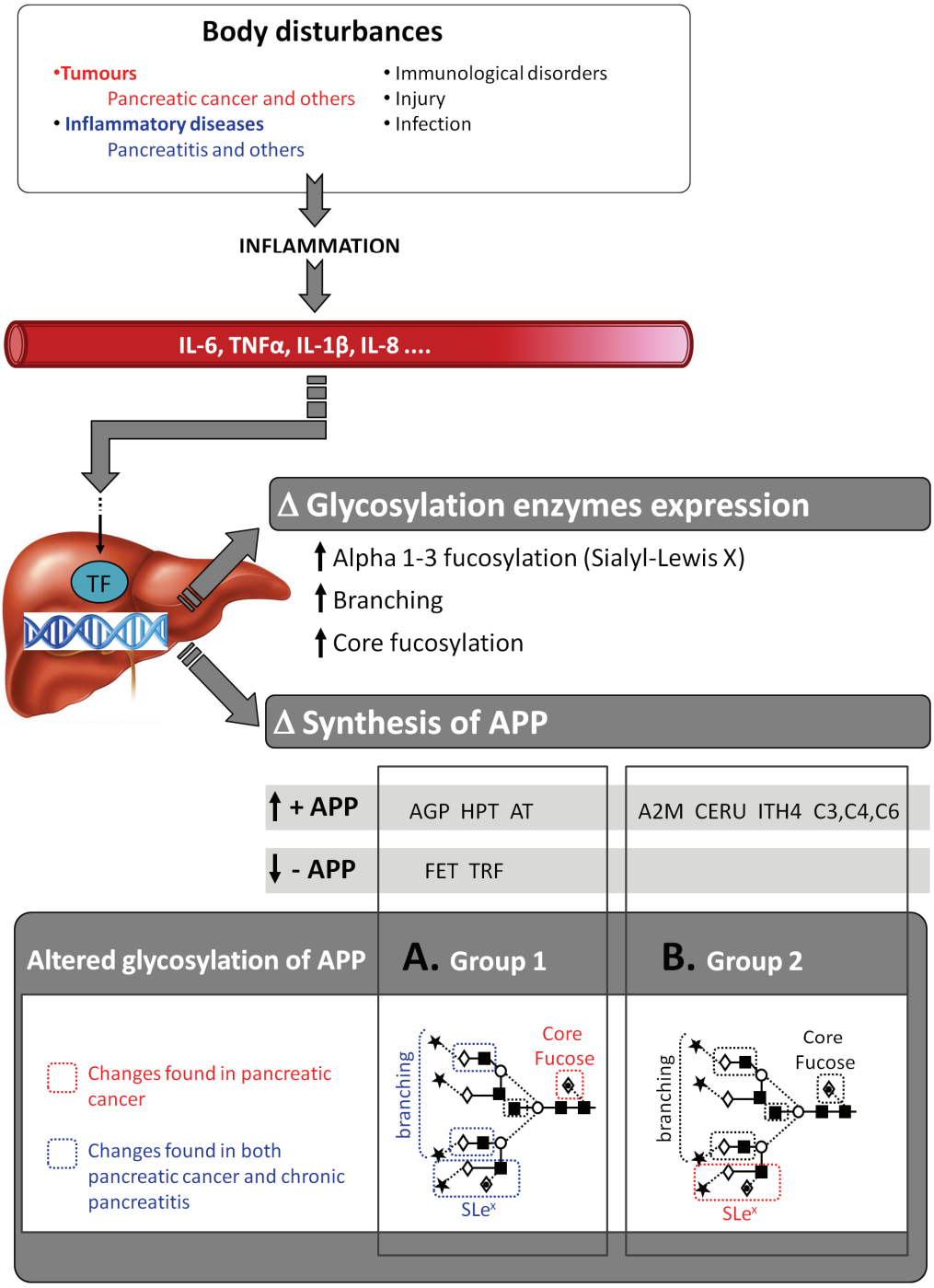


Figure 2. Acute-phase proteins (APP) changes in inflammation and malignant diseases. The serum levels of APP are modified as a response to several body disturbances that initiate the acute-phase response. The released inflammatory cytokines also modify the glycosylation machinery of hepatocytes and several glycosyltransferases may alter their expression levels, which lead to changes in APP glycosylation. Glycan example of a complex tetra-antennary structure, containing SLe^x and core fucose determinants is shown to illustrate glycosylation changes of studied APP in pancreatic cancer (PaC) and chronic pancreatitis patients (CP) compared to healthy controls (HC); (A.) Glycosylation of APP in group 1 was studied by anti SLe^x western-blot and *N*-glycan sequencing. Group 1 APP showed increased branching and SLe^x in both PaC and CP patients and increased core fucosylation specifically in PaC in AGP and HPT; (B.) Glycosylation of APP in group 2 was studied only by anti SLe^x western-blot, Group 2 APP showed increased SLe^x in PaC compared to HC and CP. Key to abbreviations: A2M, alpha-2-macroglobulin; AGP, alpha-1-acid-glycoprotein; APP; acute-phase proteins; AT; alpha-1-antitrypsin; CERU, ceruloplasmin; C3, complement C3; C4, complement C4; C6, complement component C6; FET; fetuin; HPT, haptoglobin; IL, interleukin; ITH4, inter-alpha-trypsin inhibitor heavy chain H4; TF, transcription factors; TNF, tumour necrosis factor; TRF, transferrin. Extracted and modified from Peracaula *et al.* 2010.

Increased branching creates more sites for terminal sialic acid residues and together with the up-regulation of sialyltransferases results in increased sialylation (Orntoft and Vestergaard 1999). The increase of APP tetraantennary structures in pancreatic cancer patients and pancreatitis may consequently contribute to the enhanced SLe^x of these glycoproteins.

As commented before, APP are synthesized mainly by the hepatocytes. There are two kinds of secretion pathways in the system of hepatocytes. One pathway is to an apical surface of the hepatocyte followed by secretion into bile ducts. The other is to basolateral surface followed by secretion into blood vessels. Both core and alpha 1-3 fucosylation have been proposed to increase the secretion of liver AT, HPT and AGP into the bile ducts as increased fucosylated glycans of these APP were detected in this fluid compared to serum samples of the same patients. In addition, FUT 8 deficient mice showed decrease levels of biliary AT and AGP but unaffected sera levels of these proteins (Nakagawa *et al.* 2006). However, the role of increased APP core fucosylation in cancer still remains unknown and it would be an interesting subject matter of further studies.

Tumour-derived and Host-response glycoproteins as tumor markers

Cancer cells show altered glycosylation of their cell surface and their secreted glycoconjugates (Varki *et al.* 2008). This altered glycosylation is thought to be caused by factors such as epigenetic silencing of the genes involved in normal glycan synthesis and tumour hypoxia (Kannagi *et al.* 2010). For example, the epigenetic silencing of the sialyltransferase gene responsible for α2-6 sialylation of the GlcNAc moiety is involved in the transition from the normal glycan, Disialyl Lewis A, expressed in non-malignant epithelial cells to the cancer-associated glycan, Sialyl Lewis A (Miyazaki *et al.* 2004). Similarly, it was found that the transcription of several genes involved in glycan sulphation is repressed in cancer cells but not in non-malignant epithelial cells, including those for 6-sulphotransferase, which explains the transition from the normal glycan epitop Sialyl 6-Sulpho Lewis X to Sialyl Lewis X in malignant transformation (Miyazaki *et al.* 2008). Tumor hypoxia further accelerates abnormal glycan expression in cancer cells due to transcriptional induction of a set of genes involved in glycan synthesis. For instance, genes for some sialyltransferases, fucosyltransferases, and sugar transporters are induced by hypoxia, which are involved in the synthesis of Sialyl Lewis A and Sialyl Lewis X glycans (Koike *et al.* 2004).

In addition, inflammatory cytokines can modulate the expression of certain genes involved in glycan synthesis. As discussed before, stimulation of hepatoma cell lines with certain

interleukynes lead to an enhanced expression of certain sialyltransferases, fucosyltransferases and other fucosylation related genes involved in Sialyl-Lewis antigens synthesis (Higai *et al.* 2005; Narisada *et al.* 2008).

The behavior of cancer cells results from a multi-complex process of host and environmental factors interactions. It is well established that interactions between tumor cells and the host tissue stroma play a key role in cancer progression (Tlsty and Coussens 2006). More recent advances have revealed that tumor-host interactions extend well beyond the local tissue microenvironment and that tumors not only respond to, but actively perturb host organs at distant anatomic sites (McAllister and Weinberg 2010). This indicates that many aspects of tumor biology can only be explained by a detailed understanding of both local and systemic interactions. Thefore, not only alteration of cancer cells may be useful tumour markers but also changes in other host organs as a result of cancer. In this regard, healthy cells of a cancer patient may also show changes of glycosylation as a result of altered glycosylation machinery induced by the presence of the tumour, probably due to circulating cytokines. Thus, serum glycoproteins of cancer patients may show altered glycosylation either if their biosynthesis is taking place in the cancer cell itself or if they are being synthesized by another healthy organ.

Serum Prostate-specific antigen shows altered glycosylation in prostate cancer patients compared to benign prostatic hyperplasia patients at fucosylation and sialylation level. This protein in produced specifically in the prostate tissue, thus this PSA with altered glycosylation would be produced by the prostate cancer cells and then it could reach the blood stream easily due to disruption of the prostate basal membrane.

On the other hand, some acute-phase proteins (APP) show altered glycosylation in pancreatic cancer compared to chronic pancreatitis patients and healthy individuals. This is the case of AGP and HPT that present an increase of core fucosylation and A2M, CERU, ITH4, C3, C6, and C4 that present an increase of SLe^x. These proteins are mainly produced by the liver. Thus, these APP glycan modifications are likely due to glycosylation biosynthesis alteration on hepatocytes, induced by the presence of pancreatic disorders. Although the ideal tumour marker is a glycoform of a protein secreted specifically by the tumour (because it is more likely to show high specificity in diagnosis of a certain type of cancer), glycoforms of a protein secreted by a healthy tissue of a cancer patient may reflect systemic effects of the illness and therefore be used as progression or monitoring tumour marker.

Chapter 8

Conclusions

1. The separation of serum Prostate-specific antigen (PSA), including free PSA and PSA released from ACT complex, from Prostate cancer (PCa) and benign prostatic hyperplasia (BPH) patients by two-dimensional electrophoresis resulted in 5 main subforms of the same molecular weight that were named F1 to F5 from the more acidic to the less acidic form. The different isoelectric point of F5, F4 and F5 was attributed to their sialyation degree. However, the biochemical differences among F1, F2 and F3 that lead them to show a different pI remain still unknown.

2. Relative percentage of F3 was significantly decreased in the PCa patients compared to BPH patients. This variable showed a tendency of better discriminatory power than the currently used total PSA and percentage of free PSA tests. The different proportion of F3 in the two groups of patients was attributed to different sialylation degree of PSA, which is lower in the PCa group.

3. *N*-glycan sequencing showed a decreased core fucosylation and an increase of alpha 2-3 sialic acid in the major PSA subform F3 when comparing serum PSA of a metastatic PCa patient and seminal plasma PSA from a healthy donor.

4. Assays to easily measure sialylation degree and type and core fucosylation degree of serum PSA should be developed in order to evaluate these changes in a larger cohort of PCa and BPH patients and define their potential clinical application.

5. Some serum acute-phase proteins (APP) showed increased Sialyl-Lewis X (SLe^x) in pancreatic cancer (PaC) patients and chronic pancreatitis (CP) patients compared to healthy controls. Alpha-1-acid-glycoprotein, haptoglobin and transferring showed increased SLe^x in advanced PaC and CP patients. These modifications may be linked to both cancer and inflammation and may not have diagnostic relevance. However they may be important as prognosis or monitoring markers.

6. Other APP showed an increased SLe^x only in PaC patients. SLe^x immunoreactive protein bands specifically increased in the PaC patients and not in the CP patients, contained alpha-2-macroglobulin, ceruloplasmin, inter-alpha-trypsin inhibitor heavy chain H4, complement C3, complement component C6, complement C4-A and complement C4-B. These modifications may be linked to cancer rather than to inflammation, although they may be found in other cancer types different that PaC. Thus, they may be used in the differentiation of PCa and CP but not in PaC screening.

7. Core fucosylation of alpha-1-acid-glycoprotein and haptoglobin was increased the PaC patients compared to CP and healthy controls. These modifications may be cancer-specific, although they are found in other cancer types different than PaC. Thus, they may be used in the differentiation of PCa and CP but not in PaC screening.

8. SLe^x and core fucosylation of the above reported APP should be evaluated in a larger cohort of patients, incluing CP, PaC patients of different stages and other cancer types to define their potential clinical use as diagnostic, monitoring or prognostic markers.

9. The serum levels APP precludes the detection of potential minor proteins with increased SLe^x derived from the pancreatic tumour, even after the depletion of the twelve most abundant serum proteins.

10. Serum glycoproteins of cancer patients may show altered glycosylation either if their biosynthesis takes place in the cancer cell or if they are synthesized by another healthy organ. Glycan alterations of serum PSA may reflect altered glycosylation of prostate cancer cells. Glycan alterations of APP in PaC and CP patients show hepatocytes alteration of the glycosylation synthesis induced by the presence of a pancreatic disorder.

References

Abd Hamid, U. M., Royle, L., Saldova, R., Radcliffe, C. M., Harvey, D. J., Storr, S. J., Pardo, M., Antrobus, R., Chapman, C. J., Zitzmann, N., Robertson, J. F., Dwek, R. A. and Rudd, P. M. (2008). A strategy to reveal potential glycan markers from serum glycoproteins associated with breast cancer progression. *Glycobiology* 18(12):1105-1118.

Alberts, B., Jonhson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002). Molecular Biology of the Cell. Fourth edition. New York, Garland Science.

Amado, M., Carneiro, F., Seixas, M., Clausen, H. and Sobrinho-Simoes, M. (1998). Dimeric sialyl-Le(x) expression in gastric carcinoma correlates with venous invasion and poor outcome. *Gastroenterology* 114(3):462-470.

Andriole, G. L., Crawford, E. D., Grubb, R. L., 3rd, Buys, S. S., Chia, D., Church, T. R., Fouad, M. N., Gelmann, E. P., Kvale, P. A., Reding, D. J., Weissfeld, J. L., Yokochi, L. A., O'Brien, B., Clapp, J. D., Rathmell, J. M., Riley, T. L., Hayes, R. B., Kramer, B. S., Izmirlian, G., Miller, A. B., Pinsky, P. F., Prorok, P. C., Gohagan, J. K. and Berg, C. D. (2009). Mortality results from a randomized prostate-cancer screening trial. *N Engl J Med* 360(13):1310-1319.

Apweiler, R., Hermjakob, H. and Sharon, N. (1999). On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta* 1473(1):4-8.

Arango, J. and Pierce, M. (1988). Comparison of N-acetylglucosaminyltransferase V activities in Rous sarcoma-transformed baby hamster kidney (RS-BHK) and BHK cells. *J Cell Biochem* 37(2):225-231.

Arnold, J. N., Wallis, R., Willis, A. C., Harvey, D. J., Royle, L., Dwek, R. A., Rudd, P. M. and Sim, R. B. (2006). Interaction of mannan binding lectin with alpha2 macroglobulin via exposed oligomannose glycans: a conserved feature of the thiol ester protein family? *J Biol Chem* 281(11):6955-6963.

Ashwell, G. and Harford, J. (1982). Carbohydrate-specific receptors of the liver. *Annu Rev Biochem* 51:531-554.

Azuma, Y., Murata, M. and Matsumoto, K. (2000). Alteration of sugar chains on alpha(1)-acid glycoprotein secreted following cytokine stimulation of HuH-7 cells in vitro. *Clin Chim Acta* 294(1-2):93-103.

Balk, S. P., Ko, Y. J. and Bubley, G. J. (2003). Biology of prostate-specific antigen. *J Clin Oncol* 21(2):383-391.

Barak, M., Mecz, Y., Lurie, A. and Gruener, N. (1989). Binding of serum prostate antigen to concanavalin A in patients with cancer or hyperplasia of the prostate. *Oncology* 46(6):375-377.

Bardeesy, N. and DePinho, R. A. (2002). Pancreatic cancer biology and genetics. *Nat Rev Cancer* 2(12):897-909.

Barrabes, S., Pages-Pons, L., Radcliffe, C. M., Tabares, G., Fort, E., Royle, L., Harvey, D. J., Moenner, M., Dwek, R. A., Rudd, P. M., De Llorens, R. and Peracaula, R. (2007). Glycosylation of serum ribonuclease 1 indicates a major endothelial origin and reveals an increase in core fucosylation in pancreatic cancer. *Glycobiology* 17(4):388-400.

Barry, M. J. (2009). Screening for prostate cancer--the controversy that refuses to die. *N Engl J Med* 360(13):1351-1354.

Barthel, S. R., Gavino, J. D., Wiese, G. K., Jaynes, J. M., Siddiqui, J. and Dimitroff, C. J. (2008). Analysis of glycosyltransferase expression in metastatic prostate cancer cells capable of rolling activity on microvascular endothelial (E)-selectin. *Glycobiology* 18(10):806-817.

Basu, P. S., Majhi, R. and Batabyal, S. K. (2003). Lectin and serum-PSA interaction as a screening test for prostate cancer. *Clin Biochem* 36(5):373-376.

Belanger, A., van Halbeek, H., Graves, H. C., Grandbois, K., Stamey, T. A., Huang, L., Poppe, I. and Labrie, F. (1995). Molecular mass and carbohydrate structure of prostate specific antigen: studies for establishment of an international PSA standard. *Prostate* 27(4):187-197.

Belt, K. T., Carroll, M. C. and Porter, R. R. (1984). The structural basis of the multiple forms of human complement component C4. *Cell* 36(4):907-914.

Bertozzi, C. and Rabuka, D. (2008). Structural Basis of Glycan Diversity. In: Varki, A., Cummings, R., Esko, J., Freeze, H., Stanley, P., Bertozzi, C., Hart, G. and Etzler, M. *Essentials of Glycobiology. 2nd ed.* New York, Cold Spring Harbour Laboratory press:23-36.

Bhavanandan, V. P. (1991). Cancer-associated mucins and mucin-type glycoproteins. *Glycobiology* 1(5):493-503.

Blanchong, C. A., Chung, E. K., Rupert, K. L., Yang, Y., Yang, Z., Zhou, B., Moulds, J. M. and Yu, C. Y. (2001). Genetic, structural and functional diversities of human complement components C4A and C4B and their mouse homologues, Slp and C4. *Int Immunopharmacol* 1(3):365-392.

Bracarda, S., de Cobelli, O., Greco, C., Prayer-Galetti, T., Valdagni, R., Gatta, G., de Braud, F. and Bartsch, G. (2005). Cancer of the prostate. *Crit Rev Oncol Hematol* 56(3):379-396.

Brawer, M. K., Meyer, G. E., Letran, J. L., Bankson, D. D., Morris, D. L., Yeung, K. K. and Allard, W. J. (1998). Measurement of complexed PSA improves specificity for early detection of prostate cancer. *Urology* 52(3):372-378.

Brinkman-van der Linden, E. C., de Haan, P. F., Havenaar, E. C. and van Dijk, W. (1998). Inflammation-induced expression of sialyl LewisX is not restricted to alpha1-acid glycoprotein but also occurs to a lesser extent on alpha1-antichymotrypsin and haptoglobin. *Glycoconj J* 15(2):177-182.

Carvalho, A. S., Harduin-Lepers, A., Magalhaes, A., Machado, E., Mendes, N., Costa, L. T., Matthiesen, R., Almeida, R., Costa, J. and Reis, C. A. (2010). Differential expression of alpha-2,3-sialyltransferases and alpha-1,3/4-fucosyltransferases regulates the levels of sialyl Lewis a and sialyl Lewis x in gastrointestinal carcinoma cells. *Int J Biochem Cell Biol* 42(1):80-89.

Catalona, W. J., Richie, J. P., Ahmann, F. R., Hudson, M. A., Scardino, P. T., Flanigan, R. C., deKernion, J. B., Ratliff, T. L., Kavoussi, L. R., Dalkin, B. L. and et al. (1994). Comparison of digital rectal examination and serum prostate specific antigen in the early detection of prostate cancer: results of a multicenter clinical trial of 6,630 men. *J Urol* 151(5):1283-1290.

Catalona, W. J., Smith, D. S., Ratliff, T. L., Dodds, K. M., Coplen, D. E., Yuan, J. J., Petros, J. A. and Andriole, G. L. (1991). Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. *N Engl J Med* 324(17):1156-1161.

Catalona, W. J., Southwick, P. C., Slawin, K. M., Partin, A. W., Brawer, M. K., Flanigan, R. C., Patel, A., Richie, J. P., Walsh, P. C., Scardino, P. T., Lange, P. H., Gasior, G. H., Loveland, K. G. and Bray, K. R. (2000). Comparison of percent free PSA, PSA density, and age-specific PSA cutoffs for prostate cancer detection and staging. *Urology* 56(2):255-260.

Ceciliani, F., Giordano, A. and Spagnolo, V. (2002). The systemic reaction during inflammation: the acute-phase proteins. *Protein Pept Lett* 9(3):211-223.

Chan, A. C. and Atkinson, J. P. (1983). Identification and structural characterization of two incompletely processed forms of the fourth component of human complement. *J Clin Invest* 72(5):1639-1649.

Chan, A. C. and Atkinson, J. P. (1985). Oligosaccharide structure of human C4. *J Immunol* 134(3):1790-1798.

Charrier, J. P., Tournel, C., Michel, S., Comby, S., Jolivet-Reynaud, C., Passagot, J., Dalbon, P., Chautard, D. and Jolivet, M. (2001). Differential diagnosis of prostate cancer and benign prostate hyperplasia using two-dimensional electrophoresis. *Electrophoresis* 22(9):1861-1866.

Charrier, J. P., Tournel, C., Michel, S., Dalbon, P. and Jolivet, M. (1999). Two-dimensional electrophoresis of prostate-specific antigen in sera of men with prostate cancer or benign prostate hyperplasia. *Electrophoresis* 20(4-5):1075-1081.

Chen, R., Jiang, X., Sun, D., Han, G., Wang, F., Ye, M., Wang, L. and Zou, H. (2009). Glycoproteomics analysis of human liver tissue by combination of multiple enzyme digestion and hydrazide chemistry. *J Proteome Res* 8(2):651-661.

Choi-Miura, N. H. (2001). Quantitative measurement of the novel human plasma protein, IHRP, by sandwich ELISA. *Biol Pharm Bull* 24(3):214-217.

Cohen, P., Peehl, D. M., Graves, H. C. and Rosenfeld, R. G. (1994). Biological effects of prostate specific antigen as an insulin-like growth factor binding protein-3 protease. *J Endocrinol* 142(3):407-415.

Comunale, M. A., Lowman, M., Long, R. E., Krakover, J., Philip, R., Seeholzer, S., Evans, A. A., Hann, H. W., Block, T. M. and Mehta, A. S. (2006). Proteomic analysis of serum associated fucosylated glycoproteins in the development of primary hepatocellular carcinoma. *J Proteome Res* 5(2):308-315.

Cummings, R. and Etzler, M. (2008). Antibodies and Lectins in Glycan Analysis. In: Varki, A., Cummings, R., Esko, J., Freeze, H., Stanley, P., Bertozzi, C., Hart, G. and Etzler, M. *Essentials of Glycobiology. 2nd ed.* New York, Cold Spring Harbour Laboratory press.

Daemen, M. A., Heemskerk, V. H., van't Veer, C., Denecker, G., Wolfs, T. G., Vandenabeele, P. and Buurman, W. A. (2000). Functional protection by acute phase proteins alpha(1)-acid glycoprotein and alpha(1)-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. *Circulation* 102(12):1420-1426.

Damber, J. E. and Aus, G. (2008). Prostate cancer. *Lancet* 371(9625):1710-1721.

De Graaf, T. W., Van der Stelt, M. E., Anbergen, M. G. and van Dijk, W. (1993). Inflammation-induced expression of sialyl Lewis X-containing glycan structures on alpha 1-acid glycoprotein (orosomucoid) in human sera. *J Exp Med* 177(3):657-666.

de Vries, T., Knegtel, R. M., Holmes, E. H. and Macher, B. A. (2001). Fucosyltransferases: structure/function studies. *Glycobiology* 11(10):119R-128R.

Debray, H. and Montreuil, J. (1989). Aleuria aurantia agglutinin. A new isolation procedure and further study of its specificity towards various glycopeptides and oligosaccharides. *Carbohydr Res* 185(1):15-26.

Demetriou, M., Nabi, I. R., Coppolino, M., Dedhar, S. and Dennis, J. W. (1995). Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. *J Cell Biol* 130(2):383-392.

Diamandis, E. P. (1998). Prostate-specific Antigen: Its Usefulness in Clinical Medicine. *Trends Endocrinol Metab* 9(8):310-316.

Drake, R. R., White, K. Y., Fuller, T. W., Igwe, E., Clements, M. A., Nyalwidhe, J. O., Given, R. W., Lance, R. S. and Semmes, O. J. (2009). Clinical collection and protein properties of expressed prostatic secretions as a source for biomarkers of prostatic disease. *J Proteomics* 72(6):907-917.

Duus, J., Gotfredsen, C. H. and Bock, K. (2000). Carbohydrate structural determination by NMR spectroscopy: modern methods and limitations. *Chem Rev* 100(12):4589-4614.

Eissa, S. and Sohair, S. (1998). Tumor Markers. London, Chapman & Hall Medical cop.

Fassbender, K., Zimmerli, W., Kissling, R., Sobieska, M., Aeschlimann, A., Kellner, M. and Muller, W. (1991). Glycosylation of alpha 1-acid glycoprotein in relation to duration of disease in acute and chronic infection and inflammation. *Clin Chim Acta* 203(2-3):315-327.

Feelders, R. A., Vreugdenhil, G., de Jong, G., Swaak, A. J. and van Eijk, H. G. (1992). Transferrin microheterogeneity in rheumatoid arthritis. Relation with disease activity and anemia of chronic disease. *Rheumatol Int* 12(5):195-199.

Ferlay, J., Parkin, D. M. and Steliarova-Foucher, E. (2010a). Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer*.

Ferlay, J., Parkin, D. M. and Steliarova-Foucher, E. (2010b). Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer* 46(4):765-781.

Fournier, T., Medjoubi, N. N. and Porquet, D. (2000). Alpha-1-acid glycoprotein. *Biochim Biophys Acta* 1482(1-2):157-171.

Fujii, K., Nakano, T., Kanazawa, M., Akimoto, S., Hirano, T., Kato, H. and Nishimura, T. (2005). Clinical-scale high-throughput human plasma proteome analysis: lung adenocarcinoma. *Proteomics* 5(4):1150-1159.

Fujimura, T., Shinohara, Y., Tissot, B., Pang, P. C., Kurogochi, M., Saito, S., Arai, Y., Sadilek, M., Murayama, K., Dell, A., Nishimura, S. and Hakomori, S. I. (2008). Glycosylation status of haptoglobin in sera of patients with prostate cancer vs. benign prostate disease or normal subjects. *Int J Cancer* 122(1):39-49.

Fuster, M. M. and Esko, J. D. (2005). The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat Rev Cancer* 5(7):526-542.

Gabay, C. and Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 340(6):448-454.

Ghaneh, P., Costello, E. and Neoptolemos, J. P. (2007). Biology and management of pancreatic cancer. *Gut* 56(8):1134-1152.

Gleason, D. F. and The Veteran's Administration Cooperative Urologic Research Group (1977). Histologic grading and clinical staging of prostatic carcinoma. In: (ed.), Tannenbaum, M. *Urologic Pathology: The Prostate*. Philadelphia, Lea and Febiger:171-198.

Goelz, S., Kumar, R., Potvin, B., Sundaram, S., Brickelmaier, M. and Stanley, P. (1994). Differential expression of an E-selectin ligand (SLe^x) by two Chinese hamster ovary cell lines transfected with the same alpha (1,3)-fucosyltransferase gene (ELFT). *J Biol Chem* 269(2):1033-1040.

Gold, D. V., Modrak, D. E., Ying, Z., Cardillo, T. M., Sharkey, R. M. and Goldenberg, D. M. (2006). New MUC1 serum immunoassay differentiates pancreatic cancer from pancreatitis. *J Clin Oncol* 24(2):252-258.

Greenberg, D., Earle, C., Fang, C. H., Eldar-Lissai, A. and Neumann, P. J. (2010). When is cancer care cost-effective? A systematic overview of cost-utility analyses in oncology. *J Natl Cancer Inst* 102(2):82-88.

Groskopf, J., Aubin, S. M., Deras, I. L., Blase, A., Bodrug, S., Clark, C., Brentano, S., Mathis, J., Pham, J., Meyer, T., Cass, M., Hodge, P., Macairan, M. L., Marks, L. S. and Rittenhouse, H. (2006). APTIMA PCA3 molecular urine test: development of a method to aid in the diagnosis of prostate cancer. *Clin Chem* 52(6):1089-1095.

Grote, T. and Logsdon, C. D. (2007). Progress on molecular markers of pancreatic cancer. *Curr Opin Gastroenterol* 23(5):508-514.

Gruys, E., Toussaint, M. J., Niewold, T. A. and Koopmans, S. J. (2005). Acute phase reaction and acute phase proteins. *J Zhejiang Univ Sci B* 6(11):1045-1056.

Gutman, A. B. and Gutman, E. B. (1938). An " Acid " Phosphatase Occurring in the Serum of Patients with Metastasizing Carcinoma of the Prostate Gland. *J Clin Invest* 17(4):473-478.

Hanahan, D. and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100(1):57-70.

Handerson, T., Camp, R., Harigopal, M., Rimm, D. and Pawelek, J. (2005). Beta1,6-branched oligosaccharides are increased in lymph node metastases and predict poor outcome in breast carcinoma. *Clin Cancer Res* 11(8):2969-2973.

Hansen, J. E., Iversen, J., Lihme, A. and Bog-Hansen, T. C. (1987). Acute phase reaction, heterogeneity, and microheterogeneity of serum proteins as nonspecific tumor markers in lung cancer. *Cancer* 60(7):1630-1635.

Harazono, A., Kawasaki, N., Itoh, S., Hashii, N., Ishii-Watabe, A., Kawanishi, T. and Hayakawa, T. (2006). Site-specific N-glycosylation analysis of human plasma ceruloplasmin using liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal Biochem* 348(2):259-268.

Harvey, D. J., Merry, A. H., Royle, L., Campbell, M. P., Dwek, R. A. and Rudd, P. M. (2009). Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds. *Proteomics* 9(15):3796-3801.

Harvey, D. J., Royle, L., Radcliffe, C. M., Rudd, P. M. and Dwek, R. A. (2008). Structural and quantitative analysis of N-linked glycans by matrix-assisted laser desorption ionization and negative ion nanospray mass spectrometry. *Anal Biochem* 376(1):44-60.

Haselhorst, T., Weimar, T. and Peters, T. (2001). Molecular recognition of sialyl Lewis(x) and related saccharides by two lectins. *J Am Chem Soc* 123(43):10705-10714.

Hashimoto, S., Asao, T., Takahashi, J., Yagihashi, Y., Nishimura, T., Saniabadi, A. R., Poland, D. C., van Dijk, W., Kuwano, H., Kochibe, N. and Yazawa, S. (2004). alpha1-acid glycoprotein fucosylation as a marker of carcinoma progression and prognosis. *Cancer* 101(12):2825-2836.

Heo, S. H., Lee, S. J., Ryoo, H. M., Park, J. Y. and Cho, J. Y. (2007). Identification of putative serum glycoprotein biomarkers for human lung adenocarcinoma by multilectin affinity chromatography and LC-MS/MS. *Proteomics* 7(23):4292-4302.

Herrala, A., Kurkela, R., Vihinen, M., Kalkkinen, N. and Vihko, P. (1998). Androgen-sensitive human prostate cancer cells, LNCaP, produce both N-terminally mature and truncated prostate-specific antigen isoforms. *Eur J Biochem* 255(2):329-335.

Higai, K., Aoki, Y., Azuma, Y. and Matsumoto, K. (2005). Glycosylation of site-specific glycans of alpha1-acid glycoprotein and alterations in acute and chronic inflammation. *Biochim Biophys Acta* 1725(1):128-135.

Higai, K., Azuma, Y., Aoki, Y. and Matsumoto, K. (2003). Altered glycosylation of alpha1-acid glycoprotein in patients with inflammation and diabetes mellitus. *Clin Chim Acta* 329(1-2):117-125.

Higai, K., Miyazaki, N., Azuma, Y. and Matsumoto, K. (2006). Interleukin-1beta induces sialyl Lewis X on hepatocellular carcinoma HuH-7 cells via enhanced expression of ST3Gal IV and FUT VI gene. *FEBS Lett* 580(26):6069-6075.

Hosono, J., Narita, T., Kimura, N., Sato, M., Nakashio, T., Kasai, Y., Nonami, T., Nakao, A., Takagi, H. and Kannagi, R. (1998). Involvement of adhesion molecules in metastasis of SW1990, human pancreatic cancer cells. *J Surg Oncol* 67(2):77-84.

Huber, P. R., Schmid, H. P., Mattarelli, G., Strittmatter, B., van Steenbrugge, G. J. and Maurer, A. (1995). Serum free prostate specific antigen: isoenzymes in benign hyperplasia and cancer of the prostate. *Prostate* 27(4):212-219.

Humphrey, P. A. (2004). Gleason grading and prognostic factors in carcinoma of the prostate. *Mod Pathol* 17(3):292-306.

Isono, T., Tanaka, T., Kageyama, S. and Yoshiki, T. (2002). Structural diversity of cancer-related and non-cancer-related prostate-specific antigen. *Clin Chem* 48(12):2187-2194.

Iwamura, M., Hellman, J., Cockett, A. T., Lilja, H. and Gershagen, S. (1996). Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen. *Urology* 48(2):317-325.

Izawa, M., Kumamoto, K., Mitsuoka, C., Kanamori, C., Kanamori, A., Ohmori, K., Ishida, H., Nakamura, S., Kurata-Miura, K., Sasaki, K., Nishi, T. and Kannagi, R. (2000). Expression of sialyl 6-sulfo Lewis X is inversely correlated with conventional sialyl Lewis X expression in human colorectal cancer. *Cancer Res* 60(5):1410-1416.

Jankovic, M. M. and Kosanovic, M. M. (2005). Glycosylation of urinary prostate-specific antigen in benign hyperplasia and cancer: assessment by lectin-binding patterns. *Clin Biochem* 38(1):58-65.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J. and Thun, M. J. (2009). Cancer statistics, 2009. *CA Cancer J Clin* 59(4):225-249.

Joniau, S., Goeman, L., Pennings, J. and Van Poppel, H. (2005). Prostatic intraepithelial neoplasia (PIN): importance and clinical management. *Eur Urol* 48(3):379-385.

Jorgensen, T., Berner, A., Kaalhus, O., Tveter, K. J., Danielsen, H. E. and Bryne, M. (1995). Up-regulation of the oligosaccharide sialyl LewisX: a new prognostic parameter in metastatic prostate cancer. *Cancer Res* 55(9):1817-1819.

Jorgensen, T., Kanagasingam, Y., Kaalhus, O., Tveter, K. J., Bryne, M., Skjorten, F., Berner, A. and Danielsen, H. E. (1997). Prognostic factors in patients with metastatic (stage D2) prostate cancer: experience from the Scandinavian Prostatic Cancer Group Study-2. *J Urol* 158(1):164-170.

Jung, K., Reiche, J., Boehme, A., Stephan, C., Loening, S. A., Schnorr, D., Hoesel, W. and Sinha, P. (2004). Analysis of subforms of free prostate-specific antigen in serum by two-dimensional gel electrophoresis: potential to improve diagnosis of prostate cancer. *Clin Chem* 50(12):2292-2301.

Kannagi, R. (1997). Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. *Glycoconj J* 14(5):577-584.

Kannagi, R., Izawa, M., Koike, T., Miyazaki, K. and Kimura, N. (2004). Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. *Cancer Sci* 95(5):377-384.

Kannagi, R., Sakuma, K., Miyazaki, K., Lim, K. T., Yusa, A., Yin, J. and Izawa, M. (2010). Altered expression of glycan genes in cancers induced by epigenetic silencing and tumor hypoxia: clues in the ongoing search for new tumor markers. *Cancer Sci* 101(3):586-593.

Killian, C. S., Corral, D. A., Kawinski, E. and Constantine, R. I. (1993). Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent TGF-beta and a proteolytic modulation of cell adhesion receptors. *Biochem Biophys Res Commun* 192(2):940-947.

Kim, Y. S., Itzkowitz, S. H., Yuan, M., Chung, Y., Satake, K., Umeyama, K. and Hakomori, S. (1988). Le^x and Ley antigen expression in human pancreatic cancer. *Cancer Res* 48(2):475-482.

Kochibe, N. and Furukawa, K. (1980). Purification and properties of a novel fucose-specific hemagglutinin of Aleuria aurantia. *Biochemistry* 19(13):2841-2846.

Koike, T., Kimura, N., Miyazaki, K., Yabuta, T., Kumamoto, K., Takenoshita, S., Chen, J., Kobayashi, M., Hosokawa, M., Taniguchi, A., Kojima, T., Ishida, N., Kawakita, M., Yamamoto, H., Takematsu, H., Suzuki, A., Kozutsumi, Y. and Kannagi, R. (2004). Hypoxia induces adhesion molecules on cancer cells: A missing link between Warburg effect and induction of selectin-ligand carbohydrates. *Proc Natl Acad Sci U S A* 101(21):8132-8137.

Koopmann, J., Rosenzweig, C. N., Zhang, Z., Canto, M. I., Brown, D. A., Hunter, M., Yeo, C., Chan, D. W., Breit, S. N. and Goggins, M. (2006). Serum markers in patients with resectable pancreatic adenocarcinoma: macrophage inhibitory cytokine 1 versus CA19-9. *Clin Cancer Res* 12(2):442-446.

Kosanovic, M. M. and Jankovic, M. M. (2005). Sialylation and fucosylation of cancer-associated prostate specific antigen. *J Buon* 10(2):247-250.

Kossowska, B., Ferens-Sieczkowska, M., Gancarz, R., Passowicz-Muszynska, E. and Jankowska, R. (2005). Fucosylation of serum glycoproteins in lung cancer patients. *Clin Chem Lab Med* 43(4):361-369.

Kushner, I. (1982). The phenomenon of the acute phase response. *Ann N Y Acad Sci* 389:39-48.

Lack, E. E. (2003). Pancreatic Ductal Adenocarcinoma. *Pathology of the Pancreas, Gallbladder, Extrahepatic Biliary Tract and Ampullary Region*. New York, Oxford University Press.

Laguna, P. and Alivizatos, G. (2000). Prostate specific antigen and benign prostatic hyperplasia. *Curr Opin Urol* 10(1):3-8.

Leman, E. S., Cannon, G. W., Trock, B. J., Sokoll, L. J., Chan, D. W., Mangold, L., Partin, A. W. and Getzenberg, R. H. (2007). EPCA-2: a highly specific serum marker for prostate cancer. *Urology* 69(4):714-720.

Li, C., Simeone, D. M., Brenner, D. E., Anderson, M. A., Shedden, K. A., Ruffin, M. T. and Lubman, D. M. (2009). Pancreatic cancer serum detection using a lectin/glyco-antibody array method. *J Proteome Res* 8(2):483-492.

Li, C. M. and Hutchens, T. W. (1992). Chromatofocusing. In: Kenney, A. and Fowell, S. *Practical Protein Chromatography*. Pub. Date: Apr-09-1992;:237-248.

Lilja, H., Oldbring, J., Rannevik, G. and Laurell, C. B. (1987). Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. *J Clin Invest* 80(2):281-285.

Lilja, H., Ulmert, D. and Vickers, A. J. (2008). Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. *Nat Rev Cancer* 8(4):268-278.

Linton, H. J., Marks, L. S., Millar, L. S., Knott, C. L., Rittenhouse, H. G. and Mikolajczyk, S. D. (2003). Benign prostate-specific antigen (BPSA) in serum is increased in benign prostate disease. *Clin Chem* 49(2):253-259.

Liska, J., Repiska, V., Galbavy, S., Polak, S., Varga, I., Blasko, M., Macejova, D. and Brtko, J. (2007). Prostate tumours--histological classification and molecular aspects of prostate tumorigenesis. *Endocr Regul* 41(1):45-57.

Liu, T., Qian, W. J., Gritsenko, M. A., Camp, D. G., 2nd, Monroe, M. E., Moore, R. J. and Smith, R. D. (2005). Human plasma N-glycoproteome analysis by immunoaffinity subtraction, hydrazide chemistry, and mass spectrometry. *J Proteome Res* 4(6):2070-2080.

Loeb, S. and Catalona, W. J. (2008). What to do with an abnormal PSA test. *Oncologist* 13(3):299-305.

Lowe, F. C. and Trauzzi, S. J. (1993). Prostatic acid phosphatase in 1993. Its limited clinical utility. *Urol Clin North Am* 20(4):589-595.

Lowe, J. B., Stoolman, L. M., Nair, R. P., Larsen, R. D., Berhend, T. L. and Marks, R. M. (1990). ELAM-1--dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. *Cell* 63(3):475-484.

Luderer, A. A., Chen, Y. T., Soriano, T. F., Kramp, W. J., Carlson, G., Cuny, C., Sharp, T., Smith, W., Petteway, J., Brawer, M. K. and et al. (1995). Measurement of the proportion of free to total prostate-specific antigen improves diagnostic performance of prostate-specific antigen in the diagnostic gray zone of total prostate-specific antigen. *Urology* 46(2):187-194.

Ludwig, J. A. and Weinstein, J. N. (2005). Biomarkers in cancer staging, prognosis and treatment selection. *Nat Rev Cancer* 5(11):845-856.

Magnani, J. L., Steplewski, Z., Koprowski, H. and Ginsburg, V. (1983). Identification of the gastrointestinal and pancreatic cancer-associated antigen detected by monoclonal antibody 19-9 in the sera of patients as a mucin. *Cancer Res* 43(11):5489-5492.

Martensson, S., Bigler, S. A., Brown, M., Lange, P. H., Brawer, M. K. and Hakomori, S. (1995). Sialyl-Lewis(x) and related carbohydrate antigens in the prostate. *Hum Pathol* 26(7):735-739.

Matsumoto, K., Maeda, Y., Kato, S. and Yuki, H. (1994). Alteration of asparagine-linked glycosylation in serum transferrin of patients with hepatocellular carcinoma. *Clin Chim Acta* 224(1):1-8.

Matsumura, K., Higashida, K., Ishida, H., Hata, Y., Yamamoto, K., Shigeta, M., Mizuno-Horikawa, Y., Wang, X., Miyoshi, E., Gu, J. and Taniguchi, N. (2007). Carbohydrate binding specificity of a fucose-specific lectin from Aspergillus oryzae: a novel probe for core fucose. *J Biol Chem* 282(21):15700-15708.

Mattsson, J. M., Laakkonen, P., Stenman, U. H. and Koistinen, H. (2009). Antiangiogenic properties of prostate-specific antigen (PSA). *Scand J Clin Lab Invest* 69(4):447-451.

Mattsson, J. M., Valmu, L., Laakkonen, P., Stenman, U. H. and Koistinen, H. (2008). Structural characterization and anti-angiogenic properties of prostate-specific antigen isoforms in seminal fluid. *Prostate* 68(9):945-954.

Meany, D. L., Zhang, Z., Sokoll, L. J., Zhang, H. and Chan, D. W. (2009). Glycoproteomics for prostate cancer detection: changes in serum PSA glycosylation patterns. *J Proteome Res* 8(2):613-619.

Mikolajczyk, S. D., Catalona, W. J., Evans, C. L., Linton, H. J., Millar, L. S., Marker, K. M., Katir, D., Amirkhan, A. and Rittenhouse, H. G. (2004). Proenzyme forms of prostate-specific antigen in serum improve the detection of prostate cancer. *Clin Chem* 50(6):1017-1025.

Mikolajczyk, S. D., Marks, L. S., Partin, A. W. and Rittenhouse, H. G. (2002). Free prostate-specific antigen in serum is becoming more complex. *Urology* 59(6):797-802.

Misek, D. E., Patwa, T. H., Lubman, D. M. and Simeone, D. M. (2007). Early detection and biomarkers in pancreatic cancer. *J Natl Compr Canc Netw* 5(10):1034-1041.

Miyazaki, K., Ohmori, K., Izawa, M., Koike, T., Kumamoto, K., Furukawa, K., Ando, T., Kiso, M., Yamaji, T., Hashimoto, Y., Suzuki, A., Yoshida, A., Takeuchi, M. and Kannagi, R. (2004). Loss of disialyl Lewis(a), the ligand for lymphocyte inhibitory receptor sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7) associated with increased sialyl Lewis(a) expression on human colon cancers. *Cancer Res* 64(13):4498-4505.

Miyazaki, K., Yusa, A., Kimura, N., Izawa, M. and Kannagi, R. (2008). Diminished expression of sulfate transporter DTDST induces sialyl Lewis^{x} expression and enhanced proliferation of colon cancer cells. In: Association, J. C. *Proceedings for 67th Annual Meeting of the Japanese Cancer Association*. Tokyo:426.

Miyoshi, E., Moriwaki, K. and Nakagawa, T. (2008). Biological function of fucosylation in cancer biology. *J Biochem* 143(6):725-729.

Miyoshi, E. and Nakano, M. (2008). Fucosylated haptoglobin is a novel marker for pancreatic cancer: detailed analyses of oligosaccharide structures. *Proteomics* 8(16):3257-3262.

Miyoshi, E., Uozumi, N., Noda, K., Hayashi, N., Hori, M. and Taniguchi, N. (1997). Expression of alpha1-6 fucosyltransferase in rat tissues and human cancer cell lines. *Int J Cancer* 72(6):1117-1121.

Mohamed, E., Abdul-Rahman, P. S., Doustjalali, S. R., Chen, Y., Lim, B. K., Omar, S. Z., Bustam, A. Z., Singh, V. A., Mohd-Taib, N. A., Yip, C. H. and Hashim, O. H. (2008). Lectin-based electrophoretic analysis of the expression of the 35 kDa inter-alpha-trypsin inhibitor heavy chain H4 fragment in sera of patients with five different malignancies. *Electrophoresis* 29(12):2645-2650.

Moore, K. L. (2003). The biology and enzymology of protein tyrosine O-sulfation. *J Biol Chem* 278(27):24243-24246.

Morelle, W. and Michalski, J. C. (2007). Analysis of protein glycosylation by mass spectrometry. *Nat Protoc* 2(7):1585-1602.

Morris, K. M., Aden, D. P., Knowles, B. B. and Colten, H. R. (1982). Complement biosynthesis by the human hepatoma-derived cell line HepG2. *J Clin Invest* 70(4):906-913.

Moul, J. W., Sun, L., Hotaling, J. M., Fitzsimons, N. J., Polascik, T. J., Robertson, C. N., Dahm, P., Anscher, M. S., Mouraviev, V., Pappas, P. A. and Albala, D. M. (2007). Age adjusted prostate specific antigen and prostate specific antigen velocity cut points in prostate cancer screening. *J Urol* 177(2):499-503; discussion 503-494.

Naitoh, A., Aoyagi, Y. and Asakura, H. (1999). Highly enhanced fucosylation of serum glycoproteins in patients with hepatocellular carcinoma. *J Gastroenterol Hepatol* 14(5):436-445.

Nakagawa, T., Uozumi, N., Nakano, M., Mizuno-Horikawa, Y., Okuyama, N., Taguchi, T., Gu, J., Kondo, A., Taniguchi, N. and Miyoshi, E. (2006). Fucosylation of N-glycans regulates the secretion of hepatic glycoproteins into bile ducts. *J Biol Chem* 281(40):29797-29806.

Nakamori, S., Kameyama, M., Imaoka, S., Furukawa, H., Ishikawa, O., Sasaki, Y., Izumi, Y. and Irimura, T. (1997). Involvement of carbohydrate antigen sialyl Lewis(x) in colorectal cancer metastasis. *Dis Colon Rectum* 40(4):420-431.

Nakano, M., Nakagawa, T., Ito, T., Kitada, T., Hijioka, T., Kasahara, A., Tajiri, M., Wada, Y., Taniguchi, N. and Miyoshi, E. (2008). Site-specific analysis of N-glycans on haptoglobin in sera of patients with pancreatic cancer: a novel approach for the development of tumor markers. *Int J Cancer* 122(10):2301-2309.

Narisada, M., Kawamoto, S., Kuwamoto, K., Moriwaki, K., Nakagawa, T., Matsumoto, H., Asahi, M., Koyama, N. and Miyoshi, E. (2008). Identification of an inducible factor secreted by pancreatic cancer cell lines that stimulates the production of fucosylated haptoglobin in hepatoma cells. *Biochem Biophys Res Commun* 377(3):792-796.

Nelson, D. L. and Cox, M. M. (2008). Lehninger: Principles of Biochemistry. 5th Edition.

Auvinen, A. (2009). Screening and prostate-cancer mortality in a randomized European study. N Engl J Med 360(13):1320-1328.

Nishimura, H., Kakizaki, I., Muta, T., Sasaki, N., Pu, P. X., Yamashita, T. and Nagasawa, S. (1995). cDNA and deduced amino acid sequence of human PK-120, a plasma kallikrein-sensitive glycoprotein. *FEBS Lett* 357(2):207-211.

Noda, K., Miyoshi, E., Uozumi, N., Yanagidani, S., Ikeda, Y., Gao, C., Suzuki, K., Yoshihara, H., Yoshikawa, K., Kawano, K., Hayashi, N., Hori, M. and Taniguchi, N. (1998). Gene expression of alpha1-6 fucosyltransferase in human hepatoma tissues: a possible implication for increased fucosylation of alpha-fetoprotein. *Hepatology* 28(4):944-952.

Ohyama, C., Hosono, M., Nitta, K., Oh-eda, M., Yoshikawa, K., Habuchi, T., Arai, Y. and Fukuda, M. (2004). Carbohydrate structure and differential binding of prostate specific antigen to Maackia amurensis lectin between prostate cancer and benign prostate hypertrophy. *Glycobiology* 14(8):671-679.

Okada, T., Sato, Y., Kobayashi, N., Sumida, K., Satomura, S., Matsuura, S., Takasaki, M. and Endo, T. (2001). Structural characteristics of the N-glycans of two isoforms of prostate-specific antigens purified from human seminal fluid. *Biochim Biophys Acta* 1525(1-2):149-160.

Okano, T., Kondo, T., Kakisaka, T., Fujii, K., Yamada, M., Kato, H., Nishimura, T., Gemma, A., Kudoh, S. and Hirohashi, S. (2006). Plasma proteomics of lung cancer by a linkage of multi-dimensional liquid chromatography and two-dimensional difference gel electrophoresis. *Proteomics* 6(13):3938-3948.

Okuyama, N., Ide, Y., Nakano, M., Nakagawa, T., Yamanaka, K., Moriwaki, K., Murata, K., Ohigashi, H., Yokoyama, S., Eguchi, H., Ishikawa, O., Ito, T., Kato, M., Kasahara, A., Kawano, S., Gu, J., Taniguchi, N. and Miyoshi, E. (2006). Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. *Int J Cancer* 118(11):2803-2808.

Orntoft, T. F., Harving, N. and Langkilde, N. C. (1990). O-linked mucin-type glycoproteins in normal and malignant colon mucosa: lack of T-antigen expression and accumulation of Tn and sialosyl-Tn antigens in carcinomas. *Int J Cancer* 45(4):666-672.

Orntoft, T. F. and Vestergaard, E. M. (1999). Clinical aspects of altered glycosylation of glycoproteins in cancer. *Electrophoresis* 20(2):362-371.

Park, E. I., Mi, Y., Unverzagt, C., Gabius, H. J. and Baenziger, J. U. (2005). The asialoglycoprotein receptor clears glycoconjugates terminating with sialic acid alpha 2,6GalNAc. *Proc Natl Acad Sci U S A* 102(47):17125-17129.

Pearlstein, E., Salk, P. L., Yogeeswaran, G. and Karpatkin, S. (1980). Correlation between spontaneous metastatic potential, platelet-aggregating activity of cell surface extracts, and cell surface sialylation in 10 metastatic-variant derivatives of a rat renal sarcoma cell line. *Proc Natl Acad Sci U S A* 77(7):4336-4339.

Peracaula, R., Royle, L., Tabares, G., Mallorqui-Fernandez, G., Barrabes, S., Harvey, D. J., Dwek, R. A., Rudd, P. M. and de Llorens, R. (2003a). Glycosylation of human pancreatic ribonuclease: differences between normal and tumor states. *Glycobiology* 13(4):227-244.

Peracaula, R., Sarrats, A. and Rudd, P. M. (2010). Liver proteins as sensor of human malignancies and inflammation. *PROTEOMICS - Clinical Applications* 4(4):426–431.

Peracaula, R., Tabares, G., Lopez-Ferrer, A., Brossmer, R., de Bolos, C. and de Llorens, R. (2005). Role of sialyltransferases involved in the biosynthesis of Lewis antigens in human pancreatic tumour cells. *Glycoconj J* 22(3):135-144.

Peracaula, R., Tabares, G., Royle, L., Harvey, D. J., Dwek, R. A., Rudd, P. M. and de Llorens, R. (2003b). Altered glycosylation pattern allows the distinction between prostate-specific antigen (PSA) from normal and tumor origins. *Glycobiology* 13(6):457-470.

Perez-Garay, M., Arteta, B., Pages, L., de Llorens, R., de Bolos, C., Vidal-Vanaclocha, F. and Peracaula, R. (2010). alpha2,3-sialyltransferase ST3Gal III modulates pancreatic cancer cell motility and adhesion in vitro and enhances its metastatic potential in vivo. *PLoS One* 5(9).

Poland, D. C., Schalkwijk, C. G., Stehouwer, C. D., Koeleman, C. A., van het Hof, B. and van Dijk, W. (2001). Increased alpha3-fucosylation of alpha1-acid glycoprotein in Type I diabetic patients is related to vascular function. *Glycoconj J* 18(3):261-268.

Polevoda, B. and Sherman, F. (2002). The diversity of acetylated proteins. *Genome Biol* 3(5):reviews0006.

Poon, T. C., Mok, T. S., Chan, A. T., Chan, C. M., Leong, V., Tsui, S. H., Leung, T. W., Wong, H. T., Ho, S. K. and Johnson, P. J. (2002). Quantification and utility of monosialylated alpha-fetoprotein in the diagnosis of hepatocellular carcinoma with nondiagnostic serum total alpha-fetoprotein. *Clin Chem* 48(7):1021-1027.

Prestigiacomo, A. F., Lilja, H., Pettersson, K., Wolfert, R. L. and Stamey, T. A. (1996). A comparison of the free fraction of serum prostate specific antigen in men with benign and cancerous prostates: the best case scenario. *J Urol* 156(2 Pt 1):350-354.

Pu, X. P., Iwamoto, A., Nishimura, H. and Nagasawa, S. (1994). Purification and characterization of a novel substrate for plasma kallikrein (PK-120) in human plasma. *Biochim Biophys Acta* 1208(2):338-343.

Qiu, Y., Patwa, T. H., Xu, L., Shedden, K., Misek, D. E., Tuck, M., Jin, G., Ruffin, M. T., Turgeon, D. K., Synal, S., Bresalier, R., Marcon, N., Brenner, D. E. and Lubman, D. M. (2008). Plasma glycoprotein

profiling for colorectal cancer biomarker identification by lectin glycoarray and lectin blot. *J Proteome Res* 7(4):1693-1703.

Rademacher, T. W., Parekh, R. B. and Dwek, R. A. (1988). Glycobiology. *Annu Rev Biochem* 57:785-838.

Real, F. X. (2003). A "catastrophic hypothesis" for pancreas cancer progression. *Gastroenterology* 124(7):1958-1964.

Rogers, C. G., Yan, G., Zha, S., Gonzalgo, M. L., Isaacs, W. B., Luo, J., De Marzo, A. M., Nelson, W. G. and Pavlovich, C. P. (2004). Prostate cancer detection on urinalysis for alpha methylacyl coenzyme a racemase protein. *J Urol* 172(4 Pt 1):1501-1503.

Roobol, M. J., Kerkhof, M., Schroder, F. H., Cuzick, J., Sasieni, P., Hakama, M., Stenman, U. H., Ciatto, S., Nelen, V., Kwiatkowski, M., Lujan, M., Lilja, H., Zappa, M., Denis, L., Recker, F., Berenguer, A., Ruutu, M., Kujala, P., Bangma, C. H., Aus, G., Tammela, T. L., Villers, A., Rebillard, X., Moss, S. M., de Koning, H. J., Hugosson, J. and Auvinen, A. (2009). Prostate cancer mortality reduction by prostate-specific antigen-based screening adjusted for nonattendance and contamination in the European Randomised Study of Screening for Prostate Cancer (ERSPC). *Eur Urol 56*(4):584-591.

Ros-Bullon, M. R., Sanchez-Pedreno, P. and Martinez-Liarte, J. H. (2001). Serum ceruloplasmin in melanoma patients. *Anticancer Res* 21(1B):629-632.

Rosenfeld, R., Bangio, H., Gerwig, G. J., Rosenberg, R., Aloni, R., Cohen, Y., Amor, Y., Plaschkes, I., Kamerling, J. P. and Maya, R. B. (2007). A lectin array-based methodology for the analysis of protein glycosylation. *J Biochem Biophys Methods* 70(3):415-426.

Royle, L., Campbell, M. P., Radcliffe, C. M., White, D. M., Harvey, D. J., Abrahams, J. L., Kim, Y. G., Henry, G. W., Shadick, N. A., Weinblatt, M. E., Lee, D. M., Rudd, P. M. and Dwek, R. A. (2008). HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal Biochem* 376(1):1-12.

Royle, L., Radcliffe, C. M., Dwek, R. A. and Rudd, P. M. (2006). Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions. *Methods Mol Biol* 347:125-143.

Rubin, M. A., Zhou, M., Dhanasekaran, S. M., Varambally, S., Barrette, T. R., Sanda, M. G., Pienta, K. J., Ghosh, D. and Chinnaiyan, A. M. (2002). alpha-Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. *JAMA* 287(13):1662-1670.

Rudd, P. M. and Dwek, R. A. (1997). Glycosylation: heterogeneity and the 3D structure of proteins. *Crit Rev Biochem Mol Biol* 32(1):1-100.

Saguchi, K., Tobe, T., Hashimoto, K., Sano, Y., Nakano, Y., Miura, N. H. and Tomita, M. (1995). Cloning and characterization of cDNA for inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP), a novel human plasma glycoprotein. *J Biochem* 117(1):14-18.

Saldova, R., Royle, L., Radcliffe, C. M., Abd Hamid, U. M., Evans, R., Arnold, J. N., Banks, R. E., Hutson, R., Harvey, D. J., Antrobus, R., Petrescu, S. M., Dwek, R. A. and Rudd, P. M. (2007). Ovarian cancer is associated with changes in glycosylation in both acute-phase proteins and IgG. *Glycobiology* 17(12):1344-1356.

Samadi, A. A., Davidson, S. D., Mordente, J. A., Choudhury, M. S., Tazaki, H., Mallouh, C. and Konno, S. (1999). Differential Glycosylation of Cellular Prostate Specific Antigen and the Regulatory Mechanism of Its Secretion. *Mol Urol* 3(3):147-152.

Sarrats, A., Comet, J., Tabares, G., Ramirez, M., Aleixandre, R. N., de Llorens, R. and Peracaula, R. (2010a). Differential percentage of serum prostate-specific antigen subforms suggests a new way to improve prostate cancer diagnosis. *Prostate* 70(1):1-9.

Sarrats, A., Saldova, R., Pla, E., Fort, E., Harvey, D. J., Struwe, W. B., de Llorens, R., Rudd, P. M. and Peracaula, R. (2010b). Glycosylation of liver acute-phase proteins in pancreatic cancer and chronic pancreatitis. *PROTEOMICS - Clinical Applications* 4(4):432-448.

Sarrats, A., Saldova, R., Comet, J., O'Donoghue, N., R, d. L., Rudd, P. and Peracaula, R. (2010c). Glycan characterization of total PSA 2-DE subforms from serum and seminal plasma. *OMICS* 14(4):465-474.

Satomura, Y., Sawabu, N., Takemori, Y., Ohta, H., Watanabe, H., Okai, T., Watanabe, K., Matsuno, H. and Konishi, F. (1991). Expression of various sialylated carbohydrate antigens in malignant and nonmalignant pancreatic tissues. *Pancreas* 6(4):448-458.

Sawada, T., Ho, J. J., Chung, Y. S., Sowa, M. and Kim, Y. S. (1994). E-selectin binding by pancreatic tumor cells is inhibited by cancer sera. *Int J Cancer* 57(6):901-907.

Schreiber, R. D. and Muller-Eberhard, H. J. (1974). Fourth component of human complement: description of a three polypeptide chain structure. *J Exp Med* 140(5):1324-1335.

Schroder, F. H., Hugosson, J., Roobol, M. J., Tammela, T. L., Ciatto, S., Nelen, V., Kwiatkowski, M., Lujan, M., Lilja, H., Zappa, M., Denis, L. J., Recker, F., Berenguer, A., Maattanen, L., Bangma, C. H., Aus, G., Villers, A., Rebillard, X., van der Kwast, T., Blijenberg, B. G., Moss, S. M., de Koning, H. J. and Auvinen, A. (2009). Screening and prostate-cancer mortality in a randomized European study. *N Engl J Med* 360(13):1320-1328

Sciarra, A., Mariotti, G., Salciccia, S., Gomez, A. A., Monti, S., Toscano, V. and Di Silverio, F. (2008). Prostate growth and inflammation. *J Steroid Biochem Mol Biol* 108(3-5):254-260.

Seales, E. C., Jurado, G. A., Brunson, B. A., Wakefield, J. K., Frost, A. R. and Bellis, S. L. (2005). Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by up-regulating cell motility. *Cancer Res* 65(11):4645-4652.

Seberger, P. J. and Chaney, W. G. (1999). Control of metastasis by Asn-linked, beta1-6 branched oligosaccharides in mouse mammary cancer cells. *Glycobiology* 9(3):235-241.

Seelentag, W. K., Li, W. P., Schmitz, S. F., Metzger, U., Aeberhard, P., Heitz, P. U. and Roth, J. (1998). Prognostic value of beta1,6-branched oligosaccharides in human colorectal carcinoma. *Cancer Res* 58(23):5559-5564.

Segrest, J. P. and Jackson, R. L. (1972). Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate *Methods in Enzymology* 28:54-63

Senra Varela, A., Lopez Saez, J. J. and Quintela Senra, D. (1997). Serum ceruloplasmin as a diagnostic marker of cancer. *Cancer Lett* 121(2):139-145.

Simeone, D. M., Ji, B., Banerjee, M., Arumugam, T., Li, D., Anderson, M. A., Bamberger, A. M., Greenson, J., Brand, R. E., Ramachandran, V. and Logsdon, C. D. (2007). CEACAM1, a novel serum biomarker for pancreatic cancer. *Pancreas* 34(4):436-443.

Sinn, H. P., Brown, S. A., Oberle, E. and Thompson, J. S. (1992). Analysis of the Lewisx epitope in human pancreas and pancreatic adenocarcinomas. *Int J Pancreatol* 11(2):125-135.

Smith, D. S. and Catalona, W. J. (1994). Rate of change in serum prostate specific antigen levels as a method for prostate cancer detection. *J Urol* 152(4):1163-1167.

Sobin, L. H. and Wittekind, C. H. (1997). UICC International Union Against Cancer. TNM classification of malignant tumours. 5th edition. New York Wiley-Liss.

Sobin, L. H. and Wittekind, C. H. (2002). UICC International Union Against Cancer. TNM classification of malignant tumours. 6th edition. New York Wiley-Liss.

Sokoll, L. J., Ellis, W., Lange, P., Noteboom, J., Elliott, D. J., Deras, I. L., Blase, A., Koo, S., Sarno, M., Rittenhouse, H., Groskopf, J. and Vessella, R. L. (2008a). A multicenter evaluation of the PCA3 molecular urine test: pre-analytical effects, analytical performance, and diagnostic accuracy. *Clin Chim Acta* 389(1-2):1-6.

Sokoll, L. J., Wang, Y., Feng, Z., Kagan, J., Partin, A. W., Sanda, M. G., Thompson, I. M. and Chan, D. W. (2008b). [-2]proenzyme prostate specific antigen for prostate cancer detection: a national cancer institute early detection research network validation study. *J Urol* 180(2):539-543; discussion 543.

Song, J., Patel, M., Rosenzweig, C. N., Chan-Li, Y., Sokoll, L. J., Fung, E. T., Choi-Miura, N. H., Goggins, M., Chan, D. W. and Zhang, Z. (2006). Quantification of fragments of human serum inter-alpha-trypsin inhibitor heavy chain 4 by a surface-enhanced laser desorption/ionization-based immunoassay. *Clin Chem* 52(6):1045-1053.

Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Wierzbicki, D. M., Jones, C. M., Lonblad, P. B., Magnusson, S. and Petersen, T. E. (1984). Primary structure of human alpha 2-macroglobulin. V. The complete structure. *J Biol Chem* 259(13):8318-8327.

Stanley, P. and Cummings, R. D. (2008). Structures Common to Different Glycans. In: Varki, A., Cummings, R., Esko, J., Freeze, H., Stanley, P., Bertozzi, C., Hart, G. and Etzler, M. Essentials of Glycobiology. 2nd ed. New York, Cold Spring Harbour Laboratory press.

Stanley, P., Schachter, H. and Taniguchi , N. (2008). N-Glycans. In: Varki, A., Cummings, R., Esko, J., Freeze, H., Stanley, P., Bertozzi, C., Hart, G. and Etzler, M. *Essentials of Glycobiology. 2nd ed.* New York, Cold Spring Harbour Laboratory press.

Steirer, L. M., Park, E. I., Townsend, R. R. and Baenziger, J. U. (2009). The asialoglycoprotein receptor regulates levels of plasma glycoproteins terminating with sialic acid alpha2,6-galactose. *J Biol Chem* 284(6):3777-3783.

Tabares, G., Jung, K., Reiche, J., Stephan, C., Lein, M., Peracaula, R., de Llorens, R. and Hoesel, W. (2007). Free PSA forms in prostatic tissue and sera of prostate cancer patients: analysis by 2-DE and western blotting of immunopurified samples. *Clin Biochem* 40(5-6):343-350.

Tabares, G., Radcliffe, C. M., Barrabes, S., Ramirez, M., Aleixandre, R. N., Hoesel, W., Dwek, R. A., Rudd, P. M., Peracaula, R. and de Llorens, R. (2006). Different glycan structures in prostate-specific antigen from prostate cancer sera in relation to seminal plasma PSA. *Glycobiology* 16(2):132-145.

Tajiri, M., Ohyama, C. and Wada, Y. (2008). Oligosaccharide profiles of the prostate specific antigen in free and complexed forms from the prostate cancer patient serum and in seminal plasma: a glycopeptide approach. *Glycobiology* 18(1):2-8.

Takada, A., Ohmori, K., Yoneda, T., Tsuyuoka, K., Hasegawa, A., Kiso, M. and Kannagi, R. (1993). Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. *Cancer Res* 53(2):354-361.

Takano, R., Nose, M., Nishihira, T. and Kyogoku, M. (1990). Increase of beta 1-6-branched oligosaccharides in human esophageal carcinomas invasive against surrounding tissue in vivo and in vitro. *Am J Pathol* 137(5):1007-1011.

Taniguchi, N., Miyoshi, E., Ko, J. H., Ikeda, Y. and Ihara, Y. (1999). Implication of N-acetylglucosaminyltransferases III and V in cancer: gene regulation and signaling mechanism. *Biochim Biophys Acta* 1455(2-3):287-300.

Taylor, M. and Drickamer, K. (2003). Introduction to Glycobiology, Oxford University Press.

Tei, K., Kawakami-Kimura, N., Taguchi, O., Kumamoto, K., Higashiyama, S., Taniguchi, N., Toda, K., Kawata, R., Hisa, Y. and Kannagi, R. (2002). Roles of cell adhesion molecules in tumor angiogenesis induced by cotransplantation of cancer and endothelial cells to nude rats. *Cancer Res* 62(21):6289-6296.

Thompson, I. M., Pauler, D. K., Goodman, P. J., Tangen, C. M., Lucia, M. S., Parnes, H. L., Minasian, L. M., Ford, L. G., Lippman, S. M., Crawford, E. D., Crowley, J. J. and Coltman, C. A., Jr. (2004). Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *N Engl J Med* 350(22):2239-2246.

Turner, G. A. (1995). Haptoglobin. A potential reporter molecule for glycosylation changes in disease. *Adv Exp Med Biol* 376:231-238.

Ueda, K., Katagiri, T., Shimada, T., Irie, S., Sato, T. A., Nakamura, Y. and Daigo, Y. (2007). Comparative profiling of serum glycoproteome by sequential purification of glycoproteins and 2-nitrobenzensulfenyl (NBS) stable isotope labeling: a new approach for the novel biomarker discovery for cancer. *J Proteome Res* 6(9):3475-3483.

van Gils, M. P., Hessels, D., van Hooij, O., Jannink, S. A., Peelen, W. P., Hanssen, S. L., Witjes, J. A., Cornel, E. B., Karthaus, H. F., Smits, G. A., Dijkman, G. A., Mulders, P. F. and Schalken, J. A. (2007). The time-resolved fluorescence-based PCA3 test on urinary sediments after digital rectal examination; a Dutch multicenter validation of the diagnostic performance. *Clin Cancer Res* 13(3):939-943.

Varki, A. (1994). Selectin ligands. *Proc Natl Acad Sci U S A* 91(16):7390-7397.

Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Marth, J. D., Bertozzi, C. R., Hart, G. W. and Etzler, M. E. (2009). Symbol nomenclature for glycan representation. *Proteomics* 9(24):5398-5399.

Varki, A., Kannagi, R. and Toole, B. (2008). Glycosylation Changes in Cancer. In: Varki, A., Cummings, R., Esko, J., Freeze, H., Stanley, P., Bertozzi, C., Hart, G. and Etzler, M. *Essentials of Glycobiology. 2nd ed.* New York, Cold Spring Harbour Laboratory press.

Varki, A. and Sharon, N. (2008). Historical Background and Overview. In: Varki, A., Cummings, R., Esko, J., Freeze, H., Stanley, P., Bertozzi, C., Hart, G. and Etzler, M. *Essentials of Glycobiology. 2nd ed.* New York, Cold Spring Harbour Laboratory press.

Wan, L. and Van Huystee, R. B. (1993). Rapid determination of glycoproteins and glyeopeptides by periodic acid schiff reagent dot-blotting assay on nitrocellulose membrane *Journal of Agricultural and Food Chemistry* 41 (6):896-898

White, K. Y., Rodemich, L., Nyalwidhe, J. O., Comunale, M. A., Clements, M. A., Lance, R. S., Schellhammer, P. F., Mehta, A. S., Semmes, O. J. and Drake, R. R. (2009). Glycomic characterization of prostate-specific antigen and prostatic acid phosphatase in prostate cancer and benign disease seminal plasma fluids. *J Proteome Res* 8(2):620-630.

Wong, N. K., Kanu, N., Thandrayen, N., Rademaker, G. J., Baldwin, C. I., Renouf, D. V. and Hounsell, E. F. (2000). Microassay analyses of protein glycosylation. *Mol Biotechnol* 14(2):147-155.

World Health Organitzation. (2009). Fact sheet N°297. Cancer.

Wu, J. T., Lyons, B. W., Liu, G. H. and Wu, L. L. (1998). Production of milligram concentrations of free prostate specific antigen (fPSA) from LNCaP cell culture: difference between fPSA from LNCaP cell and seminal plasma. *J Clin Lab Anal* 12(1):6-13.

Yamashita, K., Koide, N., Endo, T., Iwaki, Y. and Kobata, A. (1989). Altered glycosylation of serum transferrin of patients with hepatocellular carcinoma. *J Biol Chem* 264(5):2415-2423.

Yamashita, K., Tachibana, Y., Ohkura, T. and Kobata, A. (1985). Enzymatic basis for the structural changes of asparagine-linked sugar chains of membrane glycoproteins of baby hamster kidney cells induced by polyoma transformation. *J Biol Chem* 260(7):3963-3969.

Zeh, H. J., Winikoff, S., Landsittel, D. P., Gorelik, E., Marrangoni, A. M., Velikokhatnaya, L., Winans, M. T., Lee, K., Moser, A., Bartlett, D., Lotze, M. T., Siegfried, J. M., Whitcomb, D., Papacristou, G., Slivka, A., Bigbee, W. L. and Lokshin, A. E. (2005). Multianalyte profiling of serum cytokines for detection of pancreatic cancer. *Cancer Biomark* 1(6):259-269.

Zhang, S., Zhang, H. S., Cordon-Cardo, C., Reuter, V. E., Singhal, A. K., Lloyd, K. O. and Livingston, P. O. (1997). Selection of tumor antigens as targets for immune attack using immunohistochemistry: II. Blood group-related antigens. *Int J Cancer* 73(1):50-56.

Zhang, W. M., Leinonen, J., Kalkkinen, N., Dowell, B. and Stenman, U. H. (1995). Purification and characterization of different molecular forms of prostate-specific antigen in human seminal fluid. *Clin Chem* 41(11):1567-1573.

Zhang, Z., Bast, R. C., Jr., Yu, Y., Li, J., Sokoll, L. J., Rai, A. J., Rosenzweig, J. M., Cameron, B., Wang, Y. Y., Meng, X. Y., Berchuck, A., Van Haaften-Day, C., Hacker, N. F., de Bruijn, H. W., van der Zee, A. G., Jacobs, I. J., Fung, E. T. and Chan, D. W. (2004). Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 64(16):5882-5890.

Zhao, J., Patwa, T. H., Qiu, W., Shedden, K., Hinderer, R., Misek, D. E., Anderson, M. A., Simeone, D. M. and Lubman, D. M. (2007). Glycoprotein microarrays with multi-lectin detection: unique lectin binding patterns as a tool for classifying normal, chronic pancreatitis and pancreatic cancer sera. *J Proteome Res* 6(5):1864-1874.

Zielie, P. J., Mobley, J. A., Ebb, R. G., Jiang, Z., Blute, R. D. and Ho, S. M. (2004). A novel diagnostic test for prostate cancer emerges from the determination of alpha-methylacyl-coenzyme a racemase in prostatic secretions. *J Urol* 172(3):1130-1133.

Zweig, M. H. and Campbell, G. (1993). Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 39(4):561-5