Freie Universität Berlin

SERUM-BASED *N*-GLYCAN BIOMARKER FOR DIAGNOSIS OF EPITHELIAL OVARIAN CANCER

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THE ASCITES *N*-GLYCOME OF EPITHELIAL OVARIAN CANCER

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1. Gutachterin: Jun.-Prof. Dr. Véronique Blanchard

2. Gutachter: Prof. Dr. Rudolf Tauber

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für meine Familie

und meinen Mann Thomas

“It always seems impossible until it´s done.”

Nelson Mandela

Publications and Conference Contributions

Publications

1. Biskup, K., Braicu, E. I., Sehouli, J., Tauber, R., Blanchard, V.

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   "Cetuximab resistance in head and neck cancer is mediated by EGFR-K521 polymorphism."

   Cancer Research **2016**.

3. Weiz, S., Kamalakumar, A., Biskup, K., Blanchard, V.

   "Enhanced detection of in-gel released N-glycans by MALDI-TOF-MS."

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4. Biskup, K.; Braicu, E. I.; Sehouli, J.; Tauber, R.; Blanchard, V.

   "The serum glycome to discriminate between early-stage epithelial ovarian cancer and benign ovarian diseases."

   Disease markers **2014**, 238197.

5. Biskup, K., Braicu, E. I., Sehouli, J., Fotopoulou, C., Tauber, R., Berger, M., Blanchard, V.

   "Serum glycome profiling: a biomarker for diagnosis of ovarian cancer."

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6. Werner, A., Horstkorte, R., Glanz, D., Biskup, K., Blanchard, V., Berger, M., Bork, K.

   „Glycoengineering the N-acyl side chain of sialic acid of human erythropoietin affects its resistance to sialidase."

   Biological chemistry **2012**, 393, 777-783.

7. Zieringer, M., Wyszogrodzka, M., Biskup, K., Haag, R.,

   „Spramolecular behavior of fluorous polyglycerol dendrons and polyglycerol dendrimers with perfluorinated shells in water."

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Conference Contributions

Poster presentations

1. **Sialoglyco2010**, Potsdam August 21-26, 2010. Karina Biskup, Jalid Sehouli, Rudolf Tauber and Véronique Blanchard "Serum glycome profiling - a biomarker for diagnosis of ovarian cancer"

2. **The 5th Glycan Forum** in Berlin March 10-12, 2011. Karina Biskup, Elena Frisch, Jalid Sehouli, Rudolf Tauber, Markus Berger and Véronique Blanchard "Changes of the serum glycome in patients suffering from ovarian cancer"

3. **21st International Congress of Clinical Chemistry and Laboratory Medicine** in Berlin Mai 15-19, 2011. Karina Biskup, Elena Frisch, Jalid Sehouli, Rudolf Tauber, Markus Berger and Véronique Blanchard "Changes of the serum glycome in patients suffering from ovarian cancer"

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5. **2012 ASCO Annual Meeting** in Chicago June 1-5, 2012. K. Biskup, E. I. Braicu, J. Sehouli, R. Tauber, M. Berger and V. Blanchard "Changes of the serum glycome in patients suffering from ovarian cancer"

6. **The 7th Glycan Forum** in Berlin March 20-22, 2013. Karina Biskup, Elena I. Braicu, Jalid Sehouli, Christina Fotopoulou, Rudolf Tauber, Markus Berger and Véronique Blanchard "Serum glycome profiling - a biomarker for diagnosis of ovarian cancer"

7. **24th Joint Glycobiology Meeting** in Wittenberg November 24-26, 2013. Karina Biskup, Elena I. Braicu, Jalid Sehouli, Christina Fotopoulou, Rudolf Tauber, Markus Berger and Véronique Blanchard "Serum glycome profiling - a biomarker for diagnosis of ovarian cancer" - **oral poster presentation**

8. **12 Jahrestagung der Deutschen Vereinten Gesellschaft für Klinische Chemie und Laboratoriumsmedizin** in Leipzig October 14-17, 2015. Biskup, K.; Braicu, E. I.; Sehouli, J.; Tauber, R.; Blanchard, V. "The ascites N-glycome of epithelial ovarian cancer patients" / "Unraveling the IgG glycome of primary epithelial ovarian cancer patients" / "The effect of preanalytical conditions on human serum N-glycome as judged by MALDI-TOF-MS" / "Tolerance induction with T cell-dependent protein antigenes induces regulatory sialylated IgGs" - **oral poster presentation**

9. **SFG 2015 Annual Meeting** in San Francisco December 1-4, 2015. Biskup, K.; Braicu, E. I.; Sehouli, J.; Tauber, R.; Blanchard, V. "The serum glycome to

discriminate between early-stage epithelial ovarian cancer and benign ovarian diseases”

10. **27th Joint Glycobiology Meeting** in Nijmegen October 16-18, 2016. Karina Biskup, Elena I. Braicu, Jalid Sehouli, Rudolf Tauber and Véronique Blanchard “The ascites N-glycome of epithelial ovarian cancer patients”

Oral presentations

1. **7th Glycan Forum** in Berlin March 21, 2013. “Serum glycome profiling - a biomarker for diagnosis of ovarian cancer, the silent killer”
2. **HJ. Staudinger Symposium** in Bad Staffelstein June 05, 2016. „Identification of tumor associated glycans of serum glycoproteins as potential biomarker for diagnosis of gynecologic malignancies”
3. **27th Joint Glycobiology** Meeting in Nijmegen October 17, 2016. “The human glycome and its diagnostic potential”

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Zusammenfassung

Glykosylierung ist eine wichtige und häufig vorkommende co- und posttranslationale Modifikation der Glykoproteine. Glykane sind von wichtiger biologischer Relevanz und beeinflussen die strukturellen Eigenschaften sowie die intrazellulären Funktionen von Proteinen. Maligne Transformationen gehen mit quantitativen und qualitativen Strukturveränderungen der Glykane von zellulären Glykoproteinen und Glykolipiden einher. Diese Veränderungen konnten in Zellen und Gewebe aller bislang untersuchten experimentell induzierten oder natürlich vorkommenden Tumore ohne Rücksicht auf den Typ, das Stadium oder die Ursache der Entstehung nachgewiesen werden. Da die Glykane den physiologischen und pathologischen Status eines Individuums reflektieren können, bieten glykanbasierte Serumtumormarker neue Ansätze zur Diagnostik bösartiger Tumore und Entzündungskrankheiten.

Serum ist eine biologische Flüssigkeit, welche man nach Zentrifugation einer geronnenen Blutprobe erhält. Im Labor hat sich das Serum vor allem wegen dessen Stabilität unter diversen Bedingungen und der hohen Proteinkonzentration als eine geeignete Quelle für die Erforschung neuartiger Biomarker bewährt. Das Ziel dieser Arbeit war es, das Serum eines Patientinnenkollektivs mit epithelialen Ovarialkarzinomen zu untersuchen, wobei die Proben alle Stadien der Krankheit umfasst. Anschließend erfolgte ein Vergleich mit alterskorrelierten Kontrollseren von gesunden Probandinnen und Patientinnen mit gutartigen Ovarialtumoren. Im Folgenden, sollte die diagnostische Leistung des vorgeschlagenen, potentiellen

Biomarkers mit der von CA125 verglichen werden. CA125 ist aktuell der einzige, klinisch erprobte und routinemäßig eingesetzte Tumormarker für die Diagnose von Ovarialkarzinomen.

Für diesen Zweck wurden die N-Glykane von zuvor denaturierten Serum-Glykoproteinen unter Verwendung von PNGase F enzymatisch abgespaltet, aufgereinigt und permethyliert. Das N-Glykanprofil wurde mittels MALDI-TOF-Massenspektroskopie charakterisiert und die Strukturisomere mit MALDI-TOF/-TOF-MS bestätigt. Die statistische Evaluierung des potentiellen Biomarkers wurde mit Hilfe von SPSS-Software durchgeführt. Die beobachteten quantitativen und qualitativen Veränderungen des Serum-N-Glykoms von Patientinnen mit epithelialen Ovarialkarzinom wurden gegenüber gesunden Kontrollen in Form eines Quotienten ausgedrückt. Trotz der Tatsache, dass die Serum-Glykoproteine überwiegend in der Leber synthetisiert werden während CA125 vom Tumor exprimiert wird, konnte der CA125 unabhängige Quotient als potentieller Tumormarker für die Diagnose von epithelialen Ovarialkarzinomen eingesetzt werden. Der als GLYCOV bezeichnete Biomarker konnte für jedes Stadium der Krankheit und für die Diskriminierung zwischen Ovarialkarzinomen und gutartigen Ovarialtumoren validiert werden.

Mehr als ein Drittel der Frauen mit Ovarialmalignomen entwickeln im Verlauf der Krankheit und bei fast jedem Rückfall Aszites. Die Gegenwart von Aszites korreliert mit einer schlechten Prognose. Aufgrund der Tatsache, dass Aszites überwiegend in Spätstadien auftritt, ist es als biologische Flüssigkeit nicht für die Erforschung neuer Biomarker geeignet. Die Erforschung des Aszites-N-Glykoms könnte aber dennoch existierende Daten aus der Bioinformatik erweitern und dazu beitragen einen geeigneten Biomarker für das Ansprechen auf die Behandlung des Ovarialkarzinoms zu identifizieren. In dieser Arbeit konnte erstmalig das Aszites-N-Glykom-Profil, welches Patientinnen mit primären, serösen, epithelialen Ovarialkarzinomen entnommen wurde, dem Serum-N-Glykom-Profil gegenübergestellt werden. Die Vergleichsseren stammten von denselben Probandinnen, um die individuum-spezifischen Unterschiede beim Vergleich beider N-Glykan-Profile auszuschließen. Den Beobachtungen zufolge konnten im Aszites dieselben N-Glykan-Modulationen nachgewiesen werden, welche zuvor in Serum von Ovarialkarzinompatientinnen beobachtet wurden. Zusätzlich konnten statistisch relevante Unterschiede hinsichtlich der Antennarität, Fukosylierung und Sialylierung herausgestellt werden.

Abstract

Glycosylation is an important co- and posttranslational modification that frequently occurs in proteins. Glycans are of biological relevance as they affect many fundamental functions and properties of proteins including the structural, intracellular and immunological aspects. Malignant transformations are associated with qualitative and quantitative changes of glycan structures of all cellular glycoproteins and glycolipids. These changes could be confirmed on cells and in tissues of all experimentally-induced or naturally occurring malignancies that were analysed so far regardless of type, stage and cause of tumors.

As glycans can reflect the normal or abnormal status of an individual, glycan-based biomarkers are of great interest for the diagnosis of multiple diseases including cancer and inflammation. Serum is the liquid fraction that is obtained after whole blood has been allowed to clot. It is considered to be a biological fluid suitable for biomarker discovery as it is relatively stable under various conditions as well as it is rich in glycoproteins.

In the present work, whole serum from patients suffering from epithelial ovarian cancer (EOC) in all disease stages was analyzed and compared with age-matched healthy subjects and women suffering from benign ovarian masses. Further, the diagnostic performance of the proposed biomarker candidate was compared with CA125, the routine diagnostic marker, to assess whether total serum glycome can be used as a biomarker for early-stage diagnosis.

For this purpose, N-glycans were digested from total serum glycoproteins, previously denatured, using the PNGase F enzyme. Samples were subsequently purified and permethylated. The characterization of total N-glycan profile was performed using MALDI-TOF mass spectrometry and glycan structural isomericity was confirmed using MALDI-TOF/-TOF-MS. The statistical evaluation of potential biomarker candidates was done using the SPSS software. Quantitative and qualitative changes in the serum N-glycome from EOC patients were expressed for the first time as a ratio that can be used as a tumor marker, which is independent from CA125 as serum glycoproteins stem from the liver whereas CA125 is expressed by the tumor itself. The proposed biomarker candidate, termed as GLYCOV, has proved its worth at any disease stage and allows discrimination between ovarian cancer and the control group consisting of patients with benign ovarian masses.

More than one third of ovarian cancer patients develop ascites in the course of the disease, and almost all of them in case of cancer recurrence. The presence of ascites is usually correlated with a bad diagnosis. As this event occurs at late stages of EOC, ascites cannot be used for early-stage biomarker discovery. The exploration of ascites glycome is rather thought to complete integrated bioinformatics data and to identify usable putative biomarkers that assess treatment response of ovarian cancer.

In this thesis are reported for the first time the *N*-glycome profiles of ascitic fluid from primary serous EOC patients that are compared with the serum *N*-glycome of the same patients to overcome individual-specific glycosylation pattern. For the first time, it could be demonstrated that the glycome modulations previously reported in ovarian cancer serum were also present in ascites. In addition, statistical differences between ascites and serum samples were observed for glycan antennarity, sialylation and fucosylation.

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1 INTRODUCTION

1.1 GLYCOSYLATION: STRUCTURAL AND FUNCTIONAL PROPERTIES

Besides phosphorylation and sulfation, glycosylation is one of the most diverse post-translational modifications (PTM) occurring in proteins and significantly affects their functions [1-3]. Glycans can exist either in a free form or can be covalently attached to proteins and lipids, forming a large family of glycoconjugates. Depending on their size and quantity, glycans may significantly contribute to the total mass of glycoconjugates. Several main groups of glycoconjugates have been identified, among them glycoproteins, glycosaminoglycans, glycosphingolipids and glycosylphosphatidylinositol (GPI)-linked proteins. Glycoproteins comprise Asn-linked *N*-glycans and Ser-/Thr-linked *O*-glycans, which are predominately found in mucins. Second, glycosaminoglycans are long polysaccharides composed of repeating disaccharide units that can be present as free chains (hyaluronan) or as a part of heavily glycosylated proteoglycans. Third, glycosphingolipids are composed of one or more oligosaccharides linked to the lipid ceramides via glycosidic bonds. Finally, GPI-linked proteins, anchored in the plasma membrane, build a complex with glycans that are covalently linked to phosphatidylinositol.

Attached glycan moieties influence both intra- and intermolecular functions of glycoconjugates [4, 5]. Moreover, they are known to play a key role in multiple mechanisms of cellular regulation that comprise nascent protein folding, molecular trafficking and clearance, cell adhesion, molecular and cellular homeostasis, receptor activation, signal transduction and endocytosis [2]. Analyses of gene sequences have shown that more than 50% of all proteins in nature possess at least one glycosylation site, whereby three quarters bear *N*-glycans, one tenth *N*- and *O*-glycans and one eighth *O*-glycans [6]. In contrast to DNA, RNA and proteins, glycans are non-linear heterogenous structures with an impressive structural diversity. Their heterogenicity is expressed through higher or lower branching, loss or addition of monosaccharides like sialic acid, fucose, and galactose or through different linkage types between monosaccharides [7]. Despite the great structural diversity, *N*- and *O*-glycans synthesized by human cells are composed of only six neutral monosaccharides residues, namely: mannose (Man), galactose (Gal), glucose (Glc),

fucose (Fuc), *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) and one charged monosaccharide *N*-acetylneuraminic acid (Neu5Ac) (Figue 1).

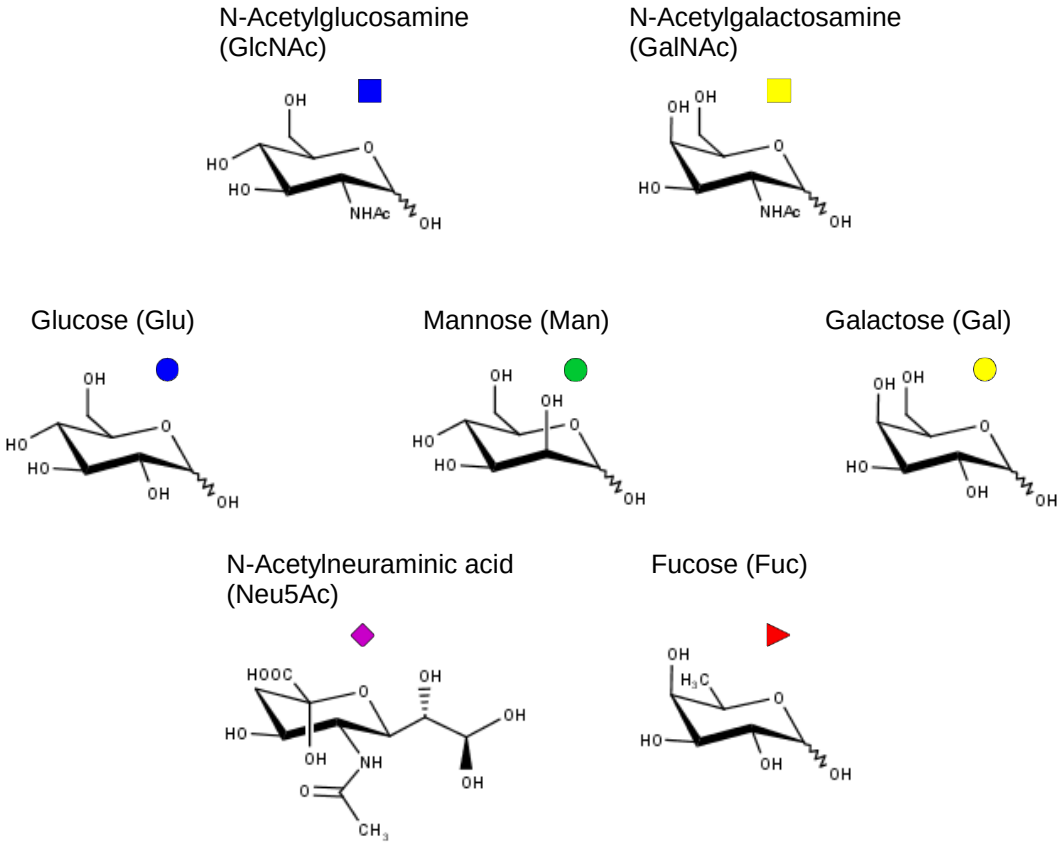


Figure 1 Monosaccharides found in mammalian glycans.

Individual monosaccharides are α- or β-linked via different carbon atoms of their ring structure, which gives rise to wide linkage diversity. Unlike proteins, the synthesis of oligosaccharides is not directed by mRNA templates. Glycan biosynthesis is initiated in the endoplasmic reticulum (ER) and is finalized in the Golgi complex (*N*-glycans) or occurs solely in the Golgi apparatus (*O*-glycans). This elaborate process requires the presence of nucleotide sugar transporters, glycosidases and glycosyltransferases as well as other enzymes necessary for biological processing of glycans [8, 9]. To date, more than 250 enzymes have been identified to be involved in processing of mammalian glycans [10].

N- and *O*-glycosylation are the two most common post-translational modifications occurring in proteins [11]. *N*-Glycans are attached co-translationally through a GlcNAc to asparagine residues of the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid with except from proline. All *N*-glycans share a common “core” structure

consisting of two GlcNAc residues and three Man. Based on further modifications of the core, *N*-glycans are divided into three groups: (A) oligomannose *N*-glycans having additional mannose units attached to the core; (B) complex-type *N*-glycans having additional GlcNAc-initiated antennae attached to the core due to the action of differential GlcNAc transferases (GlcNAc-Ts), and (C) hybrid-type *N*-glycans, which combine features of both oligomannose and complex-type *N*-glycans [8, 12, 13] (Figure 2).

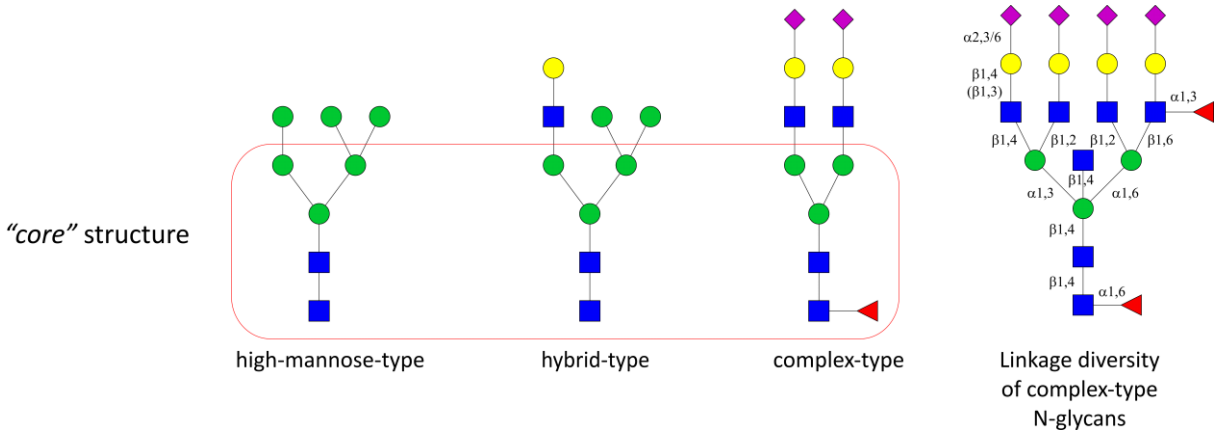


Figure 2 N-glycan types and schematic illustration of the complexity of N-glycan linkages.

Apart from *N*-glycans, *O*-glycans represent the other main group of post-translational modification occurring in proteins. They are smaller and less branched than N-glycans and have no common "core" structure. *O*-Glycans are usually linked via a hydroxyl group to either serine (Ser) or threonine (Thr) residues in proteins. The most commonly observed *O*-glycosylation form is the mucin-type glycosylation, where a GalNAc residue is covalently linked to Ser or Thr. Until now, little is known about the mechanisms determining the side occupancy of *O*-glycans, however some computer algorithms have been developed to predict *O*-glycosylation sites on glycoproteins of "higher" eukaryotes [14, 15]. The structural diversity of mucin-type *O*-glycans in mammalian glycoproteins arises predominantly from the variety of their core structures. Unlike *N*-glycans, *O*-glycans are categorized according to eight different core structures (Table 1). All those structures share a common core motif of α-GalNAc, which can then be differentially elongated by further substitution at C-3, C-6 or both those positions with β-Gal, β-GlcNAc or α-GalNAc. Core structures of type 1 and 2 (Table 1) are the most commonly observed, whereas core structures of type 3 and 4 are expressed in a more organ-specific way. Other core structures (type 5 to

type 8), including the type 4, are relatively uncommon [16, 17]. All core structures can be additionally elongated by further monosaccharide units including Gal, GlcNAc, Fuc or Neu5Ac [18]. Modifications such as sulfation, acetylation and methylation have been also reported for mucin-type *O*-glycans [17].

Core type	structure	tissue type
core 1	Gal(β1-3)GalNAc-α-Ser/Thr	most cells and secreted proteins[19]
core 2	GlcNAc(β1-6)[Gal(β1-3)]GalNAc-α-Ser/Thr	all blood cells[20]
core 3	GlcNAc(β1-3)GalNAc-α-Ser/Thr	colon tissue and saliva[21, 22]
core 4	GlcNAc(β1-6)[GlcNAc(β1-3)]GalNAc-α-Ser/Thr	mucin-secreting cell types[20]
core 5	GalNAc(α1-3)GalNAc-α-Ser/Thr	meconium[23]
core 6	GlcNAc(β1-6)GalNAc-α-Ser/Thr	ovarian tissue[24]
core 7	GalNAc(α1-6)GalNAc-α-Ser/Thr	-
core 8	Gal(α1-3)GalNAc-α-Ser/Thr	bronchia[25]

Table 1 Eight common core structures found in mucin-type *O*-glycans [26].

Several other forms of *O*-glycans could be identified so far, however, they are either rare or restricted to certain species, tissues or proteins [16, 17]. In case of cytosolic and nuclear proteins, some *O*-glycans have been found to be connected via GlcNAcβ-*O* to Ser/Thr. Serum proteins, such as factor VII and IX, protein Z and thrombospondin have been reported to contain *O*-glucose and *O*-fucose, which are attached to the hydroxyl group of Ser/Thr located in epidermal growth factor (EGF)-like repeats [16, 17, 27]. In addition, *O*-mannosylated glycans were identified on mammalian dystroglycans and brain proteoglycans [17]. Moreover, unique modifications of non-membrane bound proteins by *O*-GlcNAc were found in nuclear and cytoplasmic compartments. The single *O*-GlcNAc residue attached to proteins are generally not modified further to more complex glycan structures [8, 28].

Besides *N*- and *O*-glycosylation, another type of glycosylation namely *C*-mannosylation, has recently been unraveled in mammalian proteins [29-31]. Unlike *N*- and *O*-glycosylation, *C*-mannosylation does not involve any functional group of amino acids for the covalent binding between carbohydrate and protein. Instead, the

covalent linkage occurs between a C-atom of α-mannosyl residue and C-2 of the first tryptophan (Trp) residue being a part of the Trp-X-X-Trp consensus sequence [11, 32].

1.1.1 Functions of Glycans

Glycans play an important role in many physiological and pathological processes. They exhibit structural, protective and stabilizing features. The external orientation of glycans on glycoproteins can shield the underlying polypeptide from the recognition by antibodies and proteases. Moreover, glycans contribute to proper folding of the newly synthesized polypeptides within the ER and are important for the maintenance of protein solubility and conformation. It has been documented that incorrectly glycosylated proteins are unable to fold properly and fail to exit the ER. As a result, such proteins are degraded by proteasomes [33].

Besides, carbohydrates linked to matrix molecules, such as proteoglycans, contribute to the stability, porosity and integrity of tissue structure [34]. Oligosaccharides, on the other hand, can serve as highly specific receptors for diverse pathogens and toxins of plant or bacterial origin. They are also known to act as antigens for autoimmune and alloimmune reactions [34]. It is worth noting that, in many cases, the recognition of oligosaccharide epitopes [8, 34] is performed with impressively high specificity. For instance, influenza viral haemagglutinins localized on the surface of infected cells can specifically recognize the type, modification and linkage of the sialic acid sugar chains on the neighboring cells. Moreover, cholera toxin binds with high specificity to G_{M1} ganglioside but not to related structures [8, 34]. On the contrary, the addition of specific monosaccharides or modifications to oligosaccharides might be responsible for masking of the epitopes recognized by receptors of certain pathogens. For example, the addition of *O*-acetyl ester to the C-9 position of terminal sialic acid prevents the binding to influenza A viruses. Similarly, the autoimmune reactivity of T_n antigen can be hindered through its elongation with galactose and sialic acid [34]. Glycosylation is also known to modulate protein-protein interactions and to prevent unwanted early interactions between receptors and growth factors synthesized within the same cell [34]. Another important function served by glycans is the switching effect, visible particularly in the case of β-human chorionic gonadotropin (β-hCG). Despite the fact, that deglycosylated β-hCG is still able to bind to its receptor, it fails to stimulate and transfer the signal through adenylate cyclase, a key regulatory enzyme in all cells [8, 35]. Although

in most cases the effect of glycosylation is rather of partial than vital importance, the biological role of many growth factors and hormones appears to be highly regulated by the presence and extent of glycosylation. For example, the cytokine granulocyte/macrophage colony-stimulating factor (M-CSF) that possesses the highest activity in its recombinantly modified non-glycosylated form, occurs naturally in variety of 'glycoforms'. Interestingly, each of those glycoforms has different properties regarding binding affinity and signal transduction [34]. Finally, glycans help the immune system to discriminate between healthy and diseased cells, such as cancer cells. In this manner, cancer cells expressing different glycans, when compared to the normal cells, may consequently be recognized by the immune system. This provides new alternatives in diagnosis and therapy as well as in drug and vaccine development [36-38].

1.1.2 N-GLYCANS – BIOSYNTHETIC PATHWAY

The biosynthesis of *N*-glycans is initiated on the cytosolic face of ER membrane, where two nucleoside activated UDP-GlcNAc are transferred to the precursor dolichol phosphate (Dol-P). The resulting GlcNAcβ(1,4)GlcNAc-P-Dol sequence is commonly termed "chitobiose core". Subsequently, step by step, five mannose residues are added and the oligosaccharide precursor $Man_5GlcNAc_2$, linked to the Dol-P, is flipped from the cytosolic face into the lumen of ER [39].

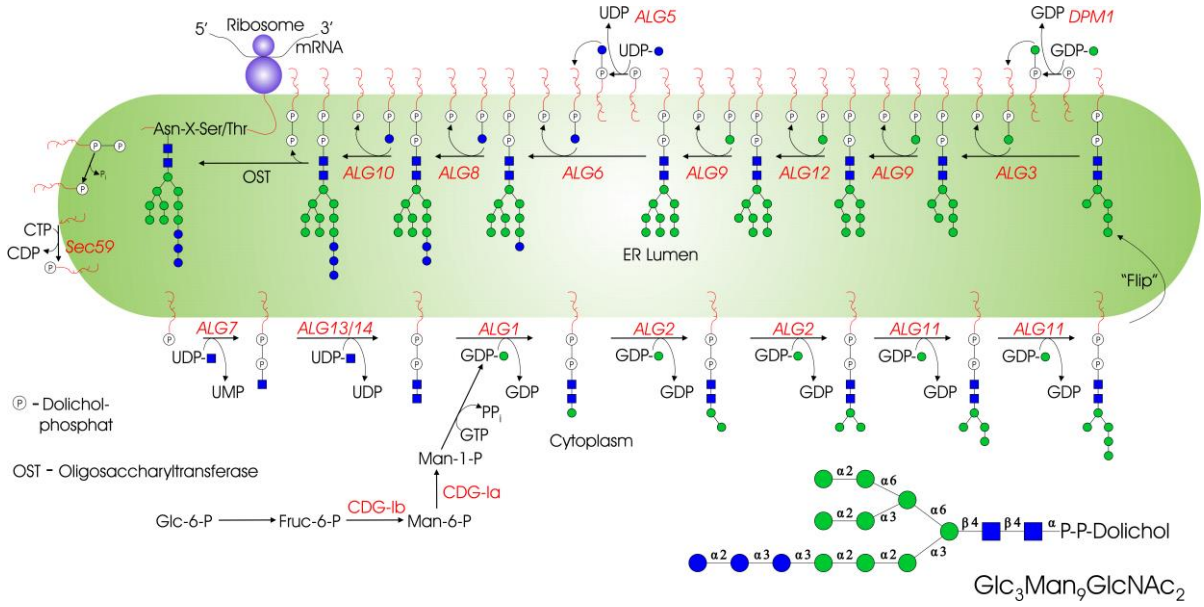


Figure 3 Synthesis of the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ on dolichol phosphate in the *Endoplasmic Reticulum*.

In the next step, the $Man_5GlcNAc_2$ structure is extended by stepwise addition of four Man and three Glc residues. Afterwards, the synthesized glycan precursor is cotranslationally transferred to the Asn residue of the consensus sequence Asn-X-Ser/Thr (X - any amino acid except of Pro), becoming a part of the nascent polypeptide chain [11] (Figure 3). The polypeptide chain adopts a loop-like conformation to enhance the nucleophilicity of the amide and thus enables the formation of the linkage between Asn and the *N*-glycan precursor. Proline is the only amino acid that prevents the loop formation and therefore cannot be a part of the consensus sequence [40]. The cleavage of the Dol-P oligosaccharide precursor and its subsequent transfer are mediated by the action of oligosaccharyltransferase [41]. During protein folding, terminal glucose residues are cleaved by α-glucosidases I and II and the terminal mannose is removed by α(1,2)-mannosidase, which then is followed by an addition of the innermost glucose when the protein is still not correctly folded [13, 42, 43]. This glycan intermediate is specifically recognized by two lectins named calnexin and calreticulin, which initiate protein refolding. If glycoproteins fail to reach their native conformation, they are directed to the cytosol and selectively eliminated in an ER-associated degradation process [44]. Nascent glycoproteins are exposed to the action of ER-resident mannosidase I, which removes the α(1,2)-linked Man from the central arm of the glycan precursor. As a result glycoproteins can exit the ER as either $Man_9GlcNAc_2$ or $Man_8GlcNAc_2$ precursor molecules [9, 45]. If the glycan precursor in the *cis*-Golgi still contains a glucose residue, it will be cleaved together with the adjacent mannose unit by endo-α-mannosidase [9]. Afterwards, α(1,2)-mannosidases IA-IC will trimmed off terminal Man residues resulting in $Man_5GlcNAc_2$, which is an intermediate for the formation of hybrid and complex *N*-glycans [9, 40]. However, N-glycans that are not completely trimmed to $Man_5GlcNAc_2$ can escape further modification in the *cis*-Golgi, resulting in oligomannose *N*-glycans bearing 5 to 9 Man residues [9].

For the processing of complex and hybrid *N*-glycans in the *medial*-Golgi two enzymes are of essential importance: GlcNAcT I/II and α-mannosidase II. The GlcNAcT I/II adds an additional GlcNAc residue to the core structure, initiating the formation of mono- and biantennary structures, whereas α-mannosidase II is responsible for cleavage of the remaining mannoses up to the core structure. Lack or incomplete action of α-mannosidase II results in the formation of hybrid *N*-glycans. In contrast, the complete action of mannosidase II leads to formation of complex structures [9, 46]. The

incompletely synthesized hybrid and complex-type *N*-glycans undergo a further modification by acting of numerous glycosidases and glycosyltransferases located in the Golgi compartments (Figure 4).

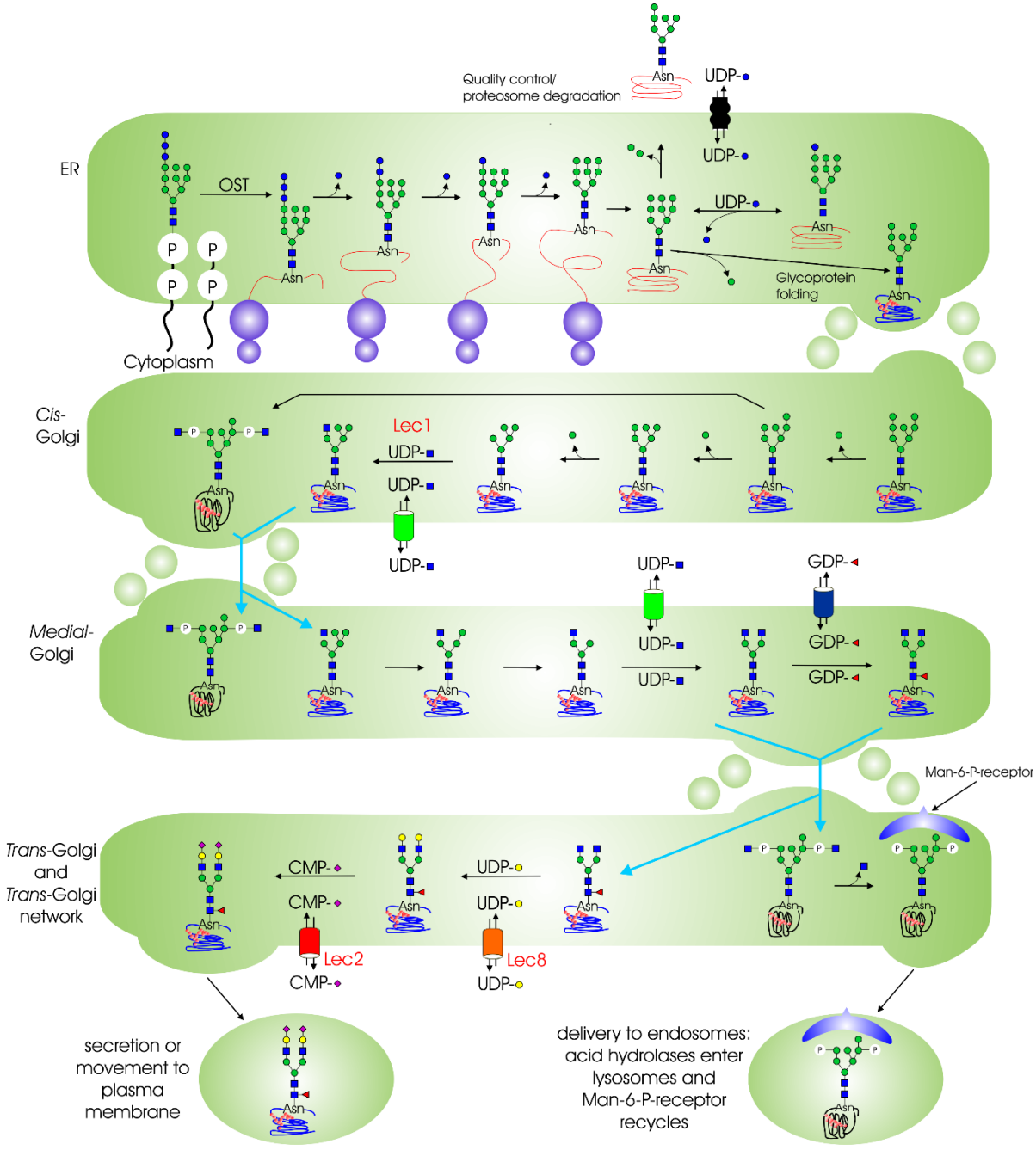


Figure 4 *N*-glycan processing in the ER and Golgi [8].

The availability of those glycosidases and glycosyltransferases depends on the type and physiological state of the cell and determines the final glycan structure [8]. Thus, the branching of biantennary glycans can be increased by the action of GlcNAc-T IV and V, resulting in up to four antennae. Complex and hybrid *N*-glycans

can undergo another modification resulting in the addition of a bisecting GlcNAc to the β-mannose of the core structure. In the *trans*-Golgi, further elongation and modification of glycan structures include the addition of neutral monosaccharides (Gal, Fuc, GlcNAc), negatively charged Neu5Ac and sulfates. In human, already synthesized truncated *N*-glycan structures can be elongated by an addition of β-linked Gal to GlcNAc residue on the antennae. This can be followed by the addition of α-linked Neu5Ac or sequential addition of Gal and GlcNAc. In case of the latter, the resulting elongation motif consisting of one GlcNAc and one Gal is referred to as *N*-acetyllactosamine (LacNAc). Additionally, a fucose residue can be attached to the innermost core GlcNAc via α(1,6)-linkage, or to the GlcNAc residue within antennae via α(1,3)-linkage [8, 40].

Glycoproteins are very heterogenous molecules. The wide diversity of *N*-glycans can be caused by many factors, such as proteins' sequence and conformation, availability of nucleotide sugars, transportation within ER and Golgi and the activity of glycosidases and glycosyltransferases localized in the Golgi. Moreover, the *N*-glycan heterogeneity might also be influenced by the character of processing enzymes (e.g. α(1,6)-fucosyltransferase (α(1,6)-Fuc-T)/GlcNAc-T IV, α-mannosidase II/GlcNAc-T I) that compete for the same substrate [8]. One particular glycosylation site on a single protein may carry a large subset of *N*-glycans, what is referred to as glycan microheterogeneity [47]. At the protein level, the different isoforms of a single protein are called glycoforms.

1.1.1 O-GLYCANS – BIOSYNTHETIC PATHWAYS

Although *O*-glycans are synthesized in the ER and Golgi, their biosynthesis is different from the one of N-glycans [14, 48]. *O*-Glycan biosynthesis initiates with the transfer of a GalNAc residue from UDP-GalNAc to the hydroxyl group of Ser/Thr on completely folded and assembled protein acceptor. This process is mediated by the action of enzymes of the polypeptide N-acetylgalactosaminyltransferase family [49]. These enzymes are substrate- and tissue-specific and act in a well-regulated manner to achieve complete *O*-glycosylation [18, 50].

O-Glycosylation may be initiated in different regions of cellular compartments (e.g. ER, intermediate ER-Golgi, proximal Golgi) and its character depends on the type of GalNAc-T present in each of them [14, 51, 52]. The elongation of the GalNAc-Ser/Thr motif

takes place within Golgi compartments and depends on the presence of glycosyltransferases, which are not only cell-specific, but also may vary during cell differentiation processes [53]. A GalNAc residue can be elongated with galactose, fucose and sialic acid, but not with mannose, glucose or xylose [8]. Moreover, *O*-acetylation of sialic acids and *O*-sulfation of galactose and GlcNAc residues significantly increase *O*-glycan heterogeneity [8, 50, 53, 54].

Serum glycoprotein *O*-glycans are made up of short, frequently unbranched chains. On the contrary, many membrane-bound glycoproteins contain multiple *O*-glycosylation sites that bear long, branched chains constituting of up to 18 monosaccharide residues [18, 55]. It was discovered that *O*-glycans present in tumors usually bear shorter and more sialylated structures than those observed in non-malignant tissues [56]. It has been also shown that T_n (*O*-GalNAc) and T (*O*-GalNAc-Gal) antigens along with their sialylated counterparts, which are a consequence of incomplete glycosylation, are expressed on mucins in many tumors [8, 57-59]. In non-cancerous tissues, these antigens were found to occur either infrequently or to be masked by a sugar chain elongation [8, 60]. It is worth noting that T_n antigens are among the most frequently occurring cancer-associated determinants as they have been shown to be expressed in more than 90% human primary adenocarcinomas [61, 62]. With the help of monoclonal antibodies, the presence of T_n antigens was also detected on mucins secreted into the blood stream of cancer patients. Remarkably, in carcinomas of epithelial origin, mucins appear to be a major carrier of altered glycosylation [8].

1.2 Pathophysiology Caused by Glycosylation Failure

Congenital Disorders of Glycosylation (CDG), i.e. inborn defects in glycosylation processing, represent a family of systematic diseases characterized by neurological and developmental deficiencies during infancy and early childhood [63, 64]. CDG are autosomal recessive genetic disorders emerging from mutations in various genes that control glycosylation processes. CDG can be caused by deficient activity of proteins, enzymes or transporters, which are responsible for the delivery and attachment of monosaccharides to the glycan chains. Defects in the synthesis of glycans may affect the production of glycoproteins and disturb the diverse functions that they serve within

the cell. This can result in a multisystemic disease, which may impair a variety of tissues and organs [8, 65].

CDG are divided into two types: type I and II. It has been shown that patients with CDG type I lack one or two glycosylation sites on transferrin (Tf) and are deficient in sialic acids. In contrast, patients with CDG type II have both Tf glycosylation sites occupied, however glycan structures are altered. Although CDG failures occur in both glycosylation types, i.e. *N*- and *O*-glycans, genetic defects affect primarily *N*-glycan assembly [8]. Enhanced UDP-GlcNAc transfer to Ser/Thr on important regulatory proteins was reported to cause hyperglycemia, which, in turn, leads to the microvascular complications and thus uncontrolled diabetic conditions [66-68]. Inactivity of the GDP-D-Man-4,6-dehydratase leads to an impaired conversion of GDP-Man to GDP-Fuc, what results in decreased import of GDP-Fuc into the Golgi apparatus in skin fibroblasts. This rare disorder, characterized by recurrent infections, is referred to as leukocyte adhesion deficiency II [69, 70]. Defects in biosynthesis of GPI-anchor in granulocytes and B lymphocytes were detected in *Paroxysmal nocturnal hemoglobinuria*, a disease characterized by destruction of erythrocytes in blood, mediated by the complement system. This destructive process results from defective formation of GPI-anchored proteins on the surface of erythrocytes, which proteins normally inhibit such immune reactions [71]. Furthermore, defects in the biosynthesis of single O-linked mannose residues were reported in muscular dystrophy and neuronal migration disorder known as *Walker-Warburg syndrome* [72, 73]. Ultimately, mutations in galactosyltransferase I, xylosyltransferase, galactosyltransferase II, or glucuronic acid transferase have been demonstrated to play a key role in the progeroid variant of Ehlers-Danlos syndrome [74, 75].

1.3 Glycan Modifications in Cancer

Glycosylation changes on cell membrane proteins are known to be associated with cancer. Nevertheless, only a limited number of all possible biosynthetic changes occurring in glycans seems to be correlated with malignant transformations and tumor progression. Particularly, modulations of sialylation, fucosylation and branching appear to be frequent cancer-related changes of glycan structures. An overview of those alterations, along with changes within the activity of corresponding glycosyltransferases that have been observed in cancer, are described in detail below.

1.3.1 GlcNAc(β1,6)Man-Branching

Increased degree of branching is one of the best-characterized glycome changes in the serum *N*-glycome of cancer patients. It is dictated by the attachment of additional GlcNAc to the chitobiose core [76]. *N*-glycan branching is mediated by the action of GlcNAc-T III, IV and V, which use biantennary glycans as substrates. GlcNAc-T IV transfers a β(1,4)-linked GlcNAc to the Manα(1,3)Man sequence on the trimannosyl core resulting in the formation of triantennary *N*-linked glycans. Subsequently, the formation of tetraantennary structures is driven by the action of GlcNAc-T V via the addition of a β(1,6)-linked GlcNAc to Manα(1,6)Man [77]. Triantennary glycans bearing a GlcNAcβ(1,4) antenna are better substrates for GlcNAc-T V, making it more preferable way for the attachment of the fourth antenna (Figure 5). On the contrary, the formation of bisecting structure through GlcNAc-T III inhibits the activity of GlcNAc-T IV and V [8, 76, 77] (Figure 5).

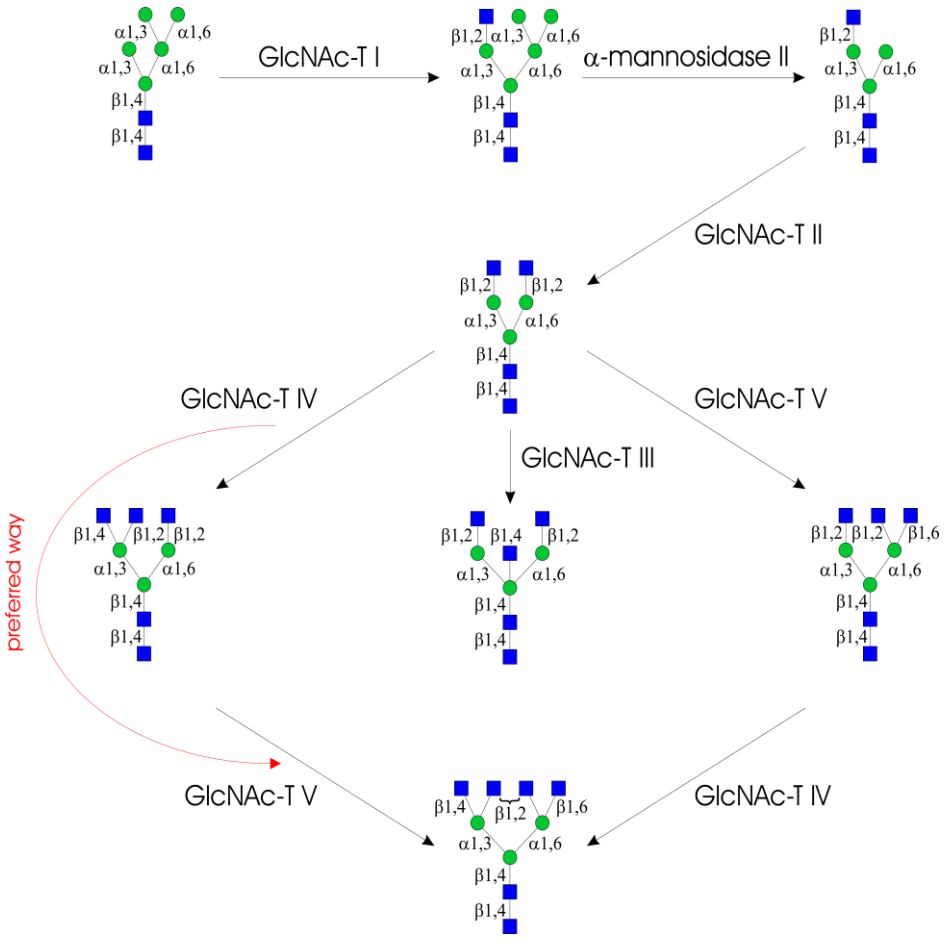


Figure 5 Branching of complex-type *N*-glycans results from the action of GlcNAc-T I to V. Blue square, GlcNAc; green circle, Man.

High expression of GlcNAc-T V leads to increased β(1,6)-branching, which has been showed to be elevated at initial stages of cancer, during its progression to advanced stages and in metastasis [78-80]. Increase of GlcNAcβ(1,6)Man branching has already been reported in tissues of breast cancer [81, 82], HCC [44], colorectal cancer [82], ovarian cancer [83], bladder cancer [84] patients as well as in cancer cell lines [85, 86].

Changed expression level of GlcNAc-T V seems to be a logical consequence of an increased transcription of the MGAT5 gene. Higher levels of GlcNAc-T V expression were positively correlated with tumor node metastasis and histological grade in patients with well- and poorly-differentiated liver tumors and in individuals with diagnosed liver cirrhosis [80]. In cell lines, elevated GlcNAc-T V expression correlates directly with increased metastatic behavior and, consequently, deficient activity of this enzyme results in the loss of metastatic phenotype. The functional role of GlcNAcβ(1,6) branching in tumor formation was first confirmed after transfection of lung epithelial cell lines with GlcNAc-T V, when transfected cells initiated the formation of solid tumors in mice. In contrast, non-transfected cells did not form tumors [78, 87]. Cell culture experiments employing MGAT5-transfected cDNA resulted in colony formation in soft agar, raised cell spreading, increased membrane invasiveness and conversion of previously normal cells to tumorigenic ones. On the opposite, mice that lack MGAT5 gene showed remarkable reduction in growth and metastatic potential of viral oncogene-induced breast tumors [88, 89]. It should also be noted that increased branching provides more sites for the attachment of charged sialic acid residues, which alone are important for cancer pathogenesis.

1.3.2 Poly-*N*-Acetyllactosamine

Galactose may be attached to the underlying GlcNAc via β(1,3) or β(1,4)-linkage thereby generating lactose motifs of type 1 or 2, respectively. Repeated addition of this motif results in the elongation of the chain and the formation of structures called poly-*N*-acetyllactosamines (poly-LacNAc) that are found in both *N*- and *O*-glycans, on mammalian glycoproteins and glycolipids [90] (Figure 6).

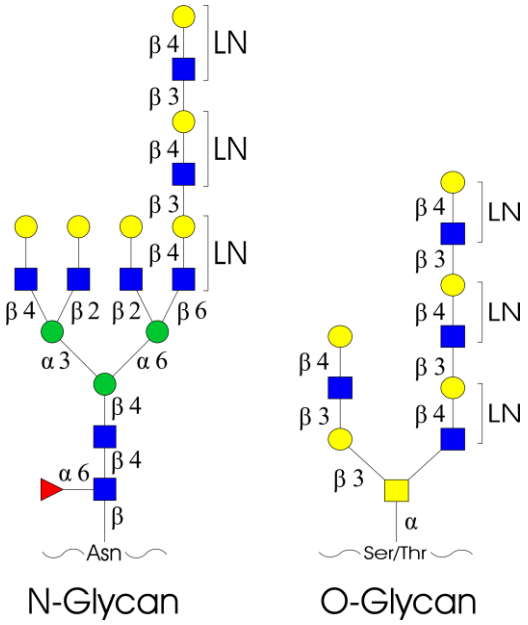


Figure 6 Examples of *N*- and *O*-glycans bearing poly-LacNAc motifs on their terminal ends [8]. Blue square, GlcNAc; yellow square, GalNAc; yellow circle, Gal; green circle, Man; red triangle, Fuc.

poly-LacNAc chains, which can be further modified by fucosylation [91], sulfation [92], sialylation [93] and numerous glycosyltransferases, are involved in specific interactions with selectins as well as other glycan-binding proteins [90, 94, 95]. In mammals, the structure of poly-LacNAc can be either linear or branched, forming the i- and I-antigen, respectively (Figure 7). The branched structure occurs when a GlcNAc residue is connected to Gal via β(1,6)-linkage [96]. The transferases responsible for the elongation or branching of poly-LacNAc are β(1,3)-GlcNAc-T that forms i-antigen and β(1,6)-GlcNAc-T that forms I-antigen [96]. Both antigens are typically found to be linked to the *N*- and *O*-glycans of glycoproteins, to the core structures of glycolipids or to keratan sulfates of proteoglycans [97, 98]. The expression of both antigens, which was reported to be onco-developmentally regulated, seems to be crucial for cancer progression. The poly-LacNAc found on *N*-glycans is indeed associated with tumor growth and metastasis [51]. Aberrant expression of i- and I-antigens was reported in pancreatic [99] and lung cancer [100], whereas decreased expression of poly-LacNAc was observed on promyelocytic leukemic cells [101] and human colon cancer cells [102].

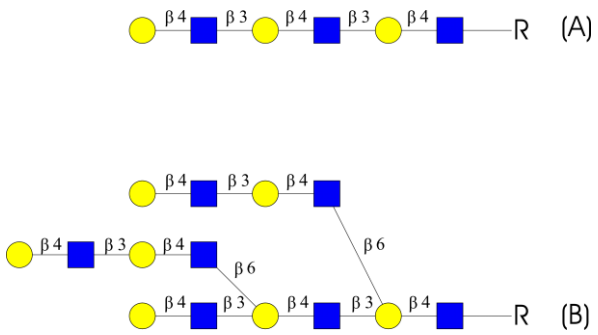


Figure 7 (A) i-antigen consisting of repeating unit (Galβ(1,4)GlcNAc)$_n$ and (B) I-antigen - a branched version derived from i-antigen. Blue square, GlcNAc; yellow circle, Gal.

N- and *O*-glycans containing the poly-LacNAc epitope were found to be elevated in metastatic murine tumor cell lines when compared to non-metastatic ones [103]. B(1,6)-GlcNAc-T V is the essential enzyme leading to formation of poly-LacNAc on *N*-glycans and its increased activity has been observed in human metastatic hepatocarcinoma [104, 105], colorectal cancer [106] as well as on metastatic cell lines. Mice that lack β(1,6)-GlcNAc-T V exhibited reduced potential for tumor growth and metastasis [89]. Moreover, the extended lactose motifs provide positions for fucose substitution, resulting in ABH blood groups and Lewis antigens formation [96]. Furthermore, long poly-LacNAc chains with attached sialyl Lewis^x (sLe^x) structure were shown to be more metastatic than their short counterparts. *N*-Glycans containing poly-LacNAc can bind adhesion molecules, which induce adhesion cascade important for tumor cell growth and invasion in surrounding tissues [107].

1.3.3 SIALYLATION

Sialic acids (Neu5Acs) are nine-carbon sugars bearing one carboxylate group, which is negatively charged at physiological pH conditions [108]. In nature, they are often found as terminal modifications on various glycoconjugates. Neu5Acs are α-linked via their second carbon atom to adjacent sugars, which can be either Gal α(2,3) or α(2,6), GalNAc α(2,6) or an additional Neu5Ac α(2,8) [93, 108]. Changes in sialic acid linkage and distribution are associated with cancer progression and metastasis [38, 108].

Until now, molecular changes occurring during tumor progression and metastasis have not yet been completely understood [38, 79, 108-110]. Nevertheless, three possible pathways have been proposed to explain aberrant sialylation in cancer: (i) enhanced expression or changed activity of sialyltransferases (STs), which leads to increased sialylation of glycans and expression of tumor-associated carbohydrate antigens such as sLe$^{x/a}$,

sT_n or polysialic acids [111, 112]; (ii) enhanced substrate availability or overexpression of genes required for Neu5Ac biosynthesis, which causes enhanced metabolic flux; (iii) altered expression of endogenous sialidases, which can cleave Neu5Ac from glycans, regulating their shedding, degradation and plasticity [113].

Abnormal sialylation promotes tumor growth and progression. Some factors such as oncogenes, hormones and exposure to chemotherapy have been shown to increase the expression of STs, and, at the same time, to reduce the expression of sialidases in tumor cells [113]. Therefore, while the synthesis of sialylated glycans in the Golgi apparatus by STs is usually enhanced, the sialidases-mediated lysosomal hydrolysis of sialylated glycans is usually decreased in cancer cells. Increased amount of such hypersialylated glycans could be found on the cell membrane of neoplastic cells [113]. Importantly, overexpression of sialylated glycans give rise to an impaired binding of sialic acids to selectins or to the extracellular matrix (ECM). This binding induces resistance to radio- and chemotherapy, promotes the invasion of cancer cells and the formation of metastasis [113]. On the other hand, sialylated glycans facilitate the apoptotic evasion. For instance, the sialylation of Fas receptor influences the interaction with Fas ligand localized on cytotoxic T lymphocytes (CTL) and integrins, and disrupt the signaling pathways leading to the suppression of apoptosis in cancer cells [113] (Figure 8).

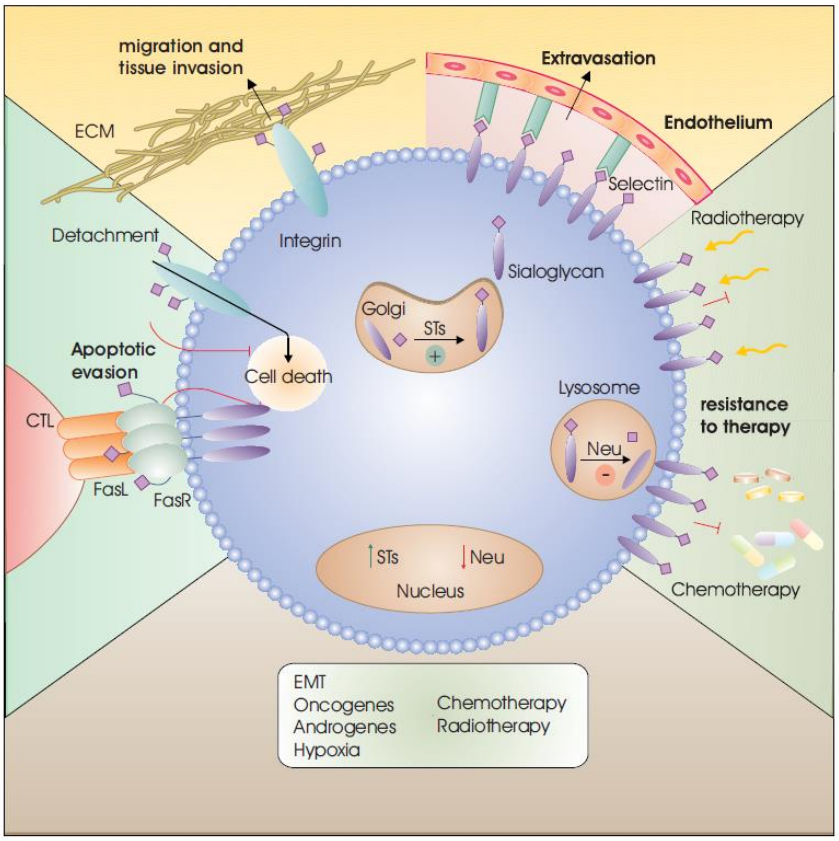


Figure 8 The role of sialic acid in cancer progression and metastasis, apoptotic evasion and in resistance to therapy [113].

There are four major ST families, which are classified according to the linkage-type formed between Neu5Ac and the adjacent monosaccharide. These are: ST6Gal (α(2,6)-linkage to Gal), ST3Gal (α(2,3)-linkage to Gal), ST6GalNAc (α(2,6)-linkage to GalNAc), ST8Sia (α(2,8)-linkage to another Neu5Ac)[93]. Overexpression of STs is generally linked to cancer progression and poor patient survival [109]. The prevalence of either α(2,3) or α(2,6) sialylation in tissues and cancer cell lines has been studied for various cancer types. For instance, ovarian cancer cell lines have been found to contain decreased level of glycoproteins bearing α(2,6)-linked Neu5Acs. In contrast, β1 integrin with increased α(2,6)-sialylation was found to promote metastasis and adhesive properties [114, 115]. The overexpression of α(2,6)-linked Neu5Acs on glycoprotein glycans was also observed in liver. It has been found to result from the overexpression of ST6Gal I in hepatocytes, occurring as a consequence of their malignant transformation [116, 117]. In breast cancer, the overexpression of α(2,6)-ST (ST6GalNAc I) has been found to enhance the tumorigenicity of cancer cells [118]. Surprisingly, it was also associated with better prognosis of patients suffering from the disease [119]. On the other hand, overexpression of ST6GalNAc II that have been

detected in tissues of colorectal carcinoma, heralded poor patients' survival [120]. In melanoma cell lines, both α(2,3) and α(2,6)-linked Neu5Acs were found to influence the metastatic potential of cells. In colorectal cancer, α(2,3)-sialylated structures showed a positive correlation with lymph node metastasis and lymphatic invasion [121]. Also, the upregulation of ST3Gal III was found to be involved in tumor progression in pancreatic cancer cell lines [122, 123] and breast cancer [124, 125]. On the contrary, enhanced expression of ST3Gal I was reported to promote mammary tumorigenesis in transgenic mice [122].

Moreover, changes within the expression of STs have been observed during patients' treatment. For instance, ST6Gal I was found to be overexpressed in cells that are resistant to cisplatin. Moreover, in cancer stem cells, the elevated expression of ST6Gal I was associated with the development of resistance to chemotherapy [126, 127]. Moreover, there is an evidence that overexpression of ST6Gal I, both in cancer and normal cells, can be induced by exposure to radiation [128, 129]. Those discoveries are of particular significance, since the resistance of tumor cells to radio- and chemotherapy prevent an effective cancer therapy.

Changes in Neu5Ac expression have also been reported in the serum of cancer patients. Altered expression levels of both α(2,3) and α(2,6)-linked Neu5Ac have been detected in the serum of cancer patients using mass spectrometry. Increased levels of α(2,6) sialylation was reported in breast [130] and lung cancer [131], whereas increased α(2,3) sialylation was found in patients with prostate cancer [132], ovarian cancer [133] and malignant brain tumors [134]. On the contrary, in lung cancer, α(2,3) sialylation was observed to be decreased [130]. In serum *N*-glycans, two STs are responsible for the attachment of Neu5Ac to the adjacent Gal: ST6Gal I that attaches α(2,6) Neu5Ac to Gal, and ST3Gal IV that attaches α(2,3) Neu5Ac to Gal. Hepatocytes, responsible for the synthesis of acute-phase serum proteins, are known to express both STs, i.e. ST6Gal I and ST3Gal IV. The overall Neu5Ac linkage in serum is therefore strongly associated with the expression level of both STs and the antennarity of acceptor glycan structures [76]. A(2,6)-linked Neu5Ac are preferably attached to biantennary glycans, whereas α(2,3)-linked Neu5Acs are more frequently found on tri- and tetraantennary *N*-glycans [135]. A shift in sialic acid linkage from α(2,3) to α(2,6) has been observed in serum glycoproteins of ovarian cancer patients that correlated with a decreased expression of α(2,3)-ST mRNA and an increased expression of α(2,6)-ST mRNA [133,

[136]. There is an evidence that α(2,6)-linked Neu5Ac negatively regulates binding of the pro-apoptotic galectins, thereby promoting tumor cell survival [33, 108].

1.3.4 FUCOSYLATION

Fucose (Fuc) is a monosaccharide present in many *N*-, *O*-linked glycans and glycolipids produced by mammalian cells. The Fuc residue can be attached either to the innermost core GlcNAc residue of *N*-glycans via α(1,6)-linkage, or to antennary GlcNAcs via α(1,3)-linkage. For instance, AFP [137] and IgG [138] carries α(1,6)-linked Fuc attached to the reducing-end GlcNAc, whereas thyroidglobulin [139] (Thy) and Hpt bear α(1,3)-Fuc attached to the antennae GlcNAc. In Tf [140] the Fuc is presented on both those GlcNAc residues [141]. Two structural features differentiate fucose from other hexoses: the lack of a hydroxyl group on the C-6 position and the L-configuration. Thirteen genes encoding Fuc-T have been identified so far. FUT 1 and FUT 2 encoding α(1,2)-Fuc-T responsible for the synthesis of H blood group antigens [142, 143]. FUT 3 to 7 and FUT 9 genes mediate the attachment of fucose to the antennary GlcNAc via α(1,3)-linkage [144, 145]. FUT 8, encoding α(1,6)-Fuc-T, controls the addition of fucose to the reducing GlcNAc on the core structure [146].

1.3.4.1 B(1,6) - CORE - FUCOSYLATION

Core fucosylation was reported to be increased in liver, colon and ovarian cancer tissues [147, 148]. Increased levels of α(1,6)-Fuc on α-fetoprotein (AFP) have been observed in hepatoma tissues of HCC patients, showing good discrimination between HCC and chronic liver diseases [146]. Moreover, increased core fucosylation was found in breast cancer cell lines and on the surface of colon cancer cells [149-151]. Increased levels of core-Fuc have also been found on serum glycoproteins in other types of cancer. Highly fucosylated Hpt was detected in sera of pancreatic cancer patients, where the enhanced fucosylation reflected the progression of the disease [152]. An elevated level of fucosylated Hpt has also been shown in lung [153], breast [154] and ovarian [141, 154] cancer patients. Moreover, studies on colorectal cancer cell lines confirmed the important role of core fucosylation in E-cadherin-dependent adhesion [150, 151]. Abnormally increased Fuc levels were also found on serum glycoproteins: Tf in liver cancer [140], thyroglobulin in thyroid cancer [139] and IgG in myeloma [138].

1.3.4.2 TERMINAL FUCOSYLATION - LEWIS EPITOPES

Lewis antigens (Le) are mono- or difucosylated disaccharides attached to GlcNAcβ(1-3/4)Gal sequences that can be terminated with Neu5Ac residue (sLe). These fucosylated antigens are usually positioned at the non-reducing end of the *N*- or *O*-glycan structures as well as on glycosphingolipids. Lewis antigens are subdivided into two types: type 1, including Lewis a (Le^a) and Lewis b (Le^b) antigens, which share a common basis structure consisting of β(1,3)-linked Gal-GlcNAc disaccharide, and type 2, which include Lewis x (Le^x) and Lewis y (Le^y) containing the β(1,4)-linked Gal-GlcNAc disaccharide [8] (Figure 9).

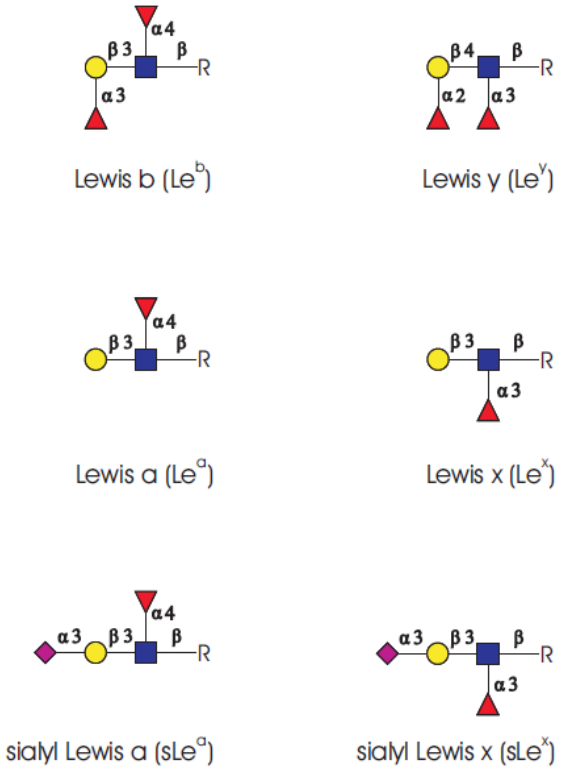


Figure 9 Lewis antigens found in malignant transformations. Blue square, GlcNAc; yellow circle, Gal; red triangle, Fuc; purple diamond, Neu5Ac [155].

In healthy individuals, Lewis antigens are normally expressed as terminal epitopes on glycoproteins and glycolipids of the secretory system and plasma as well as on erythrocytes, leukocytes and selectins. But they are also overexpressed in numerous carcinomas including lung[44], colon [156, 157], stomach [158], and kidney [159], for which they were found to be frequently shed into the blood stream [160]. In general, cancers have been found to be associated with reduced expression of type 1 Lewis antigens and elevated expression of type 2 antigens [161, 162]. In breast cancer tissues, loss of Le^b and

enhanced expression of Le^a were directly correlated with the malignancy progression, whereas overexpression of sLe^x was observed primary in advanced and recurrent cancer [163-166]. Moreover, enhanced expression of Le^y and sLe^x was reported in both primary and metastatic ovarian carcinomas [167, 168]. Overexpression of Le^b was observed in 60-90% of all epithelial cancers, including breast and ovarian cancers [169]. The presence of Le^y was directly linked to poor prognosis and decreased survival of lymph-node negative breast cancer patients. Similarly, in ovarian cancer patients its presence correlated with the increase of the malignancy [170, 171]. Increased expression of Le^x and sLe^x was observed in colon cancer tissues and cell line models, where the concomitant presence of both epitopes was correlated with shorter life expectancy [172]. SLe^x and sLe^a were defined as putative tumor markers in colon [173], breast [161, 162, 174] and pancreatic cancer [175], whereas Le^y were found to be overexpressed in ovarian cancer [176, 177].

Enhanced expression of Lewis antigens was also measured in the sera of cancer patients using immunohistochemical methods. Several glycoproteins bearing sLe^x epitopes were identified in patients with advanced breast cancer, where their increased amount correlated with the disease progression. A two-fold increase of *N*-glycans containing sLe^x was observed in the serum of breast cancer patients. Serum *N*-glycans of patients with advanced ovarian cancer have also shown elevated level of sLe^x on numerous acute-phase proteins such as Hpt, α1-acid glycoprotein (AGP) and α1-antichymotrypsin (ACT) [178-181]. Lewis antigens Le^y, Le^x and sLe^x have been detected in sera of lung cancer patients [182]. In case of tumors of epithelial origin, the expression of those antigens is usually associated with tumor progression, metastatic spread and general poor prognosis [88]. Moreover, sLe^x and sLe^a antigens acts as ligands for E-selectins and therefore contribute fundamentally to the adhesion of cancer cells to surface of endothelial cells. For this reason, sLe^x and sLe^a antigens may facilitate the event of metastasis formation [96, 183]. It has been shown that tumor cell lines of epithelial origin adhere to the endothelium via E-selectins, but on one hand the adhesion of colonic and pancreatic cancer cell lines are mediated mainly through an interaction with sLe^a. On the other hand, the adhesion of the breast, ovarian, lung and liver cell lines is promoted by the interaction with sLe^x [96, 183, 184].

Recently, it has been revealed that $sLe^{x/a}$ expression is associated with epithelial-mesenchymal transition (EMT), which is responsible for invasion of cancer cells in

surrounding tissues and tumor metastasis. It has been shown that EGF-induced EMT leads to elevated expression of sLe^x and sLe^a in colon cancer [185].

1.3.5 Blood Group Antigens

The ABH blood group antigens, distributed in body fluids, normal tissues and tumors, are typically found covalently attached as terminal epitopes to proteins, sphingolipids, or glycans. Their structures are depicted in Figure 10. ABH antigens that are located on human erythrocytes determine the type of the blood group [186]. Thus, individuals with blood group of type A possess A antigen on their erythrocytes, while those with blood group of type B possess the B antigen. Consequently, people with blood group AB carry both those antigens. Individuals with blood group 0 express the H antigen, which is also found at low level in other blood group types.

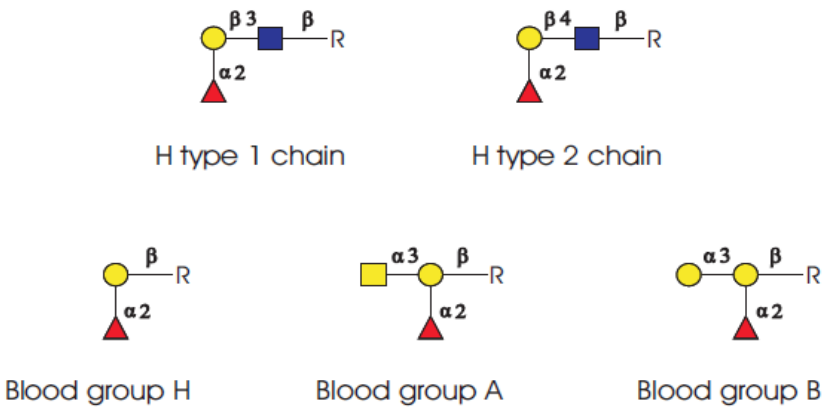


Figure 10 Structures of blood group antigens on human erythrocytes [186]. Blue square, GlcNAc; yellow square, GalNAc; yellow circle, Gal; red triangle, Fuc.

Depending on the blood group type, ABH antigens are differentially expressed within the proximal fetal human colon after birth. In colon cancer, enhanced expression of these antigens shifts to distant part of the colon [187]. Altered expression of ABH antigens in cancer comprises: (a) loss of antigens that are normally present in tissue of adults, (b) re-expression of fetal antigens, which are normally absent in adults and (c) non-consistent expression of ABH antigens in an individual [96].

Changes in the expression of ABH antigens have been reported in many carcinomas. The deletion or incomplete expression of particular blood group antigens in cancer tissues have been found in pancreatic cancer [188], neoplasia of bladder and oral tract [189, 190] as well as in ovarian cancer [191]. Moreover, loss of those antigens were reported

in lung cancer, which correlated with metastatic potential and poor prognosis [192]. In contrast, the neoexpression of ABH blood group antigens was observed in hepatocellular carcinoma [193].

Moreover, there are two cancer-associated oligosaccharides epitopes containing a fucose unit, namely antigens H and antigen Le^y. Elevated expression of H and Le^y antigens were found in tissues and tumor cell lines of oral mucosa cancer [194], colon adenocarcinoma [195], lung cancer [196, 197] and bladder tumors [198]. The overexpression of these antigens is generally associated with poor clinical prognosis. The increase of these glycan epitopes was accompanied by concomitant decrease of A and B blood group antigens.

1.4 Ovarian Cancer

1.4.1 Pathophysiology of the Disease

Ovarian cancer, being the sixth most common cancer-related malignancy worldwide, is the leading cause of death from all gynecological malignancies. It may originate from the ovary, fallopian tube or peritoneum [199]. The most common form of ovarian cancer is the epithelial ovarian cancer (EOC), which is initiated either in the ovarian epithelium itself or in cysts located on the ovary [200]. The morphological heterogeneity of EOC and the anatomical position of ovaries in the abdominal cavity are the main reasons for the frequent asymptotic course of the disease. Lack of sensitive screening methods and suitable biomarker for an early-stage dianosis are the main causes of poor survival rate [201, 202]. Only about 20% of all affected woman are diagnosed at an early disease stage I and II. The 5-years survival rate ranges from 60-90% when primary diagnosis occurs at an early-stage and it strongly fluctuates with the degree of tumor differentiation [203, 204]. Although improved treatments led to a 45% increase of the 5-years survival, an enhancement of the overall survival rate could not be observed [203] as most cancers of the ovary are diagnosed at late stages, when metastases are already present in distant organs.

The highest proportion of FIGO stages I and II are mucinous, clear cell and endometroid carcinomas, whereas serous carcinomas, mixed carcinomas and carcinosacromas are mostly present as FIGO stages III and IV [205]. Four histological subtypes of EOC differ in their clinical behavior and molecular characteristics, namely serous, mucinous, endometroid and clear cell carcinoma [206]. The serous subtype

represents over 75% of all cases and has the lowest 5-years survival rate ranging from 10-20% [207, 208]. The treatment usually consists of a cytoreductive surgery with subsequent platinum-based chemotherapy [201, 202]. Around 80-90% of patients with advanced disease will initially respond to chemotherapy but less than 15% will remain in permanent remission [209].

1.4.2 GLYCOSYLATION

Several approaches have been developed so far to monitor glycosylation changes in ovarian cancer. First, immunohistological staining was used to show differential expression of T_n and sT_n antigens in ovarian cancer tissue compared to control tissue and facilitate the characterization of tumor subtypes of the ovary [171, 210, 211]. Glycans bearing LacdiNAc motif and Lewis antigens were proposed as putative biomarkers for ovarian carcinoma using SKOV3, PA-1, OV4, OVM, m130, GG and RMG-1 as model cell lines [115, 212, 213].

Recently, glycan arrays printed with chemically synthesized carbohydrate epitopes were used for the detection of antiglycan antibodies in serum of women with diagnosed ovarian cancer, non-mucinous ovarian borderline tumors and healthy controls. Numerous glycan epitopes were proposed as highly discriminative for differentiation between tumors with low-malignant potential and healthy controls. Among all of them the P_1 structure, consisting of Galα(1,4)Galβ(1,4)GlcNAcβ, shows the highest discriminatory ability. Unfortunately, both sensitivity and specificity are moderate and comparable with those of CA125. A combination of both P_1 and CA125 improves neither sensitivity nor specificity [214].

Changes in serum glycosylation were also reported for total serum and serum glycoproteins. Using differential glycoprotein-lectin affinity methodologies, changes in glycosylation pattern of serum glycoproteins such as α1-proteinase inhibitor (α1PI) [215], Hpt [215], AGP [216], ACT, α2-macroglobulin (A2M) [217], Tf [217] and IgG [218] were observed. Several research groups reported N-glycome changes in the serum of EOC patients using different analytical approaches including high- and ultra-performance liquid chromatography (HPLC/UPLC), capillary electrophoresis (CE), and more recently MS-based techniques [136, 219-229].

Increase in triantennary, trisialylated *N*-glycan containing outer antennae linked fucose was detected in total serum using HPLC [136] and MALDI-TOF mass spectrometry [219, 230]. Elevation of tri- and tetraantennary fucosylated *N*-glycans was also found in EOC serum as well as on Hpt, AGP, α1PI and ACT by MALDI-TOF-MS, CE-LIF and HPLC [219, 222, 224, 231, 232]. Decrease of high-mannose structures was reported in total serum [219, 233]. Furthermore, increased levels of core fucosylated agalactosylated biantennary *N*-glycans were detected using MALDI-TOF-MS, HPLC and CE-LIF [136, 224, 230]. In contrast, decreased levels of *N*-glycans containing bisecting GlcNAc have been shown for total serum and IgG by MALDI-TOF-MS and CE-LIF, respectively [222, 224]. To conclude, MS-based glycomics is a very useful method for the determination of putative tumor markers for the diagnosis and monitoring of ovarian cancer.

1.5 Malignant Ascites

Ascites is an excessive accumulation of body fluid in the peritoneal cavity and it is usually a manifestation of advanced-stage diseases [234]. The presence of ascites in early-stage EOC correlates with poor prognosis and is directly linked to high-grade serous ovarian cancer, being an aggressive malignancy often accompanied with high-volume ascites [235].

Common factors leading to malignant ascites are increased capillary permeability, increased surface area for filtration as well as differences in increased hydraulic pressure and decreased oncotic pressure [236]. Several mechanisms have been proposed so far to explain the formation of malignant ascitic fluid (MAF) including lymphatic obstruction, activation of mesothelial cells in course of malignant metastatic process and increased vascular permeability caused by shedding of vascular endothelial growth factors (VEGF) and other cytokines [237-241]. Two exemplary mechanisms explaining the formation of malignant ascites from malignant cells are shown in the schema below (Figure 11).

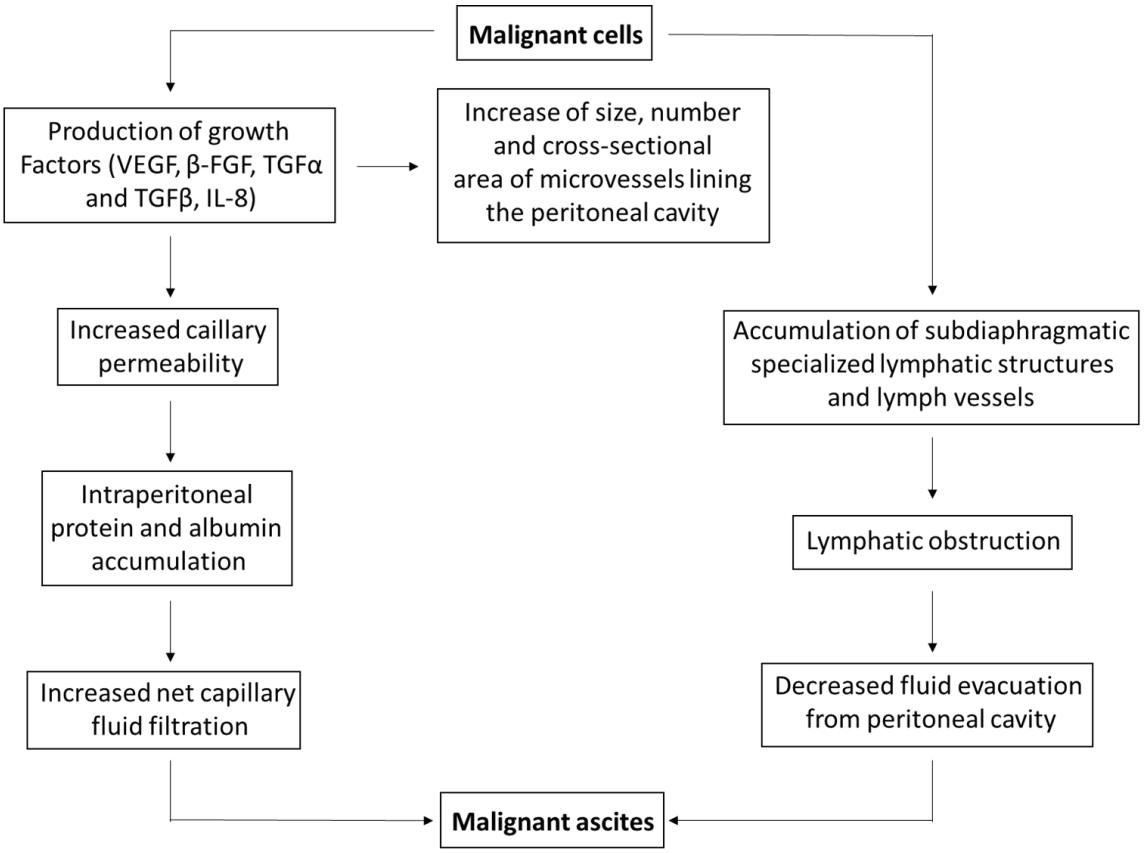


Figure 11 Proposed pathogenesis leading to malignant ascites [242].

Because MAF accumulation often occurs close to the targeted tissue, its similarity to ovarian cancer with respect to the cellular composition is high. MAF is generally a voluminous exudative event consisting of an acellular and a cellular fraction containing malignant cells originating directly from tumors, lymphocytes and mesothelial cells. It has been shown that especially ascites neoplastic spheroid cells may adhere to the ECM via β1 integrins and thus may play a role in the disease dissemination [243]. The acellular ascitic fraction includes angiogenic factors such VEGF [238] as well as other growth factors [244-247]. There is an evidence that MAF can affect the growth and invasiveness of EOC cells *in vivo* and *in vitro* [248]. Thus, investigation of ascites microenvironment based on their protein and glycan content may lead to better understanding of tumor behavior and progression.

Both proteomics and glycomics can serve as tools in the search for valuable EOC tumor markers. Because the accumulation of MAF arises predominantly when the disease is already at late-stage, exploration of disease-related proteins or glycans may not contribute to early-stage detection. Analysis of malignant ascites glycomics rather thought to extend the already existing bioinformatics data sets obtained from serum

proteome profile and to propose putative EOC tumor markers with potential use in patients' surveillance, prediction of disease outcome and response to treatment [235]. Even though a few MS-based studies provided molecular insights into ascites composition and revealed their protein and peptide content, the glycomic profile of EOC ascites is still unexplored. More than 6500 proteins and glycoproteins could be detected by analyzing the proteome of cancer cells isolated from ascites of serous EOC patients and from EOC-derived cell lines. The obtained profiles showed similarities regarding their protein composition, suggesting an extensive shedding of cell surface proteins and release into circulation [206]. Additionally, cellular and soluble fraction of ascites from EOC patients were collected and their protein profile was compared. Among proteins found exclusively in ovarian cancer ascites samples, 80 putative biomarker candidates could be proposed and 18 of them had been previously reported as proteins secreted in human plasma [234]. Moreover, a panel of 52 proteins, mainly glycosylated, isolated from the soluble fraction of ascites has been proposed as biomarker candidates for diagnosis of EOC [249].

1.6 CANCER BIOMARKERS

Cancers belongs to the leading causes of morbidity and mortality all over the world. The high morbidity to mortality rate is a result of lack of effective screening methods on early-stages or restrictions in effective treatment interventions. Cancer mortality can be reduced if persons concerned are detected and treated early, therefore, investigation of biomarkers for early-stage diagnoses are a main focus of interest in the cancer research.

According to National Cancer Institute, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of normal or abnormal processes, or of a condition or disease". Tumor markers can be produced by the tumor itself or as a response to the presence of cancer and other benign conditions. The currently available glycoprotein biomarkers are predominantly used for screening, monitoring of disease progression, prediction of disease outcome and response to treatment. Thus, there is an urgent need for discovery of novel biomarkers for early cancer detection [250].

Aberrant glycosylation of glycoconjugates such as glycoproteins, glycolipids or glycosaminoglycans is one of the fundamental features of tumor cells. At the present time, most of the U.S. Food and Drug Administration (FDA)-approved cancer biomarkers are glycoproteins [34, 109]. To date, most glycoproteins used as cancer biomarkers in the clinical diagnostics are detected using specific antibodies in immunoassay tests [109].

1.6.1 Tumor Markers Approved for the Diagnosis of Ovarian Cancer

1.6.1.1 Cancer Antigen 125 (CA125)

Cancer antigen 125 (CA125), also known as mucin 16 (MUC16), is a member of the mucin family glycoproteins [251].

Although the functional relevance of CA125 in cancer has not been fully unraveled, several factors influencing its biological role have been proposed such as its role as a lubricant, preventing the adhesion of embryonic membranes [252]. In addition, it was shown to enhance the invasive potential of a benign endometriotic cell line [253]. Furthermore, CA125 was shown to bind specifically to lectins such as galectin-1 and mesothelin [254, 255].

CA125 is a very heterogenous molecule with respect to both size and charge [254]. It is a heavily glycosylated transmembrane protein bearing both *N*- and *O*-glycans. Its molecular weight ranges from 200 to 2000 kDa and its glycan content is estimated to be about 25% [252, 256, 257]. The N-terminal region is rich in serine, threonine and proline and the C-terminus contains a transmembrane region and one potential tyrosine phosphorylation site. The molecule has 249 potential *N*-glycosylation and over 3700 potential *O*-glycosylation sites [254, 258].

Differences in *N*- and *O*-glycosylation of CA125 isolated from serum [258] or expressed in OVCAR-3 cells [259], amniotic fluid [260], placenta [261] were reported in several studies using different methodologies. Using mass spectrometry, *O*-glycans of core type 1 and 2 were predominantly detected on CA125. Additionally, bi-, tri- and tetraantennary complex-type bisecting *N*-glycans as well as *N*-glycans of high-mannose type including $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ could be found on CA125 isolated from OVCAR-3 cells [259]. Differences in *N*- and *O*-glycosylation were also observed in CA125 extracted from

amniotic fluid and placenta. In amniotic fluid, lectins showed the strongest binding to *O*-glycans as well as to core and antennary fucosylated structures [260]. CA125 from placental fluid exhibits in addition to core- and antenna-fucosylated structures a strong binding to lectins, which recognize both α(2,3) and α(2,6)-linked sialic acids. Indication for the presence of *O*-glycans or polylactosamine motifs was also noticed[261]. Recently, changes in *N*-glycosylation pattern of CA125 isolated from sera of ovarian cancer patients could be detected using WAX-/HILIC-HPLC method. Increase of core-fucosylated, mono-sialylated, biantennary *N*-glycans could be observed with a concomitant decrease of non-fucosylated, biantennary structures containing a bisecting GlcNAc. This study demonstrated that the differentially expressed glycans are better suited for the determination of specific and sensitive tumor markers than the measurement of the protein concentration itself [258]. Elevated expression of specific antigens or glycan structures on CA125 (Le^x epitopes on *N*- and *O*-glycans, bisecting *N*-glycans and high-mannose type *N*-glycans) evidenced to induce specific immunomodulatory effects, facilitating the tumor progression in ovarian cancer [252, 259]. Moreover, glycosylation of CA125 was shown to be involved in immune suppression, enabling the protection of the embryo from immune rejection [258].

CA125 is the most widely used serum marker for the detection and management of EOC and it is actually the best clinical routine marker for screening of post-menopausal women [262, 263]. In addition, CA125 was shown to be even more predictive when used in combination with sonography [264, 265]. The concentration of CA125 in serum is low as it is shed from the ovarian tissue into the bloodstream [258]. In late-stage EOC, CA125 is not only released from cancer cells but also from mesothelial cells [266]. The antigen serum concentration is elevated above the conventional cut-off vale of 35 U/mL in 80-85% women in advanced-stage EOC, but only in 50% women with EOC at stage I. Due to this fact, about half of all potentially curable tumors cannot be detected using the standard CA125-based screening method [267]. Due to its low performance for early diagnosis, it is preferentially used for follow up [263, 265]. Thus, high level of CA125 (>250 U/mL) prior to surgery and chemotherapy is associated with a worse prognosis, but when measured after several cycles of chemotherapy it is a useful prognostic factor for patient survival [268-270]. In other words, elevated concentration of CA125 during follow-up indicates disease recurrence [271].

CA125 antigen was identified for the first time in six different EOC cell lines and tumor tissue of ovarian cancer patients using the monoclonal antibody OC125 [272]. Three groups of anti-CA125 antibodies have been created, namely M11, OC125 and OV197, whereby each of them can recognize different non-overlapping epitopes on both native and denatured CA125. It was shown that OC125 antibody has reduced binding ability after treatment with PNGase F, suggesting that *N*-glycans could be part of the antigenic determinant. So far, none of the anti-CA125 antibodies allows the detection in serum of all existing molecular species of tumor-derived CA125. The different molecular species observed result from the degradation of CA125 during its transport throughout the circulatory system [254, 258, 273].

CA125 is not used alone for the primary diagnosis of ovarian cancer because of its poor sensitivity and specificity, which were determined to be 65 and 97% respectively [274]. Moreover, the sensitivity of CA125 is strongly dependent on the histological subtype. About 85% of patients with serous histotype show significant elevation in expression of CA125 in cancer tissue of ovary. An elevated level of CA125 above the cut-off limit was detected in 68% of papillary, 65% of endometroid, 40% of clear cell and 36% of differentiated adenocarcinomas. In mucinous subtype, CA125 raises only in 12% of the affected women [267]. In addition, the specificity of CA125 is also limited, especially in pre-menopausal women. On the contrary, elevated concentrations of CA125 were measured in diverse physiological conditions such as ovulatory cycle, first trimester of pregnancy or endometriosis. Furthermore, values of CA125 can rise in pathological situations e.g. during presence of benign ovarian cysts and tumors, uterine fibroids, infectious and inflammatory diseases, congestive heart failure, cirrhosis when accompanied by ascites presence and in abdominal malignancies other than EOC in women at any age [275-277].

Significant enhancement in both sensitivity and specificity for EOC detection can be reached using combined markers or techniques complementary to CA125. Combination of CA125 II, which contains antibody M11 in addition to OC125, with CA72-4 and M-CSF leads to increased sensitivity [278].

1.6.1.2 HUMAN EPIDIDYMIS PROTEIN (HE4)

Human epididymis protein (HE4) also known as WAP Four-Disulfide Core Domain 2 (WFDC2) is a heterogenous pool of small and heat-stable proteins of divergent

functions [279, 280]. Although many members of the protein family are protease inhibitors, no protease inhibitory activity could be detected for HE4 and its real function remains still unknown [279].

HE4 is an 11 kDa protein containing two whey acid protein domains and a “four-disulfide bond core” made up from eight cysteine residues. The glycan composition of the recombinant HE4 expressed in yeast Pichia pastoris (rHE4) and mammalian HEK293-F (mHE4) cells were estimated. Whereas rHE4 exhibits exclusively high-mannose structures (Man_8-Man_{11}), which are typically found on yeasts, mHE4 bears complex, biantennary N-glycans with varying degree of galactosylation, core fucosylation and sialylation. The *N*-glycosylation of HE4 appears to be important for its biological function, as mHE4, rHE4 and deglycosylated HE4 showed different proteolytic inhibitory activity in all three cases [281]. Being overexpressed and secreted by ovarian carcinoma cells, HE4 was exclusively found in cell lines expressing endogenous HE4 RNA. HE4-expressing cells secrete the glycoprotein in the extracellular matrix. The secreted form of HE4 has a higher molecular weight (25 kDa) compared to recombinant HE4 (11 kDa) and has one *N*-glycosylation site. The differences in the molecular weight of the protein after enzymatic deglycosylation confirmed that the *N*-glycosylation site is occupied [282].

HE4 is expressed in normal surface epithelium at a low level and is limited to the epithelium of the female reproductive tract and to the epithelium of proximal airways [282]. The expression level of HE4 in EOC depends on its histological subtypes. HE4 is highly expressed in 93% of serous and in 100% of endometroid EOCs patients, for which it was proposed as robust tissue biomarker. The expression of HE4 drops to 50% in clear cell carcinomas and it is not found in tumors of mucinous subtype. Moreover, most of carcinomas other than EOC don’t express this glycoprotein [282-284]. In general, it is increased in over 90% of women with EOC at the time of diagnosis [279].

HE4 is actually the most promising biomarker proposed to aid the diagnosis of women with suspected ovarian cancer [285]. It was shown to circulate in the bloodstream of ovarian cancer patients and not in age-matched healthy controls. Moreover, HE4 shows a higher specificity than CA125 in distinguishing of patients with benign ovarian diseases from those with malignant ovarian carcinomas [279]. The presence of the

secreted glycoprotein in serum can be detected with ELISA using monoclonal murine antibody directed against an HE4 epitope [284].

As HE4 is also elevated in CA125-deficient women and has low expression levels in normal ovarian tissue, benign ovarian diseases and tumors with low malignant potential, it is more specific than CA125 (specificity 93% vs. 78%, respectively) [266, 286, 287]. However, the sensitivities of HE4 and CA125 were shown to be comparable in patients with ovarian malignancies when compared to benign gynecological diseases [286]. Elevated values of HE4 were also found in non-ovarian malignancies including endometrial adenocarcinoma and in epithelial tumors of lung, colon, breast, kidney and thyroid as well as in patients with renal failure [282, 286].

The combination of the HE4 with CA125 was shown to exceed the performance of the individual biomarkers in their ability to determine the malignant potential of ovarian tumors from pre- and postmenopausal women presenting ovarian adnexal masses. The combination of both markers is known as the ROMA test and has already been approved by the FDA as a predictive marker [288, 289].

1.6.2 Tumor Markers clinically Approved for the Diagnosis of Cancers Other Than Ovarian Cancer

1.6.2.1 Prostate-Specific Antigen (PSA)

Prostate-specific antigen belongs to the family of proteolytic enzymes of Human Kallikrein 3 and exhibits a serine-protease activity [290].

PSA is produced by prostate epithelial cells and secreted into the lumen of the prostate gland where its propeptide is removed to generate the active form of PSA. Active PSA can eventually undergo proteolysis and produce inactive PSA. The occurrence of prostate cancer (PC) and other prostate diseases such as benign prostate hyperplasia (BPH) and prostatitis can lead to the disruption of the membrane and shedding of PSA into the peripheral circulation, where it can be detected using immunometric assay [291, 292].

PSA is a 28 kDa glycoprotein consisting of 237 amino acid residues with a single *N*-glycosylation site. The *N*-linked PSA glycans of seminal plasma from healthy

subjects are partially sialylated biantennary structures, which are mostly core fucosylated and only a few percentages are terminated with GalNAc residue [293, 294]. Recently it was evidenced that altered PSA glycosylation pattern enables the differentiation between normal prostate and PC as well as between the aggressive form of PC and its non-aggressive form [293, 295]. Elevated levels of core fucose and GalNAc as well as completely loss of sialylation have been shown for *N*-glycans from PSA secreted by a prostate tumor cell line when compared with those from PSA from seminal plasma of healthy individuals [293, 296]. On the contrary, a different *N*-glycan profile has been reported for PSA in the serum of PC patients when compared to normal seminal plasma, where decreased level of α(1,6)-fucose and α(2,3)-linked Neu5Ac was detected [297]. Furthermore, increased sialylation of PSA in the serum of PC patients compared to patients with BPH was demonstrated using specific lectins in lectin affinity chromatography [298, 299]. Since the differential sialylation pattern was noticed in most of the reported instances, the varying degree of sialylation could be prospectively used to improve PSA specificity in benign conditions and malignancy [297]

The predominately molecular form of PSA is the active form and is found in serum as a complex with protease inhibitors, primary with ACT and A2M. The remaining PSA in blood stream (10-30%) circulating as free PSA is biologically inactive [291, 297, 300]. In men with prostate cancer, the percentage of free PSA in serum is lower and conversely, the quantity of complexed PSA is higher than healthy men with a normal prostate or BPH [301].

PSA levels of 4.0 ng/mL and lower are considered as normal. However, various pathological and physiological factors can cause a man's PSA level to fluctuate. For example, a man's PSA level often rises in prostatitis and in infections of urinary tract. While prostate biopsies and surgery may increase PSA level in serum, some drugs used in BPH treatment can lead to its decrease in serum. In general, abnormally high PSA level or its continuous increasing over time indicates the presence of PC [302]. Moreover, serum PSA concentration rises in elderly men without PC, as PSA level reflects the prostate size, which in turn grows with increasing age [303].

The serum concentration of PSA is measured using an immunometric assay and the cut-off value for an abnormal PSA level is set to 4.0 ng/mL [304]. The estimated sensitivity

of PSA is 21% for detection of any PC and 51% for recognition of high-grade cancers (Gleason ≥8) at 91% specificity [305].

1.6.2.2 A-FETOPROTEIN (AFP)

A-fetoprotein is an “oncofetal antigen” produced by the fetal liver and yolk sack during pregnancy. It is the most abundant glycoprotein found in fetal serum [306].

AFP is a 70 kDa glycoprotein consisting of 591 amino acids and its carbohydrate content represents about 3-5% of its total mass [307]. The carbohydrate composition of AFP was determined in HCC and non-seminomatous germ cell tumor (NSGCT) patients. Seven *N*-glycan and four *O*-glycan structures were described. All reported *N*-glycans are of biantennary complex-type and the microheterogenity of AFP *N*-glycans is characterized by a different chain length, monosaccharide composition and by the extent of galactosylation, sialylation and fucosylation [307, 308]. Three *O*-glycans are mucin-type glycans composed of a Galβ(1,3)GalNAc disaccharide core with a variable number of sialic acid residues attached to it. Additionally, single *O*-GlcNAc could be detected [308].

AFP has different glycoforms and the measurement of cancer-related glycoforms, instead of the measurement of AFP concentration in serum, may contribute to improved specificity for HCC. Serum AFP concentration is elevated in HCC and benign liver diseases, whereas the ratio of AFP glycoforms varies in both. Two independent assays were developed for the quantification of absolute serum concentration of tumor-specific glycoforms and of the measurement of monosialylated AFP in serum. Both have shown a potential for discrimination between HCC and liver cirrhosis in patients, for which the total AFP concentration was in the diagnostically questionable range, namely 10-500 g/L [309]. Moreover, using isoelectric focusing (IEF), different glycoforms were identified to be highly specific for HCC[310, 311], NSGCT [312] and for patients with chronic liver disease [311, 312]. In addition, the clinical value of fucosylated AFP glycoforms AFP-L1 to AFP-L3 was evaluated based on differential binding affinity to *Lens culinaris agglutinin* (LCA). The highest binding affinity to LCA was observed for AFP-L3 glycoform, which bears an additional α(1,6)-linked fucose that is usually expressed by malignant liver tumors. AFP-L2 and AFP-L1 glycoforms have shown

moderate and non-binding affinity to LCA, respectively [307, 313]. Thus, the AFP-L3 glycoform was proposed as potential HCC tumor marker for the detection of small tumors (<2cm) [307, 314-316]. Additionally, elevated level of AFP-L3 is associated with worsened liver function [317], larger tumor mass [317], disease recurrence [314], poor prognosis and metastasis to distant parts in the body [318, 319]. AFP-L3 is U.S. FDA-approved marker for liver cancer prediction [320, 321].

AFP is a tumor marker primarily used to detect HCC and NSGCT [293]. Application of the AFP marker for screening of asymptotic population remains still controversial. Nevertheless, it is possible to use it for screening of HCC in non-alcohol induced cirrhosis patients, however, only when a standard ultrasound screening has already been performed. Additionally, AFP can replace biopsy for the diagnosis of HCC in patients with elevated risk factors and patients who already developed hepatic masses [322, 323].

In adults, the normal serum concentration of AFP is lower than 10 µg/L [309]. Serum concentrations greater than 500 µg/L are indicative for HCC [309, 324]. Constantly increasing AFP serum concentration appears to correlate with the development of HCC in individuals with known risk factors such a liver cirrhosis [324]. Additionally, moderately elevated AFP serum levels were found in pregnancy, non-malignant conditions such as hepatitis C caused liver cirrhosis, in patients with acute or chronic viral hepatitis and in other gastrointestinal cancers [322, 324, 325].

Sensitivity and specificity of detection for HCC vary widely. Using a cut-off value of 20 µg/L, AFP has been found to have a sensitivity of 41-65% and a specificity of 80-90% [322]. The overall poor sensitivity for HCC detection is caused by low AFP concentrations in HCC patients in early stages. Recently, it was shown that higher sensitivity and specificity could be achieved using a combination of total AFP and its glycoform AFP-L3 [326].

1.6.2.3 Human Chorionic Gonadotropin (HCG)

Human chorionic gonadotropin (hCG) is a glycoprotein hormone normally produced by placental trophoblasts. During the first trimester of pregnancy, the hormone production is relatively high and can be measured both in serum and urine [327].

HCG consist of two subunits named α and β. Whereas the α-subunit is common for all glycoprotein hormones (hCG, luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone), the β-subunit has a hormone-specific protein sequence [327]. When both subunits are present as non-covalently associated dimeric polypeptides, the β-subunit is responsible for the biological specificity of the heterodimeric hormone [328]. Additionally, both α- and β-subunits are found at low levels in their free forms [327].

HCG is glycosylated to a content of approximately 30%, and the glycosylation profile of its urinary form has been well characterized [327]. Both hCG subunits are glycosylated, whereby the α-subunit is shorter and is exclusively *N*-glycosylated. The β-subunit is longer and carries both *N*- and *O*-glycans. The *N*-glycan decoration of α-hCG comprises mainly non-fucosylated mono- and biantennary complex-type structures and sialylated hybrid-type *N*-glycans. In contrast, the β-subunit bears mono- and biantennary complex-type structures with over 50% α(1,6)-fucosylation. Complex type *N*-glycans are partially terminated with α(2,3) linked sialic acids. The *O*-glycans found on the β-subunit have been shown to consist of disialylated core 1 *O*-glycans and core-2 type *O*-glycans [327, 329-336].

It could be shown that hCG was not only expressed during pregnancy but also during cancerogenesis, especially in trophoblastic and testicular germ cell tumors [337]. Elevated serum concentration of free β-hCG was measured in patients with non-trophoblastic cancers [337, 338]. Some isoforms of hCG were associated with malignancies. For instance, the “nicked” hCG contains several cleaved peptide bonds within the β-hCG sequence, and the “hyperglycosylated” form is the major form found in urine of patients with trophoblastic cancers such as choriocarcinoma [339]. *N*-glycan structures found in urinary hCG from choriocarcinoma patients contain one to three terminal sialic acid residues and are more fucosylated than the hCG from non-diseased individuals or patients with benign trophoblastic diseases [340].

HCG is used as a sensitive marker for diagnosis of trophoblastic tumors and when determined together with AFP, it provides an outstanding marker for monitoring of patients with testicular germ cell tumors [337, 341].

2 ANALYTICAL METHODOLOGIES

2.1 CHEMICAL RELEASE OF N-GLYCANS

Efficient de-N-glycosylation is a key to successful and accurate glycan characterization. The structural diversity of N-glycans can only be investigated after detachment from the proteins or peptides. The chemical cleavage of N-glycans from glycoproteins can be performed using anhydrous hydrazine, which is a highly flammable and toxic reagent. However, in practice, the reaction is limited to small-volume preparations [342]. Another disadvantage of this method is deacetylation of N-acetyl groups on glycans, which need to be re-N-acetylated again [343] (Figure 12).

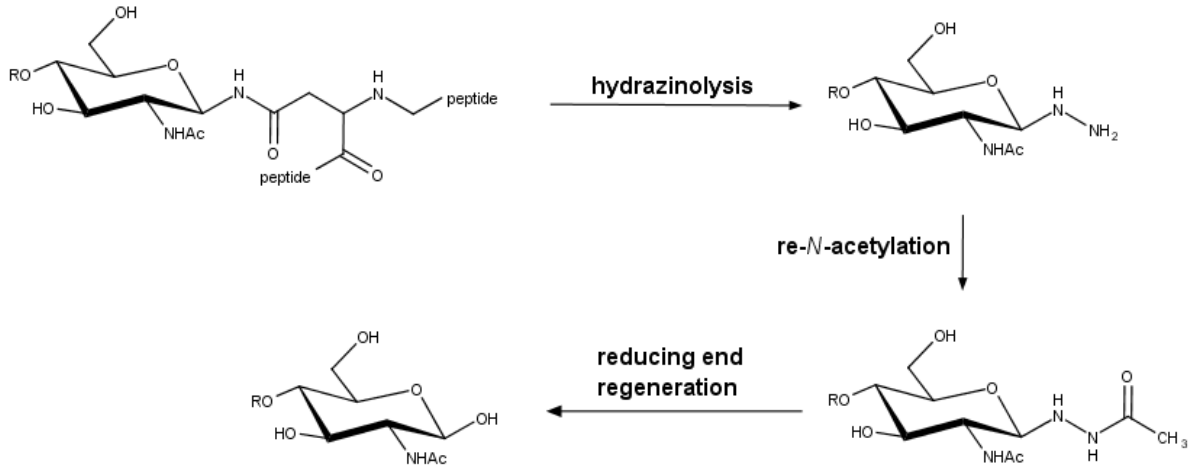


Figure 12 Proposed reaction schema for chemical removal of N-glycans from glycoproteins.

2.2 ENZYMATICAL RELEASE OF N-GLYCANS

N-Glycan release is usually performed enzymatically, since enzymes are easy to handle in any laboratory and enzymatic release of N-glycans avoids handling of dangerous organic solvents [344]. Enzymatic deglycosylation using glycosidases stemming from bacteria and plants is the most efficient method [11]. The most frequently used enzyme is peptide-N^4-(N-acetyl-β-D-glucosaminyl)asp

aragine amidase F also known as PNGase F [345]. It is a 34.8 kDa enzyme, which is secreted by *Flavobacterium meningosepticum*. It has been shown that two glutamic acids and one aspartic acid are responsible for the catalytic activity and stability of the enzyme [345, 346]. PNGase F cleaves the asparagine-linked oligosaccharides from

proteins by digestion of the β-aspartylglucosamine bond between the side chain of Asn and the first GlcNAc, thereby converting Asn into Asp [347]. The generated 1-amino-oligosaccharide intermediate hydrolyses to ammonia and a free intact N-glycan [348] (Figure 13).

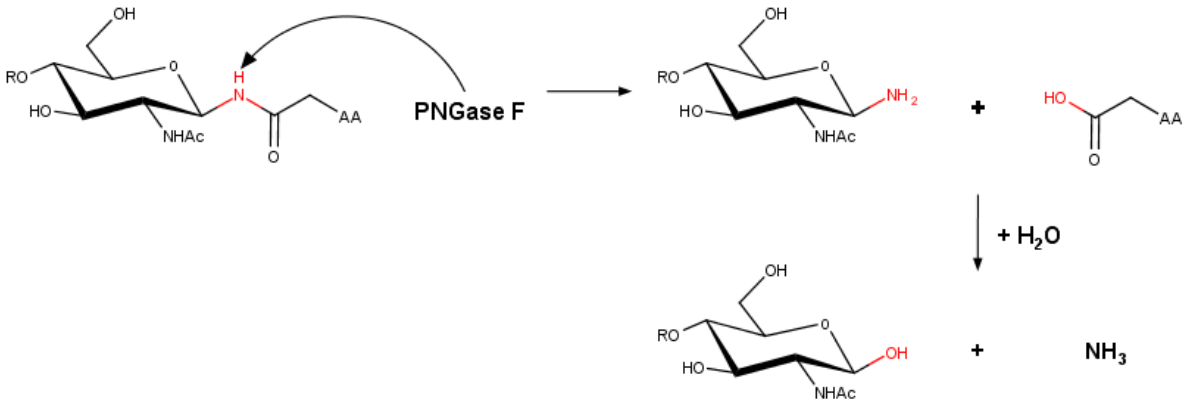


Figure 13 Proposed reaction scheme for the cleavage of N-glycans via enzymatic deglycosylation with PNGase F. A chitobiose core, consisting of two β(1-4)-linked GlcNAc residues, attached to Asn on the polypeptide chain and an active PNGase F enzyme are required for the N-glycan release from glycopeptides [345, 349].

The structural requirement for the correct cleavage is an oligosaccharide consisting of at least the N,N'-diacetylchitobiose core and a peptide linkage of amino- and carboxyl groups on Asn residue [345]. However, the accessibility of PNGase F to glycosylation sites on native, correctly folded proteins is limited, therefore the glycoprotein should be denatured before using the enzyme. This increases the efficiency of the reaction and reduces the required amount of enzyme needed for the complete cleavage of N-glycans [47]. PNGase F is active in a broad pH range (6.0-10.0), but the optimal activity can only be achieved in pH range 7.5-9.5 [350]. Due to its broad substrate specificity, the enzyme is able to detach all N-glycan types, namely high-mannose-, hybrid- and complex-type [346]. PNGase F is also able to cleave polysialylated, sulfated, phosphorylated complex N-glycans as well as α(1-6) core fucosylated N-glycans [351]. However, the processing of N-glycans bearing α(1-3) fucose on the outermost core GlcNAc is inhibited since the enzyme is unable to cleave it [345]. In this case, PNGase A enzyme can be used as an alternative. PNGase A is able to cleave high-mannose, hybrid- and complex-type N-glycans from glycopeptides [345]. Another enzyme used for the cleavage of high-mannose and hybrid-type N-glycans is endo-β-N-acetylglucosaminidase H (Endo H). The cleavage occurs between the GlcNAcβ(1-4)GlcNAc on the core-structure, thus one GlcNAc residue remains on the peptide [351].

2.3 MALDI-TOF MASS SPECTROMETRY

For better understanding of the role of glycans in complex biomolecules such as glycoproteins in biochemical processes, a complete structural characterization of glycans is of essential importance. In the glycomic field, diverse chromatographic separation methods have been used to characterize the glycome such as reversed-phase liquid chromatography (RP-HPLC), high-pH anion-exchange chromatography (HPAEC-PAD), size-exclusion chromatography. Unfortunately, the isomeric structural information about the glycan composition is only possible when coupled to powerful spectroscopic methods such as nuclear magnetic resonance (NMR) and mass spectrometry (MS). NMR provides detailed information about the structure of carbohydrate but shows limitations in terms of the required sample amount for analysis and in the complexity of data for interpretation. For determination of glycan composition, branching and linkage information of native or derivatized complex carbohydrates, mass spectrometric methods are of first choice [352]. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are sensitive measurement techniques that are widely used to characterize glycans. ESI produces different types of ions, which vary with the composition of the solvent and the type of ion source used. The produced ions provide complex glycan profiles and make the data difficult to interpret [353]. Moreover, ESI allows the sample measurement in lower throughput than MALDI [353]. The big advantage of MALDI towards ESI method is the relative ease of data analysis of the structural profile, as multiple charged ions are not generated during the measurement. Additionally, it has high tolerance towards salts and allows measurement of multiple samples over a broad measurement range within a short time period. Thus, MALDI-TOF-MS is the most convenient method used for glycan analysis [352-356].

To obtain a signal, minimal sample amount in a range pico- to femtomole is required. The analyte is mixed with a matrix compound, usually a small, ultraviolet (UV) absorbing, organic molecule and allowed to cocrystallize on a ground steel target by evaporation of the solvent. For glycan analysis, DHB (2,5-dihydroxybenzoic acid) remains the most commonly used matrix [357]. Recently, it has been shown that DHB mixed with additives such 2-hydroxy-5methoxy-benzoic acid (sDHB) gives even more homogenous mixture of samples with matrix and thus better quality of acquired spectra [358]. The cocrystallized sample is subjected to a pulsed laser and matrix crystals start

to desorb together with the analyte ions, absorbing UV-light produced by nitrogen laser beam (λ=337 nm). Most of the laser energy, which is usually in the UV range, is absorbed by the matrix and transferred to the analyte, which is ionized by processes such as hydrogen or alkali metal. The created ions are accelerated in the electric field with a constant kinetic energy [359]. Because of the pulsed ion generation, MALDI mass spectrometers are often equipped with time-of-flight (TOF) analyzer. The acceleration of the ions towards the detector occurs under vacuum with a constant kinetic energy and depends solely on their mass and charge [356, 360]. Ions exhibiting the same energy but different masses speed up and reach the detector at different measurement points. Overall, small molecules will reach the detector first [359]. As a consequence, one signal per each molecule will be created [359] (Figure 14).

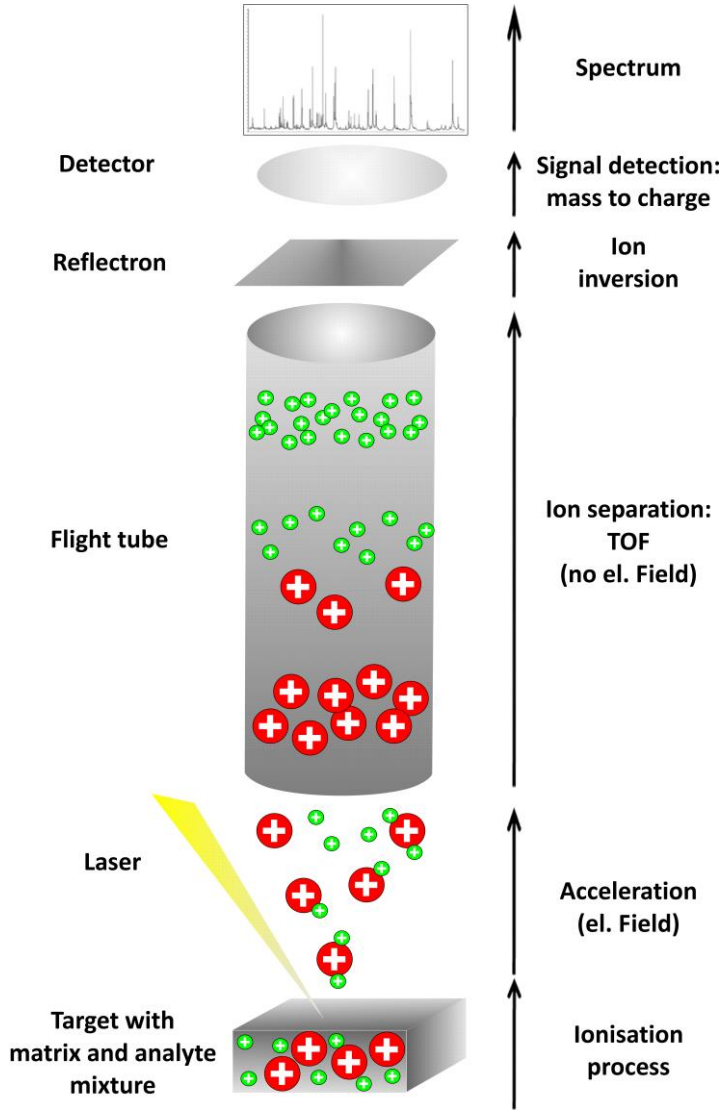


Figure 14 Schematic illustration of MALDI-TOF-MS measurement procedure.

The ionized species are mainly single charged molecule ions. For neutral glycans, alkali metal doped adducts such $[M+Na]^+$, $[M+H]^+$ and $[M+K]^+$ are preferably created. Other cations can also be produced by adding of appropriate salt to the matrix. In contrast, sialylated glycans provide adducts: $[M-H]^+$, $[M+Na]^+$ and $[M-nH+(n+Na)]^+$, whereby the latest is formed due to the salt formation [353].

Collision-induced dissociation (CID) is a useful tool for glycan fragmentation. The high-energy collision-induced activation leads to the formation of specific ion fragments, which allows the determination of sequence, degree of branching and gives hint about the linkage form of monosaccharides [352, 361].

For the simultaneous profiling of neutral and acidic structures in the positive ion mode, the negative charge of the glycans should be neutralized prior to measurement. One of the most common methods is the permethylation reaction. The reaction allows forming methyl ether from free hydroxyl groups and methyl esters from the carboxyl groups of sialic acids. For quantitative conversion, the hydroxyl groups must be deprotonated prior to the methylation step. Their sodiated forms react further with methyl iodide, providing a quantitative conversion to permethylated glycans [362]. The permethylation reaction improves glycan ionization, stabilizes sialic acids and prevents salt formation [353, 356]. Moreover, it could be shown that the profiling of permethylated glycans by MALDI-TOF-MS is similar the profile obtained in HPLC, allowing a reliable quantification of the permethylated glycans [363].

3 SCIENTIFIC GOALS

This thesis contributes to the research on novel tumor-associated serum glycan biomarkers for the detection of ovarian carcinomas. Epithelial ovarian cancer (EOC) is the most common cause of death within all gynecologic malignancies in women of all ages. One of the manifestations of advanced ovarian cancer diseases is the accumulation of ascitic fluid, which is a build-up of large volume of peritoneal fluid from infiltrated circulating serum.

The high mortality rate in ovarian cancer is caused on one hand by the absence of early symptoms, especially at early disease stages, and on the other hand by the lack of sensitive biomarkers for early diagnosis. Survival rate is strongly correlated with the disease stage at the time of diagnosis, and the early detection of cancer is therefore essential for long-term patients' survival. The discrepancy between survival rates in early and advanced stages (80-90% in early-stage vs. 15-20% in the late-stage) has reinforced the need for biomarkers with higher diagnostic accuracy to distinguish early-stage malignancies from benign pelvic masses. Incorporation of patients with benign ovarian diseases (BOD) as a control group is essential, since they are a common source of potentially false-positive cases.

The main goal of this work was the in-depth analysis of the serum N-glycome from EOC patients and its comparison with the glycomic profile from control groups, consisting of healthy individuals and patients with BOD, in order to develop a glycan-score for the diagnosis of EOC at any disease stage. Subsequently, the glycome of ascitic fluid has been unraveled for the first time.

The first part of the thesis focuses on the discovery of novel glycan-based biomarker from serum of EOC patients at all disease stages. For this purpose, N-glycans were cleaved from proteins using PNGase F, purified, permethylated and subsequently measured by MALDI-TOF-MS. The procedure was carried out using a standardized efficient and reliable protocol leading to the obtainment of highly reproducible results. Measured spectra were interpreted for their glycan composition and the corresponding areas of each glycan peak were calculated. The calculated N-glycans areas were normalized prior to statistical evaluation by setting them to a total of 100%. Using the statistical software SPSS 21.0, a glycan-based biomarker named GLYCOV was generated for late-stage ovarian cancer and the best cut off-value was calculated.

Subsequently, the diagnostic performance of the biomarker was evaluated and compared to the one of CA125, which is the only approved tumor marker for the diagnosis of ovarian cancer in the clinical routine diagnostics.

In the second part, GLYCOV was tested for its ability to detect ovarian cancer at an early-stage using a cohort consisting exclusively of ovarian cancer samples of stage I and II. Its diagnostic performance was compared to CA125, especially in order to distinguish between malignant ovarian cancer and benign ovarian diseases.

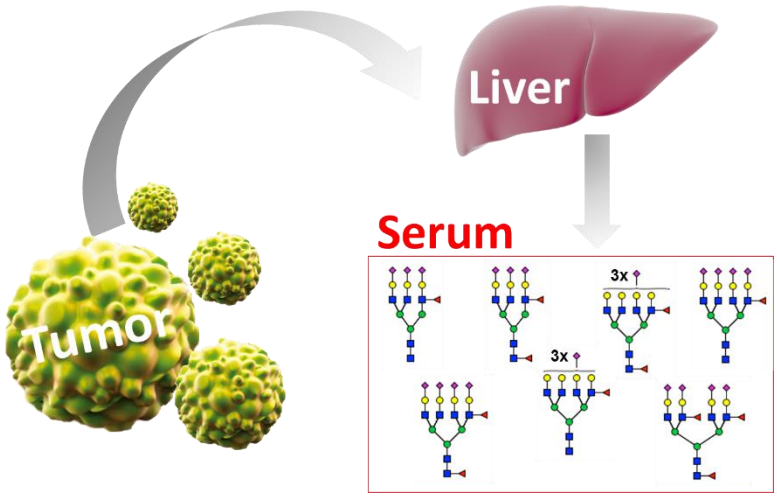
The third part of the thesis involves the first report on the total N-glycome of ascitic fluid from patients suffering from ovarian cancer. In addition, the resulting N-glycan profile was compared to the one derived from serum proteins of the same patient group, which is beneficial to overcome the patients' specific glycosylation profile. As ascitic fluid glycoproteins may originate from the tumor itself or be an infiltrate of serum, the analysis of protein glycans derived from ascites of autogenic subjects may contribute to a better understanding of general mechanisms occurring during carcinogenesis. The obtained glycomic information could prospectively be used to extend the already existing genomic and proteomic data and to evaluate novel prognostic biomarkers.

4 PUBLICATIONS

4.1 SERUM GLYCOME PROFILING: A BIOMARKER FOR DIAGNOSIS OF OVARIAN CANCER

This chapter was published in the following journal:

Karina Biskup, Elena I. Braicu, Jalid Sehouli, Christina Fotopoulou, Rudolf Tauber, Markus Berger, and Véronique Blanchard, *Journal of Proteome Research* **2013**; 12 (9), 4056-4063.



The original article and supplementary data are available at:

http://dx.doi.org/10.1021/pr400405x

DOI: 10.1021/pr400405x

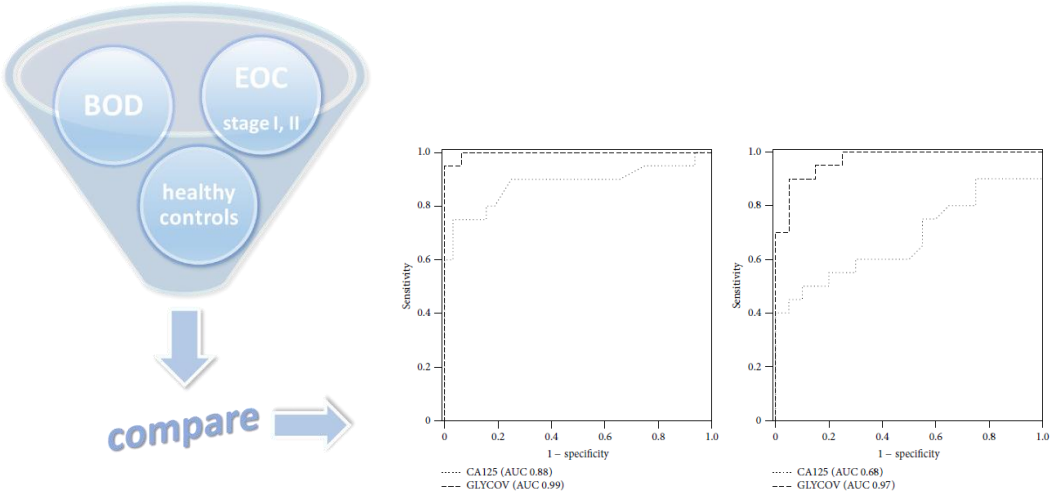
Author contribution:

- Preparation and MALDI-TOF-MS measurement of all samples
- Data analysis
- Discussion and statistical evaluation of all results
- Preparation of the manuscript

4.2 The Serum Glycome to Discriminate Between Early-Stage Epithelial Ovarian Cancer and Benign Ovarian Diseases

This chapter was published in the following journal:

Karina Biskup, Elena I. Braicu, Jalid Sehouli, Rudolf Tauber, and Véronique Blanchard, *Dis Markers* **2014**; 2014:238197.



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DOI: 10.1155/2014/238197

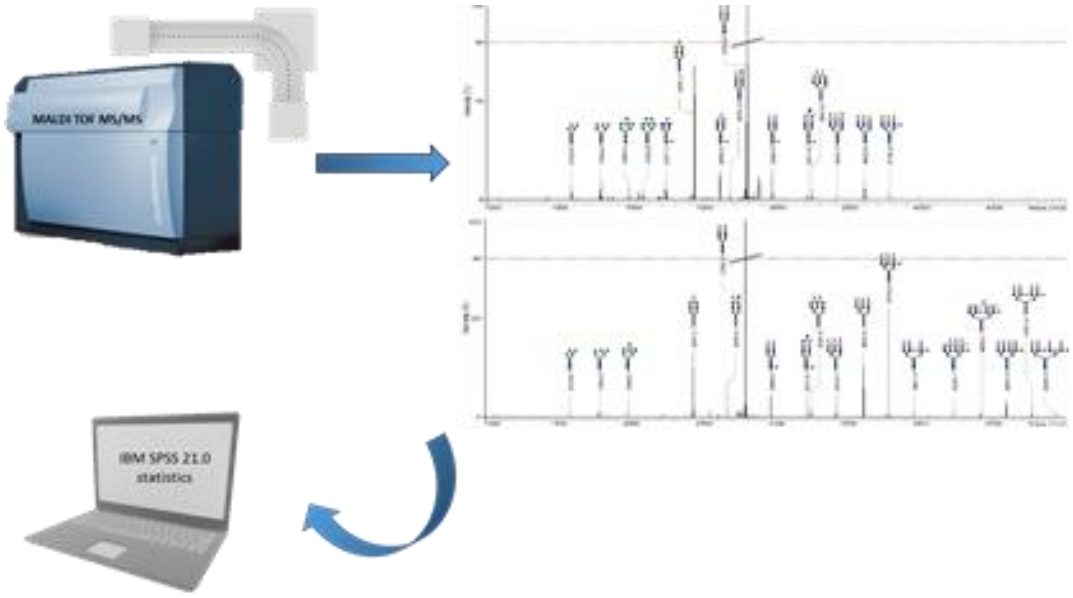
Author contribution:

- Preparation and MALDI-TOF-MS measurement of all samples
- Data analysis
- Discussion and statistical evaluation of all results
- Preparation of the manuscript

4.3 The Ascites N-Glycome of Epithelial Ovarian Cancer Patients

This chapter was published in the following journal:

Karina Biskup, Elena I. Braicu, Jalid Sehouli, Rudolf Tauber, and Véronique Blanchard, *Journal of proteomics* **2017**; 157, 33-39.



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DOI: 10.1016/j.jprot.2017.02.001

Author contribution:

- Preparation and measurement of all samples (MALDI-TOF-MS/ HPLC)
- Data analysis
- Discussion and statistical evaluation of all results
- Preparation of the manuscript

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6 APPENDICES

6.1 ABBREVIATIONS

%	percent
ABH	blood group antigens A, B and H
ACT	*α*1-antichymotrypsin
AFP	*α*-fetoprotein
A2M	*α*2-macroglobuline
Asn	asparagine
BPH	benign prostate hyperplasia
BOD	benign ovarian diseases
CA125	cancer antigen 125
cDNA	complementary DNA
CDG	*Congenital Disorder of Glycosylation*
CE-LIF	capillary electrophoresis-laser-induced fluorescence
Da	Dalton
DNA	deoxyribonucleic acid
Dol-P	dolichol phosphate
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EOC	epithelial ovarian cancer
ER	endoplasmic reticulum
FDA	U.S. Food and Drug Administration
FIGO	the International Federation of Gynecology and Obstetrics staging system for cancer
Fuc	fucose
Fuc-T	fucosyltransferase
FUT	gene encoding the Fuc-T
Gal	galactose
GalNAc	*N*-Acetylgalactosamine
GDP	guanosine diphosphate
Glc	glucose
GlcNAc	*N*-acetylglucosamine

GlcNAc-T	*N*-acetylglucosaminyl-transferase
GPI	glycosylphosphatidyl-inositol
HCC	hepatocellular carcinoma
hCG	human chorionic gonadotropin
HE4	human epididymis protein 4
HILIC	hydrophilic interaction liquid chromatography
Hpt	haptoglobin
HPLC	high-performance liquid chromatography
IgG	immunoglobulin G
kDa	Kilodalton
LacdiNAc	GalNAcβ(1,4)GlcNAc
LacNAc	*N*-Acetyllactosamine
LCA	*Lens culinaris agglutinin*
MAF	malignant ascitic fluid
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
Man	mannose
MGAT	gene encoding the GlcNAc-T
mL	Milliliter
MS	mass spectrometry
Neu5Ac	*N*-Acetylneuraminic acid/ sialic acid
NSGCT	non-seminomatous germ cell tumors
PC	prostate cancer
pH	negative of the logarithm to base 10 of the molar concentration
poly-LacNAc	poly-*N*-acetyllactosamine
PNGase F	peptide-*N4*-(*N*-acetyl-beta-glucosaminyl) asparagine amidase
PSA	prostate-specific antigen
RNA	ribonucleic acid
sLe	sialyl Lewis epitope
Ser	serine
ST	sialyltransferase
T, T_n	tumor antigens
Thr	threonine
Tf	transferrin
Trp	tryptophan

U	unit
UDP	uridine diphosphate
VEGF	vascular endothelial growth factors
WAX	weak anion exchange chromatography
WFDC2	whey-acidic-protein (WAP) four-disulfide core domain protein 2

6.2 CRRICULUM VITAE

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.