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# GLYCANS AS BIOMARKERS: STATUS AND PERSPECTIVES

## GLIKANI KAO BIOMARKERI: STATUS I PERSPEKTIVE

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**Summary:** Protein glycosylation is a ubiquitous and complex co- and post-translational modification leading to glycan formation, i.e. oligosaccharide chains covalently attached to peptide backbones. The significance of changes in glycosylation for the beginning, progress and outcome of different human diseases is widely recognized. Thus, glycans are considered as unique structures to diagnose, predict susceptibility to and monitor the progression of disease. In the »omics« era, the glycome, a glycan analogue of the proteome and genome, holds considerable promise as a source of new biomarkers. In the design of a strategy for biomarker discovery, new principles and platforms for the analysis of relatively small amounts of numerous glycoproteins are needed. Emerging glycomics technologies comprising different types of mass spectrometry and affinity-based arrays are next in line to deliver new analytical procedures in the field of biomarkers. Screening different types of glycomolecules, selection of differentially expressed components, their enrichment and purification or identification are the most challenging parts of experimental and clinical glycoproteomics. This requires large-scale technologies enabling high sensitivity, proper standardization and validation of the methods to be used. Further progress in the field of applied glycoscience requires an integrated systematic approach in order to explore properly all opportunities for disease diagnosis.

**Keywords:** biomarkers, cancer, diagnostic, heterogeneous analytes, glycans, glycome

**Kratak sadržaj:** Glikozilacija proteina je univerzalna i složena ko- i post-translaciona modifikacija koja dovodi do formiranja glikana, tj. oligosaharidnih lanaca koji su kovalentno vezani za polipeptidnu kičmu. Dobro je poznat značaj promena u glikozilaciji proteina za nastanak, razvoj i krajnji ishod različitih bolesti kod ljudi. Glikani se smatraju jedinstvenim strukturama za dijagnozu, i praćenje toka bolesti. U »omics« eri, glikom, glikanski analog proteoma i genoma, predstavlja mogući izvor novih biomarkera. Kreiranje strategije za otkriće biomarkera zahteva nove principe i platforme za analizu relativno malih količina brojnih glikoproteina. Očekuje se da glikomske tehnologije, koje su još u razvoju, a koje obuhvataju različite tipove masene spektrometrije i afinitivnih tehnika, rezultiraju novim analitičkim procedurama u oblasti određivanja biomarkera. Najveći izazovi za eksperimentalnu i kliničku glikoproteomiku su: pretraga različitih tipova glikomolekula, odabir potencijalnih markera i njihova selekcija ili prečišćavanje i identifikacija. Za ovo je neophodno razviti tehnologije koje će omogućiti visoku senzitivnost detekcije biomarkera kao i odgovarajuću standardizaciju i validaciju novih metoda, kako bi se one mogle primeniti u laboratorijskom radu. Dalji razvoj na polju primenjene glikonauke zahteva integrisani sistemski pristup sa ciljem da se na pravi način iskoriste sve njene mogućnosti u dijagnostici.

**Ključne reči:** biomarkeri, dijagnostika, glikani, glikom, heterogeni analiti, kancer

### Bioinformative potential of glycans

Over 70% of all human proteins are glycosylated and they form a major part of the human serum proteome (1, 2). Glycoproteins comprise enzymes, antibodies, hormones, cytokines, receptors and various structural proteins. Glycosylation is a ubiquitous and most important co- and post-translational modification leading to the formation of very heterogeneous and structurally complex glycans, i.e. oligosaccharide chains covalently linked to polypeptides (3–6). It is known that thirteen monosaccharides and eight amino acids are involved in glycoprotein linkages, while at least 41 bonds including anomeric configurations, phosphoglycosyl linkages, C-mannosylation as well as GPI (glycosylphosphatidylinositol anchors) can be formed. Among monosaccharides, the basic building blocks for glycans,

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the most common are hexopyranoside, acetamidosugars, 6-deoxy hexoses and sialic acid. Generally, variation in the sequence of monosaccharides in oligosaccharide chains, as well as the possible formation of different linkages, isomers and branching patterns or postglycosylation modifications of sugars, have enabled them to carry more variation than either nucleotides or amino acids, *i.e*, to have enormous bioinformative coding potential. Moreover, at the level of individual glycoproteins, both N-linked and O-linked glycans can be attached and heterogeneous glycosylation can occur. This means that the structural composition can vary from molecule to molecule, resulting in the existence of diverse glycoforms of the same glycoprotein. The presence of various glycoforms leads to functional diversity and there is no single unifying function for the carbohydrates present in glycoproteins (7).

Much potential biochemical/biological information can be hidden in the oligosaccharide chains (6, 7). Complex carbohydrates (glycans) markedly influence the physico-chemical (biochemical) properties of a molecule, in terms of maintenance of conformational stability and protection from proteolysis, but they are also important as recognitive determinants influencing antigenic properties, the control of intracellular transport, secretion (clearance and targeting) and biological activity by modifying interactions with other molecules. Moreover, glycans are considered as tags indicating the age of molecules, their origin in relation to cells, tissue or species, and in differentiation of self from non-self.

The meaning of the extreme structural diversity of glycans is explained by the introduction of the concept of a sugar code (8–10). According to this, glycans act as information-storage molecules and places for the exchange of this information, at the level of soluble, secreted or membrane-bound structures. In sharp contrast to the linear and rigid genetic or amino acid-generated codes, the glycocode implies correct two-dimensional combinations of monosaccharides. Since glycan structure is dynamically changed by many factors, such as extra- or intra-cellular stimuli, the glycocode is not strict, but flexible enough to adopt different conformations. However, the preservation of native glycan structures is essential for proper recognition by different types of carbohydrate-binding proteins, such as lectins, *i.e.* for deciphering the glycocode. Protein–carbohydrate interactions have not only biological, but also medical consequences, since they represent key steps involved in the control of cell homeostasis and its social doings (10). Thus, they are involved in sperm–egg interactions, host–pathogen interactions, leukocyte trafficking, blood clotting, apoptosis, attachment and invasion of cancer cells, etc. Consequently, understanding and implementation of basic research on human glycans in clinical applications as tools and targets for diagnosis or therapy are one focus of contemporary medical glycobiology.

## Biomedical implications of glycosylation

*Glycosylation and disease*

Glycan biosynthesis involves different types of glycosyltransferases, glycosidases and sugar nucleotides (3, 4). These enzymes and corresponding substrates are often expressed in a cell- and growth-specific manner, affecting the relative amounts and structures of glycans. Glycosylation is not template-driven, but is indirectly controlled by a number of genes, i.e. 1 % of the translated genome participates in oligosaccharide synthesis and function. Dynamic structural alterations of oligosaccharide chains have been implicated in a variety of diseases, and they reflect a reprogramming of these complex control mechanisms, at early, intermediate and mainly late glycoprotein processing steps (5, 11).

Enzyme abnormalities associated with alterations in distinct glyco-structures have been found in different types of leukemia and hematopoietic diseases, in carbohydrate deficiency diseases, leukocyte adhesion deficiencies, cystic fibrosis, diabetes, intestinal inflammatory and liver diseases, osteoarthritis, rheumatoid arthritis, thrombosis, etc. The disease implication of glycopeptide bonds was shown to be related to both N- and O- bonds, as well as glypiated linkages (5). One well-known example, including a broad clinical spectrum of patients, is congenital disorders of glycosylation (CGD) caused by defects in the synthesis and processing of N-linked glycans, mainly due to enzymatic defects responsible for dolichyl pyrophosphate oligosaccharide assembly (12, 13).

Changed activity of GDP-mannose- 4,6- dehydratase, *i.e*. lack of GDP-Fuc (fucose) resulting in impaired formation of Fuc Ser/Thr, is responsible for leukocyte adhesion deficiency type II, and closely related disorders, with subsequent recurrent infection as well as severe mental and growth retardation (14).

Formation of O-glycosidic bonds by addition of GlcNAc (N-acetylglucosamine) or Glc (glucose) on specific Thr residues in proteins that belong to the Rho family of mammalian GTP-ases, is known to be caused by microbial (clostridial) cytotoxins. They are connected with pathophysiological conditions of botulism, gas gangrene, antibiotic-associated diarrhea and pseudomembraneous colitis (15).

Similar changes, involving an increase in the presence of Ser/Thr-GlcNAc on regulatory proteins are found in diabetic conditions, and may be associated with overexpression of the key enzyme for Glc to GlcNAc conversion *i.e*. glutamine: fructose-6-phosphate amidotransferase, but also with a high level of GlcNAc-transferase transcripts in the beta cells of islets of Langerhans (16, 17).

The defect in biosynthesis of the GPI-anchor of granulocytes and B lymphocytes, i.e. in glypiation is found in paroxysmal nocturnal hemoglobinuria (18).

Profound changes in the expression and structure of carbohydrate components, resulting from rear-

rangements of glycan biosynthetic pathways in the Golgi apparatus (19–21) or activation of particular oncogenes (11, 22) are hallmarks of all types of experimental and human cancers and are of special interest for their detection and prevention. A number of changes that occur are difficult to classify, but some generalizations can be deduced.

*Cancer-associated glycan structures*

There are a wealth of literature reports on glycans and cancer, but their role and complex regulatory mechanisms in their biosynthesis are still not elucidated and the reports are often conflicting. Generally, a cancer-related oligosaccharide pattern is found to be associated either with expression and secretion of inappropriate, for example, incompletely glycosylated molecules, or the appearance of new antigens. Many molecular alterations accompany malignant transformation: changes in sialylation, fucosylation, galactosylation, N-glycan branching and truncation of the O-glycans of mucins, abnormalities in the expression of blood group and tissue antigens, etc. (23). In general, low activity of α2-FucT (fucosyltransferase) and increased activity of serum ST (sialyltransferases), serum α3-FucT and serum GalT (galactosyltransferase) are observed in cancer.

Increased sialylation is found on both N-linked and O-linked glycoconjugates (11, 24–26). For instance, mucin production and secretion which is elevated in cancer cells, predominantly comprises sialomucin (generally undersulfated). This results in enhanced activity of differentially expressed α3- (ST3) and α6-sialyltransferases (ST6). Sialic acid (Neu) can occur in various linkages and derivatives such as O-acetyl and N-glycolylneuraminic acid (Neu5Gc). Increased Neuα6Gal and decreased O-acetyl sialic acid are found in colon cancer, whereas increased O-acetyl sialic acid is observed in gastric cancer. In addition, reduction of Neu5Gc was noted in breast cancer and an elevation in colon cancer. Sialylation of blood group related carbohydrate antigens comprising T and Lewis antigens may also be typical of cancer. For instance, increased sialyl Tn is found in colon, gastric, pancreatic and cervical cancers and this is typical of well differentiated advanced stage cancers and poor prognosis.

Fucosylation is significantly altered in cancer (11, 27–29). It may be related to greater branching of oligosaccharide chains, as well as changes (decrease or increase) in fucosyltransferase (FucT) activity. Diminished α2-FucT activity influences the expression of human blood group H or human blood group Lewis: $Le^b$ and $Le^y$ antigens. α3-FucT is involved in the synthesis of $Le^x$ and $Le^y$ is higher in cancer than in normal cells. Increased fucosylation is found in breast, choriocarcinoma, endometrial carcinoma, and higher serum fucose levels in ovarian cancer.

As for galactosyltransferase (GalT) activity, this is elevated in bladder cancer and liver metastasis but decreased in hepatoma (11, 30, 31). Specifically, the activity, size, glycosylation and secretion of β4-GalT are all altered in cancer, i.e, decreased in colon and increased in lung, cervical and parotid gland cancer.

Important changes are also related to N-acetylglucosaminyltransferase (GnT) isoenzymes. Among the six GnT (I-VI), which add βGlcNAc at different positions on the trimannosyl core, three (βGnT-III, –IV and –V) play roles in the structural alterations of complex-type sugar chains during cancer (11, 32–34). In addition, GnT-V ectopically produced in epithelial cells is responsible for morphological transformation and tumor growth. An increase in β1,6-branched complex type sugar chains and the appearance of terminal Lewis antigens sequences have been observed in some cancers and are supposed to be the basis for metastasis correlating with poor prognosis. For instance, increased branching of N-glycans is found in colon and hepatoma cancer, and unusual O-glycans in choriocarcinoma and endometrial cancer.

As for O-glycans, mucin-type oligosaccharides are often truncated and express blood group associated antigens: T, sialyl T, Tn and sialyl Tn (11, 19, 35–38). Increased T and Tn antigens are found in bladder, breast, colon and gastric cancer, whereas elevated Tn is observed in liver metastasis, pancreatic and salivary gland cancer, and increased T antigen in lung cancer.

During malignant transformation, blood group antigens can be increased, decreased or have aberrant structures, whereas some tumors can express histo-blood groups which are not compatible with the erythrocytic blood group due to aberrant synthesis by blood-group-dependent glycosyltransferases (11, 19, 39, 40). These carbohydrate antigens can be added to different substrates: N-glycans, O-glycans and glycosphyngolipids. Loss of blood group antigens is associated with bladder cancer, decreased A and B antigens with lung cancer, diminished ABO antigen with squamous cell carcinoma, incompatible A and A-like antigen with gastric cancer, changed blood groups with prostate cancer and reduced sulfation of mucin blood groups antigens with colon cancer.

The appearance and sialylation of Lewis antigens are also dramatically changed in different cancers (11, 41–45). Altered Lewis antigens are found in lung cancer, and decreased sulfation is typical of breast and colon cancer. Breast cancer is also accompanied with a reduction in $Le^b$ antigen. Elevated $Le^y$ was found in hepatoma and colon cancer. $Le^x$ is associated with secretor types of cancer and it changes in colon, kidney, liver and prostate cancer. Sialyl Lewis a or CA19-9 antigen is highly expressed on the surface of various tumor cells, and its serum level is altered in gastrointestinal cancers. Enhanced expression of glycosyl-

transferases *i.e.* sialyl- and fucosyl-transferases can be responsible for changes in Lewis antigen expression, but this can also be due to incomplete synthesis of the sugar chains of different glycoproteins produced in normal cells.

*Carbohydrates in therapy and diagnostics*

Use of native glycoconjugates or chemically synthesized glycomimetics, for replacing an enzyme deficiency or as medicinal agents for inhibition of pathogen invasion or metastasis, are the basis of various glycan-based therapies (46–50). Since pathogen infection and colonization are mediated through the interaction of adhesins (bacterial lectins) or viral hemagglutinins and carbohydrates on host tissues/cells, carbohydrates can act as inhibitors of these interactions and, therefore, be used in the form of polysaccharide haptens for production of different carbohydrate-based vaccines. Thus, the well-known anti-influenza drugs are based on the inhibition of influenza virus neuraminidase, whereas routine vaccines against *Haemophilus influenzae* type b are generated to polysaccharide-protein conjugates.

Glycoconjugate vaccines are also emerging as a therapy in the battle against specific cancers (51). Carbohydrate-based anti-cancer vaccines have been prepared (experimental trials) against Lewis y, which is overexpressed in a variety of human carcinomas, against sialyl-Tn for breast and ovarian cancer, or globo H antigen for the treatment of prostate cancer.

Since inflammatory reactions are often triggered by carbohydrates, they are also used as part of the anti-inflammation strategy, some of which rely on the inhibition of selectin-ligand interactions by mimicking sialyl Lewis x (treatment of rheumatoid arthritis), or inhibition of Man 6P receptor (52).

Glycans are also involved in immunological rejection in xenotransplantation and one *in vivo* therapy is based on washing out anti-α-galactose antibodies as the primary cause of the immune response.

Carbohydrates as therapeutics must fulfill strict requirements in terms of homogeneity and purity and their preparation is associated with different technical problems regarding their chemical synthesis, the biological techniques used etc (53, 54). Several carbohydrate-based drugs are already on the market, and many others are in various phases of clinical trials (55).

In contrast to their clinical application as therapeuticals, carbohydrates of viral, bacterial, fungal or parasite pathogens or human cancer cells have been widely used diagnostically (56, 57). For instance, differential diagnosis of infectious mononucleosis and serum sickness from the healthy state is based on the reactivity of heterophil antibodies to specific viral carbohydrate antigens, Paul-Bunnell antigen, N-glycolylneuraminic acid and Forsmann antigen (57–59).

Haemagglutination assays or immunological assays for diagnosis of bacterial infection detect, for example, carbohydrates from group A strep for *Streptococcus pyogenes*, cell wall C-polysaccharide of *Streptococcus pneumoniae*, genus-specific lipopolysaccharide of different *Clamydia* species, surface carbohydrates of beta-hemolytic A, B, C, D, F and G streptococci or bacterial strains that can cause meningitis, like *Neisseria meningitides* group A, B, C, Y, *etc*. (57, 60).

Diagnosis based on antibodies or antigens associated with fungal diseases including invasive aspergillosis and candidiasis detects distinct carbohydrate antigens, such as galactomannan or β1,3 glucan, respectively (57, 61). The same holds true for the parasite-specific carbohydrates of *Leishmania, T. brucei, T. cruzi*, *Echinococcus multilocularis* and *Trichinella* species (57, 62).

Well known examples are also glycosylated hemoglobin in monitoring diabetic patients (63), or abnormally glycosylated bands of erythrocyte membranes, as well as carbohydrate-deficient transferrin for diagnosis of inherited or acquired disorders of glycosylation and alcohol abuse (64, 65).

Concerning malignant transformation, tumor-associated antigens, oncofetal or *de novo* synthesized antigens are well known as targets for diagnosis of different types of tumors. They comprise: carcinoembryonic antigen (CEA; colonic, breast, bladder), CA15-3 (breast cancer marker), CA19-9 (pancreatic and colonic), CA125 (ovarian cancer), prostate-specific antigen (PSA; prostate cancer), α-fetoprotein (AFP; hepatocellular carcinoma), as well as a number of sialylated/non-sialylated Lewis antigens (T, Tn, TF).

## Glycoproteins as analytes in clinical chemistry

Many assays, widely used in everyday laboratory practice, actually detect or measure the activity or concentration of different serum or tissue glycan/glycotope/glycoproteins, but without taking into account the specificities of their structure. There are 109 diagnostic tests currently approved by the US FDA to measure protein concentration alone, and among 18 additional assays which assess posttranslational modifications, six detect carbohydrate structures: LCA (*Lens culinaris*) – reactive α-fetoprotein, the glycated form of albumin, bone specific alkaline phosphatase, LDL, CA19-9 and carbohydrate deficient transferrin (66).

As one specified molecule should fulfill some criteria to be considered as a disease marker, the corresponding assay must meet certain analytical requirements for reliable use for clinical purposes (67, 68). Glycoproteins as analytes directly influence the analytical performance of corresponding assays due to their heterogeneous nature (67, 69). In general, the source of variation of heterogeneous analytes can be

related to: splicing variants, sequence/isoforms, chemical modifications or degradation, oligomeric state, ligand binding (metal, other proteins and cofactors), degree of structuration or different conformational states. This implies that one test must actually measure/detect different molecular forms and poses the question of inter-assay comparability. Thus, there is no WHO reference material which is specified to consist of a distinct form but they may contain a mixture of molecular forms. In addition, due to their heterogeneous nature, reference materials may react differently in different assays depending on the specificity of the antibody reagents.

In contrast to diagnostic analytes falling in category A and comprising chemically well-defined compounds where the results of measurements are traceable to SI units, glycoproteins belong to category B (67). Tests for biological substances from category B are based on their functional activity, immunoreactivity or nucleic acid amplification. This means that assay results are usually expressed as arbitrary units, *i.e.* WHO international units.

Striking examples of heterogeneous analytes are human chorionic gonadotrophin (hCG) and prostate-specific antigen (PSA). The former includes a complex heterogeneous family of intact, dissociated or degraded hCG-related polypeptides differing in carbohydrate content (70, 71). The cross-reactivity of these forms varies among different antibodies used in commercially available tests. Similarly, experimental evidence indicates at least 30 immunoreactive forms of free PSA in seminal plasma or serum. It is known that metrological traceability and standardization of immunometric assays for free or total PSA is an actual problem and a still unresolved issue in laboratory practice, having a clinical impact on information about prostate cancer, its risk and detection rate (72–74).

When considering glycans/glycoproteins as analytes, one important issue is that they can be related to the genetics and age of patients, which influences the reference range for particular pathophysiological conditions. For instance, the glycan epitope CA 19-9 or sialyl $Le^a$, exhibits genetic variation within populations, so secretor type persons may have a high background level of these epitopes normally with values exceeding up to 6-fold the established upper reference limit (75).

Generally, clinical expectations are primarily related to discovering highly sensitive and specific biomarkers, in order to eliminate any doubts and controversies, which are often encountered when interpreting assay results. This is of special importance when considering tumor marker assays of low specificity and sensitivity. This makes them almost completely inappropriate for early detection of and differentiating between benign and malignant conditions (76). However, due to the intrinsic heterogeneity this cannot be solved, but it can be improved or partially overcome by introduction of additional tests as already achieved for some analytes.

## The »omics« era: The glycome as a source of new biomarkers

In the »omics« era, the glycome, a glycan analogue of the proteome and genome, holds considerable promise as a source of new biomarkers (77). The glycome refers to the entire set of glycans in one organism, and the term glycomics is derived from the chemical prefix for sweetness or a sugar, »glyco-«, and was formed to follow the naming convention established by genomics (which deals with genes) and proteomics (which deals with proteins). Thus, glycomics, as the systematic study of all glycan structures of a given cell type or organism, is expected to answer many open questions and fill phenotype-genotype knowledge gaps (78). In the first place, this is a question of the biological meaning of diversity and constant dynamic structural changes in biological systems. However, unlike genomics and proteomics, where molecules with similar chemical properties are examined using single analytical platforms, glycomics must cope with an inherent level of glycan complexity, *i.e.* a huge chemical diversity and broad dynamic range not seen in other areas of applied biology (79).

In spite of this, glycomics offers considerable possibilities for the translation of basic discoveries to clinical practice in general or personalized medicine. Designing a strategy for biomarker discovery, new principles and platforms for the analysis of relatively small amounts of numerous glycoproteins and accurate monitoring at the level of the glycoproteome are gradually being developed (80–82). Screening of different types of glycomolecules, selection of differentially expressed components, their enrichment and purification or identification are the most challenging parts of experimental and clinical glycoproteomics.

The importance of glycomics has been fully recognized and many national and international research centers and consortiums have already been founded (83, 84). Under the Human Proteome Organization (HUPO), the Human Glycome/Proteomics Initiative (HGPI) was established in 2002, with one task related to biomarker discovery, especially tumor markers (85, 86).

Unfortunately, reliable biomarkers are not available for the majority of cancers and there is also a lack of secreted biomarkers that can be detected through non-invasive assays, such as blood tests. Among at least 100 cancer biomarkers used today, most are glycoproteins and glycolipids. Measurement of serum concentrations of tumor markers in different types of immunometric assays employing various monoclonal antibodies to the protein portion of the molecule, is often associated with problems of low specificity for cancer detection. One reason is that concentrations can be elevated during both benign and malignant processes.

Comparative studies of the sugar chain structures of different tumor markers have indicated specific

structural alterations associated with malignant transformation, in relation to glycan branching, sialylation and fucosylation. For instance, intensive structural investigations aimed at improving diagnostic usefulness have pointed to extreme heterogeneity, *i.e.* the existence of many different molecular glycoforms of PSA and CA125.

PSA comprises heterogeneous molecules differing in their polypeptide backbone as well as in carbohydrate composition, resulting in the existence of diverse PSA isoforms (87–92). Besides the major PSA subpopulation bearing biantennary N-glycan, PSA molecules with uncommon N-glycan structures, such as monoantennary glycan chains or different outer chain moieties, have been detected, and PSA of cancer origin was found to show decreased glycosylation compared to PSA from BPH and seminal plasma.

Moreover, the primary structure of CA125 contains numerous potential glycosylation sites. Data on glycosylation of OVCAR-3 cell line-derived CA125 in combination with data on both pregnancy- and cancer-associated CA125, obtained by lectin-affinity chromatography as a method for structural assessment, point to the existence of glycosylation differences (93–97).

These glycan modifications should be better in distinguishing between benign and malignant conditions than the measurement of marker concentrations alone, which is widely used in practice. Thus, cancer-associated changes in glycosylation could improve the clinical utility of known tumor markers (98–100).

The diagnostic significance of microheterogeneity of glycoproteins has been confirmed by the FDA approval of fucosylated alpha-fetoprotein (L3 fraction) as a marker of primary hepatocarcinomas in 2006 (101, 102). Investigation of the structural properties of tumor markers as possible targets for the improvement of diagnostics is gaining more attention and expanding nowadays.

*Glycomics: strategies and technologies*

Introducing the concept of glycomics offered potential for rediscovering glycans as biomarkers and enabled new strategies for their application in clinical chemistry (103–105). The experimental workflows are aimed at either the discovery of a new marker or finding specific disease-related structural alterations of known markers. Although most reported studies deal with an individual specific molecule (glycoprotein) as a diagnostic indicator, focus has been moved to the investigation of general changes in total glycans, *i.e.* glycoprotein profiling at the level of cells, tissue and biological fluids as more informative.

Different biological sources, such as serum, urine, saliva, or various cell or tissue extracts are objects of glycoprofiling. In spite of being the most complex, serum is most often used (106–108). Most serum proteins are glycosylated with common glycan structures. Since biomarkers are usually among low abundance proteins and, in structural terms, are supposed to be associated with uncommon glycans, removal of the most abundant proteins and enrichment at glycoprotein or glycopeptide levels are part of various experimental strategies (109). Thus, the main endeavors have been to create strategies how to a) pick up relevant changes relating to the heterogeneous molecules themselves; b) catch the early appearance (preceding the disease) of glyco-markers, *i.e.* how to detect relevant glyco-changes occurring at a very low level.

Consequently, enrichment of glycoproteins by affinity or chemical selection has been developed. Carbohydrate-binding proteins, such as lectins or antibodies, are used for affinity selection in different experimental techniques. A single lectin with preferred narrow specificity or multiple lectins can be employed to increase selectivity during the enrichment phase (110–113). Hydrazine or boronic acid chemistry is mostly used for chemical selection (114–116).

The enrichment step is followed by release of glycans for further structural characterization. They can be cleaved either enzymatically (N-linked) or chemically by reductive beta elimination (O-linked) or hydrazinolysis (N- and O-linked glycans) from the target and subjected to analysis (117–119). Glycan pools can be analyzed directly by mass spectrometry or be prefractionated.

Commonly used techniques in glycan analysis are high resolution mass spectrometry (MS) and high performance liquid chromatography (HPLC). Routine HPLC (reversed phase, normal phase and ion exchange) analyses of N- and O-glycans are performed after tagging the reducing end of the sugars with a fluorescent compound (reductive labeling) (120, 121). Fractionated glycans can be further analyzed by MALDI-TOF-MS (matrix-assisted laser desorption/ionization time of flight mas spectrometry) to gain information about structure and purity. To increase their ionization, glycans can be chemically derivatized using heavy isotopes and permethylation or methylation (122–125). By choosing porous graphitic carbon as the stationary phase for liquid chromatography, even non-derivatized glycans can be analyzed. Mass spectrometry is used for detection here but, in contrast to MALDI-MS, with an electrospray ionization (ESI) interface.

Thus, mass spectrometry, such as MALDI-TOF has a central place in experimental workflows for glycomics, due to the low detection limit and specificity (126–129). At present, new MS-based approaches are being developed, like multiple reaction monitoring (MRM), with the potency to quantify target proteins in unfractionated samples (130–132), and stable isotope standards, as well as capture by anti-peptide (or anti-carbohydrate) antibodies (SISCAPA) to enhance MRM (133, 134).

In addition to MS, lectin and antibody arrays also provide high-throughput screening of many samples containing glycans (135–137). They are based on reactions between chip-immobilized naturally occurring lectins or artificial monoclonal antibodies, and labeled glycoprotein samples. Although array technologies are less complicated than MS, interpretation of the results is tedious work and there is a constant need for improvement or development of new bioinformatical tools (138).

Existing methodology for glycome analysis cannot be compared to that supporting genomics and proteomics, owing to lack of cloning, amplifying or sequencing techniques, and this is one limitation for more rapid progress in the field.

## Concluding remarks

The official NIH definition of a biomarker is: »a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention«.

Glycoproteins have been used as disease and prognostic markers for a long time. Nowadays we are aware that glycans are unique for their bioinformative potential *in vivo*, and as targets to yield sensitive and discriminative *in vitro* diagnostic tests, including use singly or among a panel of different tumor markers in multiplex platforms. Biomarker discovery is a multistep process and most current glycomic data are mainly related to the so-called discovery phase, as the first one proceeding to validation and standardization steps. As additional disease-associated structural and functional changes are to be defined, the number of molecular techniques intended to be used in clinical chemistry will expand. There is a constant need for cooperation between scientists in glycobiology and glycotechnology aiming at the development of a rapid and cost-effective platform accommodated to everyday laboratory practice. Glycomics still awaits full exploration in biomedicine, especially in the deliverance of new analytical protocols in the field of biomarkers.

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## Conflict of interest statement

The author stated that there are no conflicts of interest regarding the publication of this article.

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