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Clinical application of quantitative glycomics

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Abstract

Introduction: Aberrant glycosylation has been associated with many diseases. Decades of research activities have reported many reliable glycan biomarkers of different diseases which enable effective disease diagnostics and prognostics. However, none of the glycan markers have been approved for clinical diagnosis. Thus, a review of these studies is needed to guide the successful clinical translation.

Area covered: In this review, we describe and discuss advances in analytical methods enabling clinical glycan biomarker discovery, focusing only on studies of released glycans. This review also summarizes the different glycobiomarkers identified for cancers, Alzheimer's disease, diabetes, hepatitis B and C, and other diseases.

Expert commentary: Along with the development of techniques in quantitative glycomics, more glycans or glycan patterns have been reported as better potential biomarkers of different diseases and proved to have greater diagnostic/diagnostic sensitivity and specificity than existing markers. However, to successfully apply glycan markers in clinical diagnosis, more studies and verifications on large biological cohorts need to be performed. In addition, faster and more efficient glycomic strategies need to be developed to shorten the turnaround time. Thus, glycan biomarkers have an immense chance to be used in clinical prognosis and diagnosis of many diseases in the near future.

Keywords

Biomarker; Cancer and other diseases; Clinical application; Diagnosis; Prognosis; Glycomics

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1. Introduction

Glycosylation is an important post-translational modification of proteins. More than half of proteins in humans are glycoproteins [1-3]. Nearly all membrane proteins, secreted proteins and intracellular proteins are modified by oligosaccharides [4,5]. Glycosylation is a dynamic process in cells that determines cell-cell interaction, cell-extracellular communication and cellular metabolism [6-9]. Glycans that are covalently attached to proteins play key roles in determining the structures and functions of proteins. The biofunctions of glycans can be broadly classified into structural and modulatory roles, intrinsic and extrinsic recognition, and molecular mimicry of host glycans [10]. If the biological system is an analogy to a switchboard, glycans are “on” and “off” switches in modulating glycoproteins [11].

Although more than 700 monosaccharides are found in nature [12], human glycans are mainly built using eight monosaccharide units, including glucose (Glc), galactose (Gal), mannose (Man), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), L-fucose (Fuc), sialic Acid (Sia, Neu5Ac), and glucuronic Acid (GlcA). However, glycan structures are far more complex than the configuration of proteins and nucleic acids, largely because the biosynthesis of glycans is a template-free process controlled by metabolic enzymes, such as glycosyltransferases, exoglycosidases, and glycan modifying enzymes [13,14]. Unlike amino acids in proteins or nucleotides in nucleic acids connected in linear sequences, monosaccharides are linked by either α or β configuration, at multiple possible hydroxyl groups and in linear or branched patterns. The anomers, positional and linkage isomers diversify and complicated glycan structures [12,15,16]. According to the attachment to different amino acid residues, glycans are mainly categorized as N-linked or O-linked glycans. N-glycans are attached to an asparagine amino acid residue while O-glycans are commonly linked to a serine or threonine residue of proteins [17].

The initial study, revealing the association of glycosylation change to cancer, dates back to 1969, which reported for the first time a distinct difference between the membrane glycoproteins of healthy and transformed mouse fibroblasts [18]. Since then, many studies have shown both overexpression and/or underexpression of glycans as well as size increase, branching and modifications in the core structure of N-linked and O-linked glycans [19,20]. More cancer-associated glycans and their effects on transformation, metastasis and tumor progression were studied [21-23]. Currently, most of the FDA approved disease biomarkers are glycoproteins [6]. Glycan targeted therapeutics is becoming prevalent in recent years but have not been extensively explored yet. The discovery of glycan biomarkers would facilitate the understanding of disease pathology and prompt more selective treatment and diagnosis. However, for this to happen, reliable analytical methods are needed.

Advancement in mass spectrometry (MS) and separation techniques facilitate the identification of glycan structures and quantitative analysis of glycans derived from biological samples. Electrospray ionization (ESI)-MS and matrix-assisted laser desorption ionization (MALDI)-MS are the most widely used ionization techniques for glycan analysis. However, MS technique alone is not adequate to characterize glycan isomers. Thus, separation techniques such as liquid chromatography (LC) and capillary electrophoresis

(CE) are often coupled with MS to achieve high throughput and comprehensive quantitative glycomic analysis.

Glyco-science can be classified into several categories, including the studies of glycogene, glycoprotein, glycopeptide, released glycan, glycolipid, free oligosaccharide, *etc.* In this review, we are focusing on the clinical glycan biomarker discovery through glycomic studies, which only analyze the released glycans. we will describe and discuss MS methods with and without separation which are employed in glycome profiling to discover and validate potential glycobiomarkers of several diseases. The potential glycobiomarkers of different cancers and diseases would be summarized and discussed.

2. Quantitative glycomics techniques

Absolute quantitation often refers to the precise amounts of the analytes from a calibration curve constructed using known amount of standards. On the other hand, relative quantitation is usually acquired through dividing individual glycan abundance by total glycome abundance (or by the abundance of the most intense glycan). However, glycan standards are not easily acquired, which inhibits the use of absolute quantitation in disease glycomic studies. Therefore, almost all clinical glycomics studies are presented in relative quantitation, which can readily exhibit the up- or down- regulation of glycans between diseased and healthy cohorts. Quantitation methods in glycomics field have been previously reviewed by Packer and coworkers [24]. This section will introduce the techniques that are currently being used in clinical glycomic research.

2.1. Lectin microarray-based techniques

Lectin binding was widely used to analyze different glycan compositional moieties in biological systems. A variety of specific lectins have been used to facilitate clinical diagnosis or prognosis of different cancers. *Lens culinaris* agglutinin has been employed to target α 1,6 core fucosylation level of α -fetoprotein (AFP) to diagnose hepatocellular carcinoma [25]. By immobilizing different lectins on a solid surface, lectin microarrays prompt high-throughput characterization of the glycosylation pattern of target analytes, including intact glycoproteins or even whole cells [26]. With less sample preparation steps, binding patterns of glycans to the lectin array prompts the differentiation between glycan profiles [27]. The lectin-glycan conjugates can be targeted either through fluorescence labeling of analytes before applying to the microarray, or by applying a fluorescence-labeled antibody interacting specifically with a certain analyte. Details of glycomics studies using lectin microarrays were previously discussed [27,28]. However, lectin-based methods could not acquire the intact glycan structural information while MS-based analyses could.

2.2. MALDI-MS-based techniques

MALDI-MS is the most commonly used MS analysis method in clinical glycomics, MALDI-MS has advantages in its simple sample preparation procedure and fast analysis as well as sufficient glycan structural information [29]. Numerous studies of MALDI matrix have been conducted to increase ionization efficiency and decrease background noise, among which, 2,5-dihydroxybenzoic acid (DHB) is the most popular matrix used in

MALDI-MS of glycans. However, MALDI can easily fragment labile glycans such as sialic acid [30]. Therefore, derivatization techniques such as methylamidation/esterification or permethylation are commonly performed to avoid such loss in ionization. Additionally, methylamidation/esterification of sialic acid permits the identification of linkage isomers [31].

Normalization to the total intensity or most abundant structure is usually utilized to quantitatively assess the abundance of glycans since MALDI intensity cannot be used to interpret the abundance difference between each sample. Nevertheless, a direct comparison of peak intensities in multiplexed samples has also been previously demonstrated to be efficient in investigating the changes of single glycan structure [32]. Additionally, taking advantages of the capability to spatially resolve sample spots, MALDI imaging has been used to reveal glycan distribution changes in tissue sections [33,34]. Details in recent MALDI imaging of glycomics studies have been reviewed by Drake *et al.* [35]. However, MALDI-based glycomics is performed without separation, which makes the spectra complex when analyzing biological samples. Also, the competitive ionization of analytes decreases the sensitivity, demanding the combination of other efficient separation techniques with MS.

2.3. MS coupled with separation techniques

Separation of glycans prior to MS analysis is a commonly used method to reduce ion suppression during the ESI process. Benefiting from the compatibility with MS and high separation resolution, LC-MS is widely used in clinical glycomic profiling [29]. A variety of LC techniques have been employed to characterize glycosylation patterns, including hydrophilic interaction liquid chromatography (HILIC) [36-43], reverse phase liquid chromatography (RPLC) [44-47], high-pH anion exchange chromatography (HPAEC) [48,49] and liquid chromatography using porous graphitic carbon (PGC) [15,45,50-55] have been previously demonstrated and elaborated reviewed [56,57].

Different derivatization methods have also been employed to enhance glycan stability and ionization efficiency. Among these methods, fluorescence labeling of glycans through reductive amination using different labeling reagents, such as 2-aminobenzamide (2-AB) [58,59] and 2-aminobenzoic acid (2-AA) [60], enables simultaneous spectroscopy quantitation and identification of glycans. Permethylation can significantly enhance MS signals, stabilize labile glycan residues such as sialic acids and fucoses, thus ensuring accurate structural information by tandem MS experiments [15]. Esterification distinctly modifies sialic acids and form intramolecular lactone and ester of α 2,3 linked and α 2,6 linked sialic acids, respectively, thus distinguishing sialic acid linkage isomers [61].

Capillary electrophoresis (CE) is also widely used in glycan separation and provides a mean to resolve isomeric structures. Glycans are often derivatized with a charged chromophore or fluorophore tag to be efficiently separated by CE. Despite the instability of ESI caused by the low flow rate of CE which is commonly amended by a sheath liquid, CE-MS facilitates glycomics with isomeric separation [62,63]. Also, the high-resolution of CE makes it suitable to analyze low sample amounts.

Quantitation in MS based glycomics is usually attained by defining the peak areas of extracted ion chromatograms (EIC). Relative quantitation is achieved by comparing the peak areas of the glycans to the sum of EIC peak areas of the entire glycome [52,64] or peak area of the most intense glycan [65]. Fluorescence response has also been used for quantitation in the case of reducing end labeled glycans. Another commonly used technique in glycomics quantitation is multiple reaction monitoring (MRM) tandem MS. Since the quantitation is based on the intensities of selected transitions, MRM analyses are less influenced by the coeluting contaminants relative to MS quantitation methods, thus resulting in high sensitivity and wide dynamic range. Glycomics analysis by MRM has been previously reviewed by Lebrilla and coworkers [66]. Using these aforementioned quantitative glycomic techniques, many promising glycan biomarkers have been discovered in different diseases. Some of the most potential glycan biomarkers in different diseases are summarized in Table 1 for N-glycobiomarkers, Table 2 O-glycobiomarkers, and Table 3 isomeric glycobiomarkers.

3. Glycans as potential biomarkers in cancer

Cancer is the leading cause of death in developed countries and even more serious in less developed countries [67,68]. The incidences of cancer are increasing due to the growth and aging of the population [67]. As discussed above, along with the development of glycomics technologies, glycomics has become more essential and significant in cancer studies. Glycosylation has been proved to be associated with carcinogenesis such as tumor adhesion [8,69,70], malignant transformation [71,72] and metastasis [73-75]. Advanced glycomics methods have facilitated glycobiomarker discovery in cancers. The ultimate goal of such studies is to quantitatively identify unique N- and O-glycans and glycan patterns in cancer samples relative to healthy samples, thus facilitating the use of glycans as biomarkers in clinical prognosis, diagnosis or therapy.

3.1. Potential N-glycobiomarkers of different cancers

3.1.1 Breast cancer—Breast cancer is the most common cancer in females, which accounts for 25% of all cancer cases [76]. Breast cancer represents the major cause of death in women [76]. Breast cancer is also considered as the second metastatic cancer following lung cancer [76]. It is estimated that 10-16% of breast cancer patients are suffering from malignant brain metastasis [77]. Also, the numbers of breast cancer brain metastasis is increasing because of the longer survival period of primary breast cancer patients and the improvement of diagnostics methods [78].

Rudd and coworkers [79] investigated the glycome expressions in breast cancer sera relative to healthy subjects. After 2D gel electrophoresis, N-glycans were released by in-gel PNGase F digestion and labeled with 2-AB. Labeled glycans were then identified and quantified using weak anion exchange liquid chromatography (WAX-HPLC), then amide-normal phase (NP)-LC-MS/MS. Tri- and tetra-sialylation of glycans were higher in breast cancer sera, as well as the branch fucosylation level. The linkages of observed glycans were studied using an array of exoglycosidases digestion. N5H4F1 glycan (N, N-acetylhexosamine; H, hexoase; F, fucose; and S, sialic acid; F listed before N, core fucosylation; F listed after N, branch fucosylation; *F, the position of fucose is not defined; see Table 1 for more details) exhibited

a significant increase (>2 folds) in advanced breast cancer patients. The levels of N5H4F1 glycan was assessed in a longitudinal study involving 10 breast cancer patients from early stage to late metastatic stage to define the potential of using the level of N5H4F1 as a breast cancer indicator. The increasing levels of N5H4F1 in most late-stage breast cancer patient suggested that such glycan structure was a useful and reliable indicator of breast cancer progression and metastasis than cancer antigen (CA) 15-3 and carcinoembryonic antigen (CAE) (which are the most commonly used biomarker for breast cancer).

Also, similar glycomics was performed on circulating tumor cells (CTCs) derived from 27 advanced breast cancer patient sera (16 with CTCs < 5/7.5 mL, 11 ≥ 5/7.5 mL) relative to 13 healthy women [80]. CTCs were separated through CellSearch™ System (Veridex, LLC), and N-glycans were released by PNGase F, followed by 2-AB labeling. NP-HPLC coupled to serial exoglycosidases digestion were applied to separate and identify glycan structures. Sial Lewis^x (SLe^x) contained bi-, tri- and tetra-antennary N-glycans were separated after treating with different enzymes, which resulted in only SLe^x branch left (without sialic acid). Total SLe^x abundance increased along with other three SLe^x glycans (N5H4F1, N6H4F1, and N5H5F2) in CTCs ≥ 5/7.5 mL group, which suggested a potential indicator for breast cancer metastasis. Moreover, 4 N-glycan structures, especially Man9 were found elevated during breast cancer progression using MALDI-FTICR [81].

Glycan sialylation and fucosylation of sera from 82 breast cancer patients with different stages and 27 cancer-free people was defined using MALDI-MS [82]. The patient sera glycomics was compared to that of seven different breast cancer cell lines, including MCF10A (normal breast cell line), MCF-7(non-invasive) and MDA-MB-231 (invasive) [83]. The increase of sialylation and fucosylation observed in breast cancer sera is an indicative glycobiomarkers for breast cancer progression. Also, significant alterations in the expressions of 8 glycan structures were observed in breast cancer sera, and 6 of them were consistent with what observed in cell lines [82].

Recently, Ju *et al.* [84] reported five bifucosylated N-glycan as potential biomarkers in initial breast cancer and recurrent breast cancer patients. The authors investigated 134 serum samples including 91 breast cancer sera and 43 healthy controls using LTQ-ESI-MS. The identification of reduced and permethylated glycans released from 50 µL serum sample was achieved using MSⁿ (n was up to 7) technique. Overall, 11 N-glycans demonstrated significant expression changes in breast cancer patients while 5 bifucosylated glycans, which exhibited higher increase, were selected for receiver operation characteristic (ROC) curve analysis and the area under the curve (AUC) calculation to estimate their diagnostic capacity. The bifucosylation degree and carcinoembryonic antigen (CEA) level had a combined AUC of 0.963 while CEA alone has an AUC of only 0.794, suggesting an increase of breast cancer diagnostic capacity by measuring bifucosylation degree and CEA together. Also, increased bifucosylation degree level could be utilized as an indicator for recurrent breast cancer since it was proved to outperform commonly used breast cancer biomarkers, such CEA, CA 15-3 and CA 125.

More recently, Terkelsen *et al.* [85] have reported N-glycan signatures from tumor interstitial fluid and serum of breast cancer patients and their association with clinical outcomes. The

authors investigated 85 tumor interstitial fluids (TIFs) (luminal A = 43, luminal B = 12, luminal B HER2-enriched = 8, HER2 = 9, and TNBC = 13) and 54 normal interstitial fluids (NIF) samples, as well as their paired serum samples. There were 54 TIF-NIF paired samples (108 samples in total). N-glycans from TIF and NIF samples were released using the high-throughput automated method by a liquid-handling robot, labeled with 2-AB, and analyzed using HILIC-UPLC with fluorescent detection, calibrated by 2-AB labeled dextran ladder and identified by their glucose units (GU). Then the peaks were assigned to glycan structures based on the in-house database established before. The N-glycan analysis strategy allowed 165 N-glycans assigned to 46 glycan peaks. The glycan peaks were then associated with clinical outcomes such as breast cancer phenotypes, clinical variables, and tumor infiltrating lymphocytes (TILs). The increase in the expression levels of nine bisection N-glycans (N5H3, N5H4, F1N5H4, F1N5H5, N5H5S1, F1N5H5S1, F1N4H5S2, N5H6F1S3, and F1N5H5S2) were found to contribute to tumor suppression in NIF. Correlations between several glycan patterns and proportion/composition of infiltrating lymphocyte populations were observed. Five N-glycans (F1N4H4, F1N4H4 (branch isomer), F1N4H5, F1N5H5S1, and F1N2H3) levels in TIF correlated with that in paired serum. Importantly, F1N5H5S1 and F1N2H3 were shown to have the prognostics potential through validation using an independent serum cohort.

3.1.2. Liver cancer—Liver cancer is not only one of the most common cancer, but also has the second highest cancer death cases [86]. Hepatocellular carcinoma (HCC) counts for more than 80% of malignancies of liver cancer, and the survival rate of HCC is only 2-7% [87]. Such a low survival rate is caused by the lack of early diagnosis [88]. Most serum glycoproteins are of hepatic origin, suggesting the importance of glycomics studies in liver diseases [89].

In the early stages of liver diseases, high-mannose glycan levels have been exclusively associated with HCC condition [90]. Goldman *et al.* [91] investigated 202 serum samples representing 73 HCC cases, 77 cancer-free, and 52 with chronic liver disease using MALDI-TOF. A total of 83 N-glycans were identified while 57 glycan expression alterations were found in HCC cases. The combination of the levels of 3 glycans (N5H6S2, N6H7S3 and bisecting N5H4S1) effectively predicted HCC with 90% accuracy. Moreover, the investigation of salivary glycomics in liver diseases were performed [92]. Although, several glycans were observed the expression alterations in different liver diseases, the increase of total fucosylation level and decrease of total sialylation level were more promising to be considered as the HCC indicators in this study.

Recently, Kamiyama *et al.* [93] investigate N-glycan profiles from 369 patients who underwent a hepatectomy procedure for HCC and 26 healthy people. Released N-glycans were purified by glycoblotting using BlotGlyco H beads (Sumitomo Bakelite, Tokyo, Japan) coupled to on beads methyl esterification and then analyzed using MALDI-TOF. With this fast sample preparation and analysis strategy, 67 N-glycans were identified and quantified. The correlation between identified N-glycans and different clinical indexes of HCC patients was measured. Receiver operating characteristics (ROC) analysis between HCC samples and disease-free samples revealed 14 N-glycans with a definite increase in HCC (AUC>0.8). Among the observed structures, F1N5H6S2 and F1N6H7S3 were elevated in more than 70%

of HCC patients. Additional correction analyses of these two glycans identified F1N5H6S2 as a significant HCC recurrent indicator and F1N6H7S3 as a significant prognosis indicator.

Lubman and coworkers [94] investigated the alterations in fucosylation degree of serum haptoglobin from a cohort containing 104 individuals representing 14 healthy controls, 40 cirrhosis and 50 HCC patients using MALDI-QIT-TOF. The total fucosylation degree significantly increased in HBV- and ALC-related HCC, suggesting the diagnostic potential of the total fucosylation degree for distinguishing HBV- and ALC-related HCC from their corresponding cirrhosis. In addition, the significant elevation of bifucosylation degrees of different cohorts were also observed. High AUC value in ROC analysis (more than 0.82 with specificity and sensitivity more than 70%) indicated that bifucosylation degree could be a promising biomarker in liver cancer progression of patients suffering from cirrhosis. Combining bifucosylation degree and clinical marker AFP facilitated a significant improvement in the ability to discriminate HCC from cirrhosis patients. The fact that bifucosylation degree increased in all early stage HCC cases indicated that it could be a potential indicator for early stage HCC patient with cirrhosis.

MALDI imaging mass spectrometry (MALDI-IMS) for tissue N-glycan profiling was initially developed by Drake and coworkers [34]. They recently utilized this technique to assess the glycome of liver cancer tissue microarray (TMA) [95]. Tissues of TMA blocks were initially sectioned at 5 μ m and mounted on a positively charged slides, followed by thermo fix, deparaffinization, and rehydration. N-glycans were then released by PNGase F spray and analyzed using MALDI-FTICR. Figure 1 depicts the TMA tissue position and two N-glycans which could distinguish liver tumors from normal tissues [95]. These two glycans were selected out from 26 identified glycans because of the high AUC (>0.8 with $p < 0.05$) in ROC analysis. The authors reported that up-regulation of N6H7 and down-regulation of Man8 might be promising indicators for liver cancer. The expression changes of these two glycans were also observed in another study using similar MALDI-IMS strategy [96].

In a recent study, Kim *et al.* [97] introduced a MALDI-MS based quantitative targeted glycomics (MALDI-QTaG) strategy to analyze α -fetoprotein (AFP) glycans from normal human serum and HCC cell line Huh7. Sialic acids of released glycans were neutralized using acetohydrazide. Then glycans were labeled by Girard's reagent P and analyzed by MALDI-TOF-MS. The comparison of AFP glycans from normal serum and HCC cells revealed a significant increase of core-fucosylated glycans in HCC cells (including an obvious increase of F1N4H5S2 and F1N4H5S1). Although the sources were different specimens, the comparison exhibited the different glycan expressions in different sources, which might also need to pay attention to.

3.1.3. Ovarian cancer—Ovarian cancer is another major cancer in females, which is the fifth leading cause of death in women [98]. Usually, ovarian cancer was diagnosed in a late stage, after metastasis occurred, which left only few treatment options and a short survival period [99]. Although prognostic indicators for early-stage ovarian cancer has been reported, such as CA125 [100], searching for more promising biomarkers in addition to CA125 is essential. Recently, more glycome expression changes in ovarian cancer have been investigated and considered as potential biomarkers.

When considering different glycan types, core-fucosylation was observed to increase in ovarian cancer side population cells [101]. The increase of ovarian cancer core-fucosylation was also reported in patient serum, along with the increase of agalactosyl biantennary glycans and SLe^x [102]. A similar increase of agalactosyl biantennary glycan expression level was reported through investigating glycome from 30 ovarian cancer patient sera [103]. Meanwhile, a decrease of bisecting structure and increase of tri- and tetra-antennary structure were observed, and particularly, N6H7S4 and N6H7F1S4 were found significantly increased in ovarian cancer [103].

Biskup *et al.* [104] compared the serum glycome from 63 preoperative epithelial ovarian cancer (EOC) patient with 33 healthy subjects. Released and permethylated N-glycans were analyzed using MALDI-TOF/TOF. Totally 47 glycans were identified and quantified, among which 11 glycans were found significantly up- or down-regulated. GLYCOV value (the ratio between average abundance of up-regulated glycans and average abundance of down-regulated glycans) was used for ROC analysis, resulting in a better sensitivity (97%) and specificity (98.4%) than the widely used CA125 biomarker. Miyamoto *et al.* [99] investigated glycan expressions in ascites fluid of ovarian cancer and normal serum samples. N-glycans N5H6, *F1N5H6S1, *F2N5H6S3, and *F1N6H7S1 were significantly over-expressed in ovarian samples. However, the reported data were based on only one patient ascites fluid.

Recently, Blanchard and coworkers [105] compared the N-glycome derived from five ovarian cancer patients with that of healthy subjects. Glycoproteins were purified by 2D gel electrophoresis and then N-glycans were released by on-gel PNGase F digestion. After desialylation and APTS labeling, glycans were analyzed by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) and mapped to pre-established database. The increase of branching and Lewis^x were observed in EOC patients on tri- or tetra-antennary glycan structures. The overexpression of core-fucosylated bi-antennary structure (F1N4H5) on alpha-1-acid glycoprotein (A1AG) could be a promising indicator for EOC. In a more recent study, Blanchard and coworkers [106] investigated for the first-time ascites and matched serum glycome from 20 EOC patients and 20 healthy women using MALDI-TOF/TOF. A total of 47 glycans were identified, among which seven glycans exhibited significant expression differences in all three cohorts (ascites EOC, EOC and healthy). The significant decrease of high-mannose and bisecting structures and increase of sialylation, branching, branch fucosylation were observed in ascites EOC when compared with serum. The discrepancy suggested that glycan biomarkers might be different in ascites relative to serum and additional investigations are warranted.

MALDI-TOF/TOF was also applied for N-glycan imaging of ovarian tumor tissue sections [107]. N-glycomics was initially performed using PGC-LC-MS/MS from three ovarian cancer patients, allowing the identification of 40 N-glycans. In *situ* released N-glycans of tissue section were analyzed by MALDI-TOF. Four N-glycans (N4H3, F1N5H6, Man8 and N2H3) were found to have significant capacities to distinguish tumor, stromal, adipose, and necrotic tissue regions. In ROC analysis, all these glycans exhibited high AUC values (0.84 to 0.99), especially the over-expressed Man8 which could be designated as a tumor marker. However, the up-regulation of Man8 was not consistent with another independent research

of serum glycomics [104], which highlighted the different glycan expressions in different biological specimens.

3.1.4. Colorectal cancer—Colorectal cancer (CRC) is the third prevalent cancers in both men and women [98]. With a poor prognosis, nearly half of CRC patients are diagnosed when the disease in its metastatic state [108]. A new generation of screen test, including molecular biomarkers in biological samples, is necessary. However, despite the increasing efforts of biomarker searching, reliable biomarkers in clinical diagnosis has not been identified yet. The most widely used biomarker for CRC is CEA but lacks sensitivity for early-stage CRC [109]. Therefore, glycans as potential biomarkers were investigated in colorectal cancers.

Early studies revealed that glycan branching and sialylation were associated with colon cancer metastasis [110]. GlcNAc-caped N-glycans were also found up-regulated in colon cancer cell line HT-29 [111]. Bisecting structures were shown to exhibit up-regulation in metastatic colorectal cancer cell line LIM1215 [112]. Increased levels in colorectal carcinoma were also reported for high-mannose structures [113] and α 2,6-sialylation [114]. In contrast, a decrease of core-fucosylation was observed in CRC patients [115].

The use of DNA Sequencer in clinical glycomics analysis was reported by Callewaert *et al.* [116]. In a more recent work, Zhao *et al.* [115] studied serum N-glycan profiles from 347 individuals, including 219 CRC patients (before surgery) and 128 age- and sex-matched healthy people. N-glycans were released, fluorescently labeled and desialylated before analysis by DNA sequencer-assisted/fluorophore-assisted carbohydrate electrophoresis (DSA-FACE). A total of 9 common structures were identified in all types of samples, among which N4H5 and N5H6F1 were significantly up-regulated in CRC samples, while F1N4H4 and F1N4H5 were down-regulated. Logistic regression analysis was applied to correlate N-glycan alterations with CRC, thus permitting the construction of a mathematical model (CRCglycoA = $8.017 + 0.286 \times (\text{F1N4H3 abundance}) - 1.594 \times (\text{F1N4H4 abundance})$) with an AUC value of 0.92 in ROC analysis. The same strategy was used to establish the mathematical model CRCglycoB (CRCglycoB = $6.523 + 0.298 \times (\text{F1N4H3 abundance}) - 2.252 \times (\text{F1N5H3 abundance}) - 0.257 \times (\text{F1N4H5 abundance})$), which could differentiate CRC from adenoma with an AUC value of 0.81. The two N-glycan expression-based mathematical models exhibited better diagnostic capacities than the widely used biomarker CEA (AUC 0.81 and 0.73, respectively). In a follow-up studies after CRC patient surgery, the CRCglycoA values were significantly decrease in nearly half of patients (21 of 46), which verified the potential of CRCglycoA value as a better biomarker for CRC diagnosis.

Novotny and coworkers [117] analyzed serum glycome from 42 CRC patients and 20 controls using both MALDI-TOF and microchip electrophoresis. MALDI-TOF permitted the identification of 45 glycans. The increase of bisecting glycans N5H3 and F1N5H4S1 might be a unique feature of CRC. Microchip electrophoresis allowed the identification of 16 significantly differential glycan expressions, 9 of which agreed with MALDI-TOF. Several changes of isomers were also observed in microchip electrophoresis. The reported microchip electrophoresis results are complementary to the MALDI-MS data.

Recently, Kaprio *et al.* [118] investigated tissue N-glycome from 18 rectal carcinoma patients with different stages and five rectal adenoma patients using MALDI-TOF/TOF. Relative abundance of Man5, N3H4, sialylated glycans and paucimannose (F1N2H2, N2H3, F1N2H3, and N2H4) were increased in carcinoma samples when compared with adenoma samples. Paucimannose and sial lewis-a epitopes were further analyzed by Immunohistochemistry on 220 patient samples. The increase of paucimannose and SLe^A were associated with poor prognosis of advanced colorectal cancer.

Rudd and coworker [119] investigated plasma glycome from 633 CRC patients, and 478 age and gender-matched healthy people. N-glycans were 2-AB labeled and analyzed using HILIC-LC. Glycan structures were mapped to the database established using exoglycosidases digestion and MS characterization. In a total of 42 glycan peaks were detected and identified, among which 11 glycans showed a significant increase while seven were decreased in CRC (Figure 2). F1N4H5 and N6H7S4 (all sialic acids are α 2,3 linked) had the most significant decrease and increase in CRC, respectively. When considering the cancer stages, the decrease of F1N4H5 and F1N4H5S1 (sialic acid is α 2,6 linked) could be a consistent biomarker across all CRC stages. Discrimination models were trained and tuned (based on 625 CRC vs. 468 control data sets) to distinguish CRC from healthy cases. The combination of all peaks and clinical features achieved a 95% of specificity and 0.3649 sensitivity in discriminating CRC. Also, this model could discriminate different colon cancer stages from healthy cases. The model was then validated using another 18 individuals (8 CRC and 10 healthy) set and achieved a 77.8% accuracy with 100% specificity and 50% sensitivity.

Glycans of specific glycoproteins such as IgG in CRC were also analyzed [120]. IgG was purified from healthy (N=19), benign colorectal patient (N=23) and CRC patient (N=36). N-glycans were released from IgG, methylamidated and analyzed using MALDI-TOF. Totally 28 glycans from IgG were identified and verified by PGC-LC-MS/MS, among which 9 glycan expressions were showed significant alterations in CRC and benign samples. The decrease of 5 glycan expressions (Man5, N4H5, F1N3H4S1, N4H5S1, and F1N4H5S2) were considered as the potential biomarkers in CRC through ROC analysis and further validated on 19 cancer patients and 20 healthy people. Compositional saccharides of CEA glycan from CRC patients were also studied using lectin microarray [121]. The increase of fucose, mannose and Thomsen-Friedenreich antigen, and a decrease of N-acetylgalactosamine, N-acetylglucosamine, galactose, branched and bisecting N-glycans were observed on CRC CEA. Also, a 9-lectin assessment could be used to discriminate CRC tissues with 83% sensitivity and around 90% specificity.

3.1.5. Lung cancer—Lung cancer is one of the most common cancer worldwide and the leading cause of cancer death [76,98]. There are two groups of lung cancer, non-small cell lung cancer (NSCLC) which accounts to 80% of the reported cases and small cell lung cancer (SCLC) which accounts to 20% of the reported cases [122]. Also, lung cancer has the highest frequency of brain metastasis [123]. To date, imaging is still the major diagnostic technique for lung cancer. Although there are some biomarkers such as CEA or CA125, the lack of specificity and sensitivity inhibits their broad uses, thus demanding the need for better biomarkers [124].

In the early studies, the overexpression of SLe^x was found to be a biomarker in both serum or tissue samples of lung cancer [125,126]. However, SLe^x could be a prognosis indicator for lymph node metastasis (71% sensitivity) but not a reliable diagnosis factor for NSCLC (24% sensitivity) [126]. Therefore, SLe^x might be considered as a biomarker for lung cancer progression, but not for diagnosis, suggesting more glycan markers should be uncovered.

Rudd and coworkers [38] studied serum N-glycomes from 100 lung cancer patients and 84 healthy subjects using HILIC and WAX-HPLC. Meanwhile, the glycomes of haptoglobin purified from the same serum samples were also investigated [38]. N-glycans were released from sera or haptoglobin, labeled by 2-AB and then analyzed by HPLC. HILIC permitted 17 peaks containing more than 30 glycan structures. The analysis was based on the peak areas, among which N4H5F1S2, N6H6S2, N6H7, N5H6S2 (coeluted), F1N6H6S3, N6H7S3, N6H7S2, F1N5H6S3, N5H6F1S3 (coeluted), N7H4S4, N6H7F2S4 (coeluted) were significantly increased while N3H5S1, F1N4H4S1, F1N4H5, F1N5H4S1 (coeluted), F1N4H5S1, N5H5S1, N5H6, N3H6S1, F1N5H5S1 (coeluted) were significantly decreased in lung cancer patients. Also, the significant increase of N4H5F1S2, N6H6S2, N6H7, N5H6S2 and F1N6H6S3, N6H7S3, N6H7S2, F1N5H6S3, N5H6F1S3 were observed in all cancer stages. Therefore, these 9 glycans (identified in only two chromatographic peaks) could be lung cancer biomarker enabling effective early detection of the disease. Coupled with WAX-HPLC, the significant increase of SLe^x, mono-antennary glycans and tri-sialylated glycans were also observed in lung cancer. ROC analysis was performed based on the alteration of glycan abundances (peak areas) and provided an AUC value of 0.938 with an 85% of sensitivity and 86% of specificity for distinguishing lung cancer patients from healthy subjects. These values were even higher when glycomic expressions were combined with C-reactive protein (CRP) expression.

Recently, Ruhaak *et al.* [127] analyzed blinded pre-diagnostic serum N-glycome from 100 NSCLC patients and 199 healthy controls using nano HPLC-PGC-chip-TOF-MS. A total of 74 glycans were identified in 75% of the samples. Through ROC analysis of each glycan, four glycans were selected with AUC > 0.6 and FDR < 0.05, among which N4H3 and *F1N4H3 were overexpressed in NSCLC while *F1N4H5 and *F1N5H6 were decreased. Also, a set of 18 glycans were picked up to represent 8 different glycan compositional features (e.g., galactose number and sialic acid number). These 4 glycans and 8 features were then validated in a blind set that consisted of 108 NSCLC patients and 216 healthy cases. Among them, 4 glycan variables exhibited an AUC > 0.6. The combination of these 4 features (*F1N5H4, *F1N6H5, bi-sialylation, and tetra-galactosylation) achieved a better diagnostic result (AUC of 0.74). Glycosylation changes could be combined with other known lung cancer biomarkers such as pro-SFTPb to be the indicators for NSCLC risk assessment and progression tracking. The decrease in F1N4H5 was also observed in a previous study when analyzing lung adenocarcinoma plasma glycome [128].

Besides serum or plasma samples, glycomes derived from lung cancer tissue sections were also investigated by Wang *et al.* [129]. They analyzed released and permethylated N-glycome from 29 lung cancer tissue sections and 14 normal samples (discovery set) using MALDI-TOF. Total 58 N-glycans were identified using MSⁿ (n=3), among which 6 glycans (including 5 high-mannose glycans) showed significant up-regulation in lung cancer while 8

(6 were sialylated) showed down-regulation. All these 14 glycans received AUC values > 0.7, except Man9 (AUC of 0.686). The expressions of these glycans were validated using another sample set which consisted of 50 cancer tissues and 24 normal controls (validation set). Combining the increase of high-mannose glycans (5 glycans) and a decrease of sialylated glycans (6 glycans) exhibited AUC value of 1 in both the discovery and validation sets.

3.1.6. Pancreatic cancer—The survival rates of most cancers are increasing; however, the 5-year relative survival for pancreatic cancer remains low (8%) [98]. Pancreatic cancer has the lowest 5-year relative survival rates because the survival rate for distant stage is only 3% and over 50% of the cases are diagnosed at a distant stage [130]. The clinical management of pancreatic cancer is hampered by the rapid progression and large portion of asymptomatic individuals at an early stage of the disease [131]. Therefore, biomarkers promising accurate prognosis and early diagnosis of pancreatic cancer are needed. Currently, carbohydrate antigen 19-9 (CA 19-9) is a widely utilized biomarker in clinical practice for diagnosis, monitoring treatment and recurrence of pancreatic cancer [89,132]. However, CA 19-9 suffered from the lack of sensitivity and specificity [133].

Many studies have revealed N-glycan profile alterations in blood serum proteins of pancreatic cancer sample sets relative to the healthy control sets. Lubman and coworkers [134,135] developed lectin enrichment, and mass spectrometry-based analysis approaches for understanding glycosylation sites and N-glycome. In one of the studies, increased branching, fucosylation and sialylation of N-linked glycans were observed in the serum of pancreatic cancer [134]. A similar trend was observed in a more recent analysis of glycome on haptoglobin isolated from human serum [135]. Both core and antennary fucosylated glycans of haptoglobin increased in pancreatic cancer samples compared to benign conditions. However, these studies were conducted as proof-of-principle studies, and only small sample sizes were employed. Additionally, the increased fucosylation on the β chain of haptoglobin was also reported by Miyoshi and coworkers in other studies where larger sample sizes were employed [136,137].

Although blood serum samples are used for the majority of biomarker discovery studies [138], analysis of other specimens such as pancreatic cyst fluids [139] and cell lines [140] were also explored. Novotny and coworkers [139] have performed N-glycomics study on 20 pancreatic cyst fluid samples, from which complex N-linked glycans containing two to six fucose residues were observed. This study aided in the development of test system for early detection of pancreatic cancer. Wuhler and coworkers [140] thoroughly characterized four pancreatic cell lines with different metastatic behaviors and one healthy control cell line. They evaluated glycan profile features including sialylation, fucosylation, Lewis antigens N-acetylhexosamine content and antennary. Discrepancies between the results obtained from different cell lines highlighted the importance of avoiding generalizing conclusions for the biology of cancer based on one or few cell lines.

MALDI imaging technique was also used for the pancreatic cancer biomarker discovery [95]. The intensities of on-tissue released N-glycans were determined by MALDI-MS at discrete tissue locations and used to construct images representing the distribution of

glycans. This method was further applied in tissue slices including a human pancreas tissue [95]. The tissue block contained tumor tissue, non-tumor tissue, fibroconnective tissue representing desmoplasia surrounding the tumor tissue, and necrotic tissue. Glycan F1N6H3 was found in the non-tumor region of the pancreas, while glycan Man8 was predominant in the tumor region of the tissue. Desmoplasia is represented by F1N4H5. Additionally, in the region where necrosis was identified, N4H5 was found to elevate. In this study, glycan Man8 was also determined to be able to distinguish tumor hepatocellular carcinoma tissue from non-tumor tissue. This workflow enabled a high throughput spatial screening of the N-glycan biomarkers associated with cancers.

3.1.7. Prostate cancer—Prostate cancer is a major cancer diagnosed in man, accounting for approximately 20% of the new diagnoses and it is also one of the top 3 causes of cancer death [98]. As prostate cancer usually causes increased prostate-specific antigen (PSA) concentrate in the blood, the serum PSA level was approved by the Food and Drug Administration (FDA) as a marker for treatment monitoring of prostate cancer in 1986, and as a marker for diagnosis in 1994 [141]. However, the use of serum PSA concentration as a biomarker for prostate cancer is controversial because over-diagnosis and over-treatment remain as problems associated with the PSA test [141-143]. Many of the PSA-derived or other biomarkers combined with the conventional PSA test were proved to be valuable in improving the accuracy and specificity of the testing for prostate cancer [141,142].

Most of the prostate cancer biomarkers, including PSA, are glycosylated, herein the potential of aberrant PSA glycosylation patterns used as a diagnostic tool for prostate cancer is warranted. The profiling of PSA N-glycans using oligosaccharide sequencing and mass spectrometry has revealed distinct glycan patterns of PSA derived from normal and tumor origins [144]. Unlike normal PSA glycans, which were sialylated, the glycans derived from prostate cancer cells were all neutral and fucosylated.

The alterations of glycan profiles were observed not only in PSA but also in sera. The N-glycome of 24 prostate cancer patients and 10 healthy males was also depicted using MALDI-TOF mass spectrometry and compared using statistical tools [145]. This approach allowed the detection of over 50 N-glycans, of which 12 glycans were significantly different between cancer and healthy sample sets. The comparison of intensities according to glycan types suggested an increase in the fucosylated complex biantennary, complex tri- and tetra-antennary N-glycans associated with prostate cancer. Similarly, Lebrilla and coworkers [146] observed a decrease in the levels of high-mannose and acidic glycans, and an increase in the levels of fucosylated bi-antennary complex N-glycans in prostate cancer serum samples when comparing serum glycome between 10 patients with prostate cancer and 10 with removed prostate. A more recent study analyzed the N-glycome of 650 patients with different stages of prostate diseases [147]. The overexpression of serum tri- and tetra-antennary N-glycans in castration-resistant prostate cancer (CRPC) was observed. As a result, the tri- and tetra-antennary glycans were proposed as potential biomarkers for CRPC [147].

3.1.8. Other Cancers—Quantitative N-glycomics using multiple techniques has been utilized for uncovering better cancer biomarkers and proved to be efficient in many cancers as discussed above. Besides the aforementioned cancers, the use of glycomics as a biomarker discovery tool was also reported in other types of cancers, such as kidney cancer [148], esophageal cancer [149,150], gastric cancer [151] and brain cancer [152,153]. A significant elevation of high-mannose structures and highly sialylated structure were observed in kidney cancer [148]. The decrease of two core fucosylated glycans, F1N4H5 and F1N4H4, was considered as potential biomarkers for esophageal cancer with more than 90% sensitivity and 85% specificity [149]. The decrease of core fucosylation could be a satisfactory biomarker for early gastric cancer detection (AUC 0.923) [151]. The increase of larger, sialylated structures were observed in neuroblastoma cell line [152].

3.2. Potential O-glycobiomarkers in different cancers

The value of O-glycans as biomarkers is less studied than that of N-glycans due to the lack of an efficient method for O-glycans release. Also, the common chemical release methods employed to cleave O-glycans are time-consuming and/or prompt glycan peeling. On the other hand, the use of O-glycans as potential biomarkers for cancers is important for two reasons. First, the mucin type (GalNAc-type) O-glycans are frequently found in transmembrane and secreted portions which are altered as a result of cancer development and progression. Second, one of the most common occurring alterations associated with cancer is truncated O-glycans as a consequence of changing in the environment and transferases expression [154]. All potential O-glycobiomarkers that thus far have been reported in the literature are summarized in Table 2.

3.2.1. Breast cancer—The well-characterized mucin 1, a biomarker approved for the diagnosis of breast cancer [155], is heavily O-glycosylated. It has been known that the alterations of mucin O-glycans occur in the mammary gland during malignancy [156]. Therefore, developing comprehensive O-glycan profiling workflows is an alternative strategy for the biomarker discovery for breast cancer. As a proof-of-concept study, Miyamoto and coworkers [157] employed a MALDI-FTICR-MS-based approach to analyze O-glycans extracted from breast cancer cell line supernatant and serum samples. Similar glycan patterns were observed in the spectra of the three cell lines (MDA-MB-361, MDA-MB-468, and MDA-MB-453), which were also similar with the glycans identified in ovarian cancer serum [158]. However, some glycans identified in these three MDA-MB cell lines were not observed in the more precancerous cell line BT 474 or the normal breast cell line MCF10A. Also, they further investigated serum O-glycome obtained from a mouse model of metastatic breast cancer, as well as a small set of serum samples obtained from patients with breast cancer (n=4) and healthy individuals (n=4) [157]. Mice used in this study were transplanted at 5 weeks of age with small pieces of mammary tumors that originated from the PyMT mouse model of breast cancer. Indicated by the analysis of sera (from week 4 to 8), 21 glycan structures were detected in mice sera at all time points, while 11 more glycan structures were only detected after the tumor implantation. Moreover, it was demonstrated that the differences in O-glycan profiles were sufficient to distinguish the patients with breast cancer from those without cancer by PCA analysis, although the findings were tentative due to the small number of samples involved in the study.

3.2.2. Ovarian cancer—Lebrilla and coworkers [158] analyzed O-glycome of shed glycoproteins in 4 ovarian cancer cell cultures (including OVCAR-3, Caov-3, SK-OV-3 and ES-2) using MALDI-FTICR. Although many identified oligosaccharides were common among 4 cell lines, each cell line possessed some unique structures. In addition, they further investigated the O-glycome on a set of cancer (n=5) and normal serum samples to determine potential oligosaccharide markers for ovarian cancer (n=5). It was demonstrated that there were at least 15 unique glycans observed in all cancer patients but being absent in healthy individuals.

O-glycans of ovarian cyst fluid and tissues from patients with serous and mucinous ovarian tumor were profiled by Vitiazeva *et al.* [159]. The mass spectrometry average composition (MSAC) of oligosaccharides was used as a parameter to describe glycan profile characteristics in each sample. To calculate MSAC, all structures identified by LC-ESI-MSⁿ were first reduced to monosaccharide compositions (the number of hexose, N-acetylhexosamine, fucose, sialic acid residues and sulfate groups). The intensities of corresponding structures were multiplied by the monosaccharide compositions and summed over the entire sample. Then, the ratio between summed intensity of a monosaccharide residue and the total intensity of all oligosaccharides over the sample was defined as the MSAC. Based on the MSAC, fucosylation, sialylation and sulfation of O-glycans appeared to be important features in differentiating the type of tumors.

Karlsson, N. G., McGuckin, M. A., O-Linked glycome and proteome of high-molecular-mass proteins in human ovarian cancer ascites: identification of sulfation, disialic acid and O-linked fucose. *Glycobiology* 2012, 22, 918–929.

3.2.3. Other Cancers—Attempts in searching for O-glycan biomarkers were also made for many other cancers. He *et al.* [160] analyzed the released and 1-phenyl-3-methyl-5-pyrazolone (PMP) labeled O-glycans derived from 157 gastric cancer and 144 healthy control serum samples using an LC-MS-based approach. The MS spectra (average of retention time ranging from 19.14 to 20.88 min) of O-glycans derived from serum of gastric cancer patients and controls are shown in Figure 3a. A partial least squares-discriminant analysis (PLS-DA) model was used to evaluate the O-glycans alterations in patients with gastric cancer and healthy individuals (Figure 3b). Also, an unpaired Student's t-test and ROC of the significantly altered O-glycans were performed. The AUC value of the ROC analysis was 0.97 (Figure 3c). Several structures, including core1, core2, ST antigen, and core2 complex O-glycans were determined to be up-regulated in gastric cancer sera over the healthy control sera, whereas the expression level of *m/z* 529.75 (H1N2F2, which was a novel O-glycan extended from core 1) and diST-antigen were significantly decreased in the serum of gastric cancer compared with controls samples. Moreover, Miyamoto and coworkers [161] identified 14 sulfated O-glycans as potential biomarkers by comparing sulfated O-glycans profiles of 11 gastric and 9 pancreatic adenocarcinoma serum samples with those of 20 disease-free serum samples. Furthermore, Miyamoto and coworkers [162] adopted the selected reaction monitoring (SRM) analysis, showing the potential of core 1 sial Lewis A, whose correlation with CA19-9 varies from poor to weak depending on the type of cancer, to be considered as a diagnostic marker for cancer. Recently, Packer and coworkers [163] profiled the N- and O-glycans of serous ovarian and peritoneal cancer

tissues, suggesting that 3 O-glycans among many altered glycosylations enabled discrimination of tumor-sampling sites. Moreover, Packer and coworkers [64] also performed glycomic comparison between colon cancer cell cultures and tumor tissue. The study revealed differences of N- and O-glycome existing between different cancer cell lines and the epithelial cells from tumor tissue, hence, the discrepancies need to be considered regarding choosing cell line models for glycan biomarker discovery.

3.3. Isomeric N-glycans as potential cancer biomarkers

With the development of separation techniques, glycan isomers have been resolved, and their distribution were found to be different among different diseases. Different separation techniques have been used to analyze isomeric glycans, including ion mobility mass spectrometry [164], MALDI [165], porous graphitized carbon chromatography (PGC) [166], hydrophilic liquid interaction chromatography (HILIC) [167], and reverse phase chromatography [168]. Different glycan isomers have been considered as potential biomarkers for several types of cancer and diseases. Potential glycan isomer biomarkers that have been reported are listed in Table 3.

In the early study, the importance of glycan isomeric characterization was demonstrated by Clemmer and co-workers [169], who used ion mobility mass spectrometry to analyze derivatized serum glycans from patients with liver cancer (n=19), cirrhosis (n=20), and healthy individuals. Although the partial separation of glycan isomers or conformers were observed, distributions for individual ions were found sufficient to distinguish patients with liver cancer or cirrhosis.

Recently, Clemmer and co-workers [170] analyzed N-glycan isomers derived from serum samples related to three disease phenotypes, Barrett's esophagus, high-grade dysplasia, and esophageal adenocarcinoma, as well as a normal control group. IM-MS allowed the identification of 46 isomeric structures originating from the 11-glycan composites, which could distinguish esophageal adenocarcinoma samples from normal control group samples by principal component analysis (PCA). Also, for N4H5S1 and F1N4H5S1, the intensity ratio of high-mobility to low mobility isomers were significantly higher in esophageal adenocarcinoma patients. Although the IM-MS demonstrated an efficient separation of small glycan isomers, the separation of larger isomers or closely related isomeric structures were still limited due to the low resolution of this technique.

MALDI-MS techniques have also been used to analyze glycan isomers without separation, in which derivatization of different isomers is needed. After derivatization, different sialic acid linkage isomers can be distinguished. Alley and Novotny [171] investigated sialic acid linkage isomers in breast cancer patients using MALDI-MS. Isomeric glycans with α 2,3- or α 2,6-linked were distinguished through the amidation before permethylation. Only α 2,6-linked sialic acids could be amidated, resulting in the separation of two isomers with 13 *m/z* unit per sialic acid residue. Accordingly, amidated and permethylated tri-antennary tri-sialylated structure (N5H6S3) showed four peaks of different structural isomers. With the mass differences of different sialic acid linkage isomers, a higher level of α 2,6-linked sialic acid residues was observed in cancer patients. Although the number of samples employed in

this study (10 cancer-free and 10 late-stage breast cancer) was not large enough, the increase of α 2,6 sialylation might be a potential biomarker for breast cancer diagnosis.

Recently Wuhler and coworkers [172] investigated the distribution of α 2,3- and α 2,6-sialylated N-glycan isomers in colon carcinoma tissues using MALDI imaging coupled to *in situ* derivatization technique. During the derivatization, α 2,6-linked sialic acid formed a stable dimethylamide, whereas α 2,3-linked sialic acid converted to a lactone. Then, lactone was hydrolyzed, forming stable amide, which resulted in 28.03 Da mass difference between α 2,6- and α 2,3-linked sialic acids. Histology images acquired the clearly different distributions between α 2,6- and α 2,3-sialylated glycans across the tissue regions. The increase of α 2,6-sialylated glycan isomers in necrotic tissue, collagen-rich (tumor) areas and red blood cells suggested the association between α 2,6-sialylation and colon cancer.

A comprehensive characterization of isomeric glycan structures is achieved using MS in conjunction with highly efficient separation techniques, such as LC or CE. As discussed above, different LC techniques such as HILIC, RPLC, and PGC chromatography have been used for this purpose. Tousi *et al.* [61] used 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM)/methanol derivatization method in conjunction with nano-HILIC-Orbitrap-MS, to identify the sialic acid linkages of haptoglobin derived from patients with different types of cancers (n=4). Among different cancers (lung, breast, ovarian, pancreatic, gastric cancer and pancreatic cancer), differential expression analysis of glycan isomers showed that high level of α 2,6-linked F1N5H6S3 could be a potential marker in discrimination of pancreatic cancer from other cancers ($p < 0.05$).

PGC has been used as an efficient material in the isomeric separation of glycans due to its hydrophobic and polar interactions with glycan species and the planer morphology of PGC [57,166,173], which make it an efficient tool for glycan isomeric marker discovery in diseases. Lebrilla and coworkers [50] analyzed isomeric profiles of native glycans in serum samples from two groups of prostate cancer patients with different prognoses using PGC-chip-HPLC-TOF-MS. A total of more than 300 N-glycan species (including isomers) corresponding with over 100 N-glycan compositions were identified. Overall, 38 isomers related to 19 N-glycan compositions showed significant differences between the two groups. Both isomers of N5H3 increased in the poor prognoses group when compared with good prognoses group. For Man9, three isomers were detected, one of which showed significantly higher abundance in poor prognoses group. These increased isomers in poor prognoses group might be considered as biomarkers. A similar technique was used by Leiserowitz and coworkers [174] to analyze isomeric glycans derived from epithelial ovarian cancer. PGC-chip-HPLC-TOF-MS permitted the identification of 26 differentially expressed glycans between epithelial ovarian cancer and healthy control samples. Two isomers of N4H5F1S1 showed significantly higher abundances in cancer over the healthy samples, which might be considered as ovarian cancer biomarkers.

In a recent work, Liu *et al.* [175] explored isomeric glycans in pancreatic cancer patient group and healthy control group using PGC-nanoLC-ESI-MS system. A total of 280 glycan isomers corresponding to 72 glycan compositions were identified, of which 25 glycan isomers with different significant expressions were considered as potential biomarkers for

the diagnosis of pancreatic cancer. Among these 25 isomers, 16 isomers (mostly fucosylated and sialylated) showed higher abundances in patients with pancreatic cancer. As an example, while two of the four detected isomers of F1N4H5 showed lower relative intensities in cancer patients, one of them obtained with higher relative intensity.

Human skin N- and O-glycome and their expression changes associated with two skin cancers, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), were investigated by Möglinger *et al* [176]. PGC-nanoLC-ESI-MS and capillary gel electrophoresis with laser induced fluorescence detection were performed to reveal the over-expression of high-mannose N-glycans and core 2 type O-glycans in BCC and SCC. Moreover, the decrease of α 2,3 sialylation level was observed in SCC. Hinneburg *et al* [177] investigated N- and O-glycans released from formalin-fixed and paraffin-embedded (FFPE) tissues of hepatocellular carcinoma (HCC) patients using PGC-nanoLC-ESI-MS. A significant decrease of α 2,6 sialylation was observed in tumor tissues, while core fucosylated N-glycans and core 2 type O-glycans were increased.

Takahashi *et al* [178] studied isomeric fucosylated N-glycans derived from haptoglobin in the sera of patients with five types of gastroenterological cancers (esophageal, gastric, colon, gallbladder, pancreatic), a non-gastroenterological cancer (prostate cancer) and normal controls using PGC-LC-ESI-MS. Both core- and antennary-fucosylated glycans were detected with significantly higher abundances in gastroenterological cancers over the healthy controls, while only core-type fucosylated N-glycans were relatively increased in prostate cancer samples. The differential expressions of core- and antennary-fucosylated glycans enabled the distinction between gastroenterological and prostate cancers. PGC-nanoLC-ESI-MS was also employed to analyze the membrane glycosylation of non-cancerous ovarian surface epithelial and serous ovarian cancer cell lines, which revealed a higher level of α 2,6 sialylated glycans on ovarian cancer membrane proteins [179].

Recently, an efficient separation of permethylated glycans on PGC at high-temperature (75 °C) was reported by Mechref group [15]. The method was successfully used for the isomeric characterization of N-glycan isomers derived from normal breast cancer cell lines MDA-MB-231 and brain seeking cell line MDA-MB-231BR [15]. A total of 106 glycan isomers and 21 non-isomeric glycans were identified, among which 37 structures exhibited significant differences of isomeric distributions between the two cell lines. The increase of α 2,6 sialylations was observed in most highly sialylated structures. Glycan isomer N4H5S2(6,6) was significantly increased in brain seeking cell line 231BR, suggesting this isomer could be a possible indicator for breast cancer brain metastasis. Similar results were shown for trisialylated glycans, confirming that α 2,6 sialylation might contribute to breast cancer brain metastasis. Significant changes of isomeric distributions for some non-sialylated structures were also observed.

More recently, Mechref and coworkers [52] investigated isomeric glycosylation patterns of haptoglobin glycoproteins derived from 10 cirrhotic and 10 HCC patients. PGC-LC-MS/MS allowed the identification of 34 glycan isomers, among which 8 isomers expressions were significantly altered. Figure 4 depicts the isomeric alterations of N4H5F1S1 in different HCC and cirrhosis samples. A significant increase of N4H5F1S1(6) and N4H5S1(6) can be

observed in HCC patients. ROC analysis of 6 most significant isomers showed AUC more than 0.79, suggesting these isomers could be potential biomarkers for HCC. A higher level of α 2,6-linked sialic acid residues was observed in HCC over cirrhotic patients, which allowed distinguishing early-stage HCC from cirrhosis.

4. Glycans as potential biomarkers in Alzheimer's disease

Alzheimer's disease (AD) is the primary cause of dementia and one of the most common neurodegenerative disorder [180]. It is known that glycans play significant roles in AD development and studies confirmed variation in protein sialylation and N-glycosylation in the brain of AD patients [181]. Alterations in protein sialylation between AD (n=16) and healthy individuals (n=7) were studied by using lectin blotting analysis of cerebrospinal fluid (CSF) proteins [182]. Wheat germ agglutinin (WGA) and concanavalin A (Con A), were used to analyze the sialylation pattern between AD, and non-AD patients. The decrease of sialylation in CSF was observed in AD patients.

Recently, a comprehensive analysis of N-, O-, and O-GlcNAc-glycomes in various brain regions and the serum of AD patients (n=10) has been reported by Segal and coworkers [183]. A 45-lectin microarray was used to analyze serum glycan patterns between AD patients and healthy controls. Nine lectins which bound to fucose (lotus tetragonolobus lectin (LTL)), acetyllactosamine (lycopersicon esculentum lectin (LEL)), solanum tuberosum lectin (STL), and phytolectin americana lectin), and GalNAc (Jacalin, helix pomatia agglutinin (HPA), vicia villosa lectin (VVA), dolichos biflorus agglutinin (DBA), maackia amurensis hemagglutinin (MAH), and griffonia simplicifolia lectin I (GSL-I B4)), showed statistically higher signals in AD serum, and two lectins which bound to the sialic acid motif (maackia amurensis lectin (MAL) and phytohaemagglutinin (PHA)), showed significantly lower signals in AD patients. In addition, different brain regions, namely, frontal cortex, hippocampus, temporal cortex, and parietal cortex showed higher level of O-GlcNAc in membrane proteins for AD patients. Although lectin microarray provided a valuable approach for AD glycan biomarker discovery, other techniques such as MS-based glycomics which can acquire higher sensitivity and more structural information are necessary to reveal different AD biomarkers.

5. Glycans as potential biomarkers in diabetes

Diabetes is a disease in which the levels of glucose in the blood and urine elevated, resulting in abnormal metabolism of carbohydrates [184,185]. Individuals suffering from diabetic are subjected to develop neurological, retinal, renal and macrovascular complications. German and coworkers [186] studied the effect of gestational diabetes mellitus (GDM) on free oligosaccharides and protein-conjugated glycan profiles in woman breast milk. GDM develops during the pregnancy and identified by elevated blood glucose, affects 7% of pregnancies in the United States. Milk was analyzed for free oligosaccharides abundances, protein concentrations, and N-glycan derived from lactoferrin (the major milk glycoprotein) and secretory immunoglobulin A (sIgA, the predominant Ig in human milk). Although no significant difference in free oligosaccharides between milk from women with GDM (n=8) and without GDM (n=16) was obtained, significant differences were shown between two

groups for N-glycans derived from lactoferrin and sIgA. Abundances of mannose, fucose, and sialylated sIgA glycans were decreased in the milk samples from GDM women, while higher abundances of lactoferrin fucosylated and sialylated N-glycans (36–72%) were observed. The differential glycan expressions in milk could be potential indicators for immune functionality.

Significant changes in N-glycan composition in the sera of diabetic patients were reported by Testa *et al* [187]. N-glycomic changes was investigated among serum samples from 562 patients with type 2 diabetes and 599 healthy control subjects. The level of F1N4H4 were found significantly reduced in both male and female diabetic patients. In addition, N4H5 glycans were found significantly increased only in male diabetic patients. The decrease of biantennary N-glycans and increase of tri-/tetra-antennary structures were observed in sera of type 1 [188] and type 2 [189] diabetic patients, represented promising diagnostic and prognostic biomarkers. More recently, Packer and co-workers [190] studied N- and O-linked protein glycosylation in diabetic mouse ovarian tissues. In comparison with nondiabetic mouse ovaries, sialylated and fucosylated glycans were detected with significantly lower relative abundances, where high-mannose structures showed higher relative abundances. Moreover, O-linked released glycans from control and diabetic mice ovaries, exhibited an increase in the NeuGc:NeuAc ratio in diabetic ovarian tissues.

6. Glycans as potential biomarkers in other diseases

Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) can cause cirrhosis and hepatocellular carcinoma in humans. Blomme *et al.* [191] reported that hyperfucosylation, increase in branching and bisecting N-acetylglucosamine of glycans were observed in liver diseases. Complex N-glycan expression in sera from chronic hepatitis B virus (CHB) and C virus (CHC) infected patients compared to healthy individuals were investigated. Kang *et al.* and Kuno *et al.* demonstrated a significantly increased level of high mannose, core fucose and sialylated glycans in CHC patients than in CHB patients [192,193]. N-glycans profiles by MALDI-TOF/TOF-MS have shown that glycans F1N5H4 (bisecting), N5H5 (bisecting) and F1N6H3F1 are overexpressed in CHB patients. In contrast, glycans F1N5H4 (bisecting), N3H5, N6H5 and F1N5H6 are overexpressed in CHC patients, compared to healthy individuals [194]. The over-expression of bisecting glycan F1N5H4 was also observed in liver cancer. The correlation of glycosylation to other diseases, including but not limited to arthritis [195], psoriasis [196], and asthma [197], Huntington's disease [198], amyotrophic lateral sclerosis (ALS) [199], chronic pain [200], *etc.* were also investigated. However, most studies of these diseases were focused on intact glycoproteins while glycans were rarely reported.

7. Conclusions and future perspectives

Glycosylation is a common post-translational modification of proteins in human cells and organisms. Association of glycans with the cell surface is a ubiquitous phenomenon. The onset of cancers and diseases cause the change of glycan structures. Therefore, cracking the glycan code would reveal the cellular physiology and characteristics of disease conditions. Advances in MS and separation techniques are powerful tools, which provide highly

sensitive and reproducible methods in the comparative glycomic analysis for the discovery of potential glycan biomarker candidates. Most potential glycan biomarkers discovered in cancers and diseases are summarized in Tables 1-3.

Although there are more glycans reported in different diseases, only those glycans that were observed the outstandingly unique expressions in diseases are listed in the table. Most of the listed glycans were achieved the ROC analysis with higher AUC values than existing biomarkers, suggesting the high potential of these glycans to be indicators of corresponding diseases. Therefore, the summarized glycans should be paid more attention to in future studies. Table 1 summarizes potential N-glycan biomarkers reported in recent years. Many glycan structures, such as *F2N5H6S3 and F1N5H6S2, were observed the same up-/down-regulation in different diseases. In contrast, several glycans, such as Man5 and F1N4H5S1, exhibited opposite alterations in different diseases. The alterations of the same glycan structure in different diseases provide the hint of the role glycans played in the biological processes, which could facilitate the use of glycan biomarkers in clinical research. Compare to N-glycans, there are less cohort-based studies for O-glycans due to the lack of efficient release method and the high microheterogeneity of O-glycans. However, many effects have been made to get a deeper insight of O-glycan biomarker discovery. Table 2 summarizes potential O-glycan biomarkers, which were reported to have significant alterations in diseases and most possible to be exploited as biomarkers. Nevertheless, the work for O-glycomics in clinical cohort is not enough. More studies of O-glycomics should be performed to reveal the O-glycan biomarkers. Like O-glycan biomarker, glycan isomer study is also a challenge. With the improvement of glycan isomeric separation, more glycan isomers were observed the significant expression changes in different diseases, as shown in Table 3. However, the challenge of glycan isomer biomarker discovery is still the lack of cohort studies and the effective way of glycan isomer characterization.

Many of the overlapping glycans in different cancers should be validated prior to apply those glycans as reliable glycobiomarkers. The common trends of the increases of glycan fucosylation, sialylation, bisecting and branching in diseases (Table 1 and 2) and the increase of α 2,6 sialylation in cancers (Table 3) may provide some important glycan features in clinical biomarker studies. Figure 5 depicts Venn diagrams comparing the N- and O-glycobiomarkers reported for the most common cancers. A total of 78 N-glycan biomarkers were compared in the top six cancers (Figure 5a) while 20 O-glycans in two cancers (Figure 5b). The overlap of glycobiomarkers among different cancers indicates the need to focus on specific structures and biosynthetic pathways that appear to be highly influenced by disease development and progression. Although glycan profiles provide biological information about glycan structures, isomeric glycan structure analysis is not adequately studied or considered because of the lack of reliable analytical methods. However, this is recently been addressed. In addition, glycan biomarker discovery studies were mainly focused on cancers. There are much more researches associated with cancers than other diseases. Although aberrant glycosylation was also reported in other diseases, only limited studies were made to reveal the potential glycan biomarkers for these diseases. Therefore, a large number of glycan biomarkers are still uncovered out of cancer field, and more works are needed.

Despite of the increasing report of potential glycan biomarkers which have the better prediction ability in prognosis and diagnosis of diseases, the clinical use of glycan biomarkers in practice is quite limited. The reasons to prevent the wide use of glycan biomarkers in clinical analysis are their relatively high cost and long turnaround time. In addition, the interpretation of glycomics data is also unfriendly to clinical chemists who have little glycomics experience. Glycan focused therapeutics would improve the selectivity to diseased cells from healthy cells and amplify therapeutic opportunities. However, to widely apply glycan markers in clinical analysis and prompt diagnosis in the future, more efficient and readily conducted sample preparation procedures and faster data processing methods must be developed to satisfy large numbers of biological sample analysis. In addition, large cohort studies of glycomics is still not sufficient to have a definite conclusion of glycan biomarkers. More investigations on different diseases and clinical cohorts should be performed to verify the potential glycan biomarkers, especially for O-glycans and glycan isomers. Moreover, most of glycomics studies are focusing on cancers, leaving a large number of glycan biomarkers in other diseases uncovered. With more and more glycan structures and glycan biomarkers discovered, glycan database would facilitate automatic data processing and ultimately benefit the application of glycan biomarker in diagnosis and development of glycan targeted therapeutic and diagnostic strategies in the future.

Expert commentary

Quantitative glycomics has been applied in glycan biomarker discoveries to improve clinical prognosis and diagnosis of many diseases. Several glycan or glycan pattern markers have a better predictive or diagnostic capacity than already widely used biomarkers (such as CA125 and CEA). The glycan patterns offer higher diagnostic accuracy when combined with the existing biomarkers. Thus, glycans have a great potential to be exploited as biomarkers of disease, thus prompting the development of glycomics techniques. With the improving of glycomics strategies, glycan biomarker screening has become more sensitive and accurate, facilitating the clinical use of glycan markers.

Different glycan analysis techniques, such as LC-MS/MS or MALDI-IMS, and different labeling reagents, allow the identification of more low-abundant glycans and more reliable quantitation. These improvements provide the easier and more efficient ways to compare glycome expression among large cohorts, which can reveal more glycan alteration in diseases. Thus, glycan biomarker discovery has become possible and attracted more interest of scientists. However, to date, glycomics studies are more focused on cancers. Only a few reports have evaluated glycome changes in other diseases as discussed here. Glycan biomarkers for diseases other than cancers are starting to appear in the literature. Also, the fact that non-of these reported glycan markers are widely used in clinical diagnosis suggested that we still need more accessible, faster and more efficient techniques in glycomics.

This review is not intent to cover exhaustive glycan structures associated with all diseases, but to provide some important cases which represent the efforts of glycan biomarker discovery and their possibilities to be developed as biomarkers in clinical prognosis and diagnosis. However, there is an unbalanced distribution of glycomics study across diseases.

More works were focused on cancers while only limited reports were about other diseases. Also, most of large cohort glycomic studies were associated with different cancers. The lack of systematic glycomics for other diseases, such as arthritis or psoriasis, needs to be addressed. Large number of glycan biomarkers for these diseases were still uncovered, providing a wider direction to glycomic scientists.

Another challenge in this field is the number of samples. Although some researches of large patient cohorts have been made, glycomic studies of different diseases for a large number of biological samples (such as patient sera or tissues) are still not enough. More investigations based on big cohorts need to be performed so that sufficient clinical data can be collected to facilitate and verify the use of potential glycan biomarkers. This also requires the faster speed from sample preparation to glycan quantitation, thus decreasing the turnaround time. MALDI-IMS may provide a solution, which could be the complimentary of LC-MS/MS in high-throughput glycan biomarker discovery.

Due to aforementioned limitations, the fact that glycan biomarkers have not yet applied in clinical prognosis and diagnosis demands a wider and deeper investigation of glycan expression in different diseases, which can be screened out and verified based on a large number of clinical cases. Fortunately, more glycomic researchers are participating in the field, better glycomic technologies are introduced, and more glycan biomarker candidates are detected, which make the use of glycan biomarkers possible in the near future.

Five-year view

In the last decades, the development of glycomic technologies guarantee the screening of potential glycan biomarkers. Lectin-based technique has been applied for a long time and is still being used in medical labs due to its fast analysis speed and simple protocol. MALDI-MS has attracted much interest recently, because it can provide more structural information with a high sensitivity. In recent years, more and more potential glycan markers have been revealed using LC/CE-MS/MS, which has the highest sensitivity and is rich in structural information. The wide use of mass spectrometry in glycan biomarker discovery studies facilitate the understanding of roles glycans played in diseases. However, the relative long analysis time, high cost and complex data processing limited the use of MS-based techniques for large clinical cohorts. The technology will keep developing to overcome these issues and lead the boom of MS-based glycan marker discovery.

In the near future, more sensitive, faster and easier-to-use method for quantitative glycomic study will be developed, thus facilitating the discovery of more glycan biomarkers.

Automated glycomic analysis robot, which can take over the glycan sample preparations from human, has been reported and will be improved to achieve a high throughput glycan analysis. These developments will benefit the large cohort analysis. Another challenge besides the development of glycomic techniques is the interpretation of glycomic data. Glycomic data processing is not friendly with unexperienced people, which may hinder the use of MS-based glycomic analysis in hospitals. This demands the development of the efficient data processing software to simplify glycomic data interpretation. With many efforts, better databases and automated data process software will be established, as well as

the bioinformatics tools. These databases, softwares and bioinformatics tools will enable more research efforts in glyco-biomarkers discovery.

As discussed in the manuscript, many glycan markers have been demonstrated the higher prediction accuracy than original biomarkers for several diseases. Moreover, the combination of these glycan markers and existing glycoprotein marker even can be the better indicators for clinical prognosis. Although the investigation is still needed to be performed on more clinical cases. with the increasing studies of these potential glycan markers, the combination of new glycan marker and original glycoprotein markers will be used for a better clinical diagnosis in the future.

To date, the glycan biomarker discovery is more focusing on cancer field. There are limited researches have been made for other diseases. Thus, large number of glycan biomarkers are still untouched out of the cancer field. Nevertheless, the important role that glycans plays in disease progress has been notified and attracted more interest of glycomic scientists. The studies about glycan biomarkers in other diseases will be made by more researchers, revealing more potential glycan markers in other diseases. The use of glycan as biomarkers in the prognosis of other diseases will also become possible in the future as well as cancers.

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Key issues

- Different techniques for quantitative glycomics were developed and utilized for glycan marker discovery.
- Utilizing different techniques, many glycan expressions or glycan patterns are proved to be better biomarkers for the prognosis and diagnosis of different cancers.
- Not only glycan compositions, but also glycan isomers could be efficient indicators for diseases development and progression.
- O-glycomics studies also revealed possible O-glycans as biomarkers in clinical application.
- Although several glycan markers have been reported in several diseases other than cancer, the number of studies in this field is rather limited.

Figure 1. N-glycan profiling of tissue microarray (TMA) distinguished liver cancer from normal tissues using MALDI imaging.

A liver TMA purchased by BioChain consisting of 2 tumor tissue cores and one normal tissue core from 16 patients was imaged (200 μm raster). The H&E (a) provides the TMA location (red letters and numbers) and classifies whether the row is tumor (green bar) or non-tumor (red bar). $m/z = 2393.95$ (c) and $m/z = 1743.64$ (d) were able to distinguish between hepatocellular carcinoma and uninvolved liver tissue. An overlay of these ion demonstrates that $m/z = 2393.95$ is elevated in tumor tissue and $m/z = 1743.64$ is elevated in normal tissue (b). Reproduced with permission from [95].

Figure 2. N-glycan profiling of a large cohort revealed the differential glycan expressions between CRC patients and healthy people using HILIC-UPLC.
(a) A representative chromatogram from human plasma N-glycome and peak assignments from the CRC cohort. Significant peaks (found on training set of 625 patients vs. 468 control) are colored in red (increased in CRC) and blue (decreased in CRC). ‘*’ indicates one of the top five peak abundance changes (i.e. lowest p-value). (b) Significant peaks are marked decreased (blue) or increased (red) in all CRC and four stages of CRC. Reproduced with permission from [119].

Figure 3. Differentiation of gastric cancer and healthy control groups using O-glycan profiles. (a) MS spectra (average of retention time ranging from 19.14–20.88 min) of O-glycans from serum of normal controls (upper panel) and gastric cancer patients (lower panel). Proposed O-glycan structures were based on LC-MS/MS and the biosynthesis pathway of glycobiology. (Red triangle, fucose; yellow circle, galactose; yellow square, N-acetylgalactosamine; blue square, N-acetylglucosamine; purple diamond, N-acetylneuraminic acid; PMP, 1-phenyl-3-methyl-5-pyrazolone; 2× and 3× indicate the number of fucose.) (b) PLS-DA score plot based on LC-MS data of O-glycans derived from gastric cancer (red circles) and healthy control (black squares) serum samples. (c) ROC analysis of the significantly altered O-glycans in the patient serum samples (AUC = 0.97). Reproduced with permission from [160].

Figure 4. Profiling of isomeric glycans derived from the blood serum of HCC and cirrhotic patients.

Extracted ion chromatograms of biantennary monosialylated branch-fucosylated glycan linkage isomers derived from (a) cirrhotic and (b) hepatocellular carcinoma patients. (c) MS/MS interpretation of biantennary monosialylated branch-fucosylated glycan. Reproduced with permission from [52].

Figure 5. Venn diagram of N- and O-glycan biomarkers summarized from cancers that have most potential N-glycan biomarkers reported.
(a) Potential N-glycan biomarkers of top 6 cancers. **(b)** Potential O-glycan biomarkers

Table 1.

All potential N-glycan biomarkers reported in recent years.

Glycans	Breast cancer	Liver cancer	Ovarian cancer	Colorectal cancer	Lung cancer	Kidney cancer	Esophageal cancer	Pancreatic cancer	Prostate cancer	Brain cancer	Chronic Hepatitis B virus (HBV)	Chronic Hepatitis C virus (HCV)
FIN4H6S1	up [82]											
FIN5H5F1S2	up [82]											
FIN6H7S2	up [82]								up [145]	up [152]		
*F2N5H6S3	up [82]	up [93]	up [99,104]									
N6H7S4	up [82]		up [103]	up [119]		up [148]						
N5H6S2	down [82]	down [91]			up [38]				up [145]			
N3H5S1	down [82]	up [91,93]						up [134]				
N5H4F1 (SLe ^x)	up [79,80]											
Man3				up [118]								
Man4				up [118]								
Man5	up [83,84]	up [93]	down [104]	up [118] down (IgG) [120]								
Man6	up [83]		down [104]		up [129]	up [148]			down [146]			
Man7			down [104]		up [129]				down [146]			
Man8		down [95,96]	down [104] up [107]		up [129]				down [146]			
Man9	up [81]				up [129]	up [148]		up [134]	down [146]			
FIN4H4F1	up [84]											
FIN4H5F1	up [84]											
FIN5H5F1	up [84]	up [92]										
FIN4H5F1S1	up [84]	up [97]										
FIN5H6F1	up [84]		up [99]									
FIN4H5S1(3)	down [85]											
FIN2H3	down [85]											
N6H4F1	up [80]											

Glycans	Breast cancer	Liver cancer	Ovarian cancer	Colorectal cancer	Lung cancer	Kidney cancer	Esophageal cancer	Pancreatic cancer	Prostate cancer	Brain cancer	Chronic Hepatitis B virus (HBV)	Chronic Hepatitis C virus (HCV)
N6H5F2	up [80]											
N4H3	down [81]				up [127]			up [134]				
FIN5H6S2		up [93]			up [128]			up [134,136]	up [145]			
FIN6H7S3		up [93]							up [145]			
N3H6S1		up [93]			down [129]							
N5H4S1 (bisecting)		up [91,93]										
FIN5H3 (bisecting)		up [93]										
N3H4S1		up [91,93]										
FIN5H4 (bisecting)		up [93]							up [145]		up [194]	
N5H4 (bisecting)		up [93]										
FIN4H3	down [81]	up [93]			up [127] up (IgG) [128]				up [145]			
FIN6H7S4		up [93]	up [103]									
N5H6 F1S3		up [93]	up [104]		up [38]							
FIN4H5S2		up [97]		down (IgG) [120]				up [136]				
FIN4H5S1		up [97]		down [119]	down [129]			up [136]				
N4H5		down [91]		up [115] down (IgG) [120]					up [145]			
N6H7S3		down [91]	up [104]		up [38]				up [145]			
*F2N4H5S2			up [99]									
N5H6			up [99]									
FIN5H6S1			up [99]					up [136]	up [145]			
FIN6H3F1											up [194]	
N6H5												up [194]
FIN6H7S1			up [99]					up [134]				
N6H7F1S3			up [104]									
N6H7F1S4			up [103,104]									
N6H7F2S4			up [104]									

Glycans	Breast cancer	Liver cancer	Ovarian cancer	Colorectal cancer	Lung cancer	Kidney cancer	Esophageal cancer	Pancreatic cancer	Prostate cancer	Brain cancer	Chronic Hepatitis B virus (HBV)	Chronic Hepatitis C virus (HCV)
N6H7F3S4			up [104]									
FIN4H5	up [81]		up [106]	down [115,119]	down [127,128]		down [149]					
N3H4				up [118]								
FIN2H2				up [118]								
FIN2H3				up [118]	up [129]							
N5H6F1				up [115]								
FIN4H4				down [115]			down [149]		down [146]			
FIN3H4S1				down (IgG) [120]				up [134]				
N4H5S1				down (IgG) [120]	down [129]				up [146]			
N5H3 (bisecting)				up [117]								
FIN5H4S1 (bisecting)				up [117]								
N4H5F1S2					up [38]							
N6H6S2 (bisecting)					up [38]							
N6H7		up [95,96]			up [38]							
FIN6H6S3 (bisecting)					up [38]							
N6H7S2					up [38]				up [145]			
FIN5H6S3					up [38]			up [134,136]				
*FIN5H6					down [127]							up [194]
N5H5 (bisecting)					down [128]						up [194]	
FIN5H5 (bisecting)					down [128]							
N4H4S1					down [129]							
N4H5S2					down [129]				down [145]			
N3H5					down [129]							up [194]
N4H4								up [134]				
*FIN3H6								up [134]				
N3H7								up [134]				
N3H6S1								up [134]				

Glycans	Breast cancer	Liver cancer	Ovarian cancer	Colorectal cancer	Lung cancer	Kidney cancer	Esophageal cancer	Pancreatic cancer	Prostate cancer	Brain cancer	Chronic Hepatitis B virus (HBV)	Chronic Hepatitis C virus (HCV)
FIN3H7								up [134]				
FIN4H4S1								up [134]				
*FIN4H4F2								up [134]				
*FIN5H3S1								up [134]				
*FIN5H4F1								up [134]				
*FIN5H4F1S1								up [134]				
*FIN5H5S2								up [134]				
N5H7S2								up [134]				
FIN5H8S1								up [134]				
N5H4S1								up [134]				
N6H6S3								up [134]				
N5H5S1 (bisecting)									down [145]			
*F2N4H7S1									down [146]			
N5H6S3										up [152]		
N5H6S4										up [152]		

Note: Nomenclature of glycans: N, N-acetylhexosamine; H, hexoase; F, fucose; and S, sialic acid; numbers denote the number of relative sugar. When F is before N, it is core fucosylation; when F is after N, it is branch fucosylation; *F denotes that the position of fucose was not designated in the corresponding paper. e.g. F1N6H7F2S4 represents the tetra-antennary, tetra-sialylated, tri-fucosylated N-glycan HexNAc6Hex7DeoxyHex3NeuAc4, which has a core fucose and two branch fucoses.

N-glycan biomarker selection criteria: we only summarized N-glycans which had been proved to be the possible biomarkers of diseases through ROC analysis in a big cohort or N-glycans with most significant alterations discussed in the paper (for those glycans without ROC analysis in small cohort such as cell lines).

Table 2.

All potential O-glycan biomarkers reported in recent years.

Glycans	Pancreatic cancer	gastric cancer
H1N2F2		down [160]
H1N3		up [160]
H1N1		up [160]
H3N3		up [160]
H2N3S1F1		up [160]
H5N3		up [160]
H1N2		up [160]
H3N4F3		up [160]
H1N1S1		up [160]
H1N1S2		down [160]
H2N1F1S1 (SLe ^a)	up [162]	up [162]
H2N1 + SO ₃	up [161]	up [161]
H2N1F1 + SO ₃	up [161]	up [161]
H1N2 + SO ₃	up [161]	up [161]
H3N2 + SO ₃	up [161]	up [161]
H3N2F1 + SO ₃ ⁻	up [161]	up [161]
H3N2F2 + SO ₃	up [161]	up [161]
H2N2F1 + SO ₃	up [161]	up [161]
H2N2F2 + SO ₃ ⁻	up [161]	up [161]
H3N2dF1 + SO ₃	up [161]	up [161]
H3N2F2 + SO ₃	up [161]	up [161]

Note: Nomenclature of glycans: N, N-acetylhexosamine; H, hexoase; F, fucose; and S, sialic acid; numbers denote the number of relative sugar. e.g. H2N3S1F1 represents the O-glycan HexNAc3Hex2DeoxyHex1NeuAc1.

O-glycan biomarker selection criteria: we only summarized O-glycans which had been proved to be the possible biomarkers of diseases through ROC analysis in a big cohort or O-glycans with most significant alterations discussed in the paper (for those glycans without ROC analysis in small cohort such as cell lines).

Table 3.

All potential glycan isomer biomarkers reported in recent years.

Glycans	Breast cancer	Liver cancer
N4HS1(3)	down [171]	
N4HS1(6)	up [171]	
N4HS2(3,3)	down [171]	
N4HS2(3,6)	up [15]	
N4HS2(6,6)	up [171]	
N5HS1(3)	down [171]	
N5HS1(6)	up [171]	
FIN4HS2(3,3)	down [171]	
FIN4HS2(3,6)	up [171]	
FIN4HS2(6,6)	up [15]	
FIN5HS1(3)	down [171]	
FIN5HS1(6)	up [171]	
N5H6S2(3,3)	down [171]	
N5H6S2(6,6)	up [171]	
FIN5H6S2(3,6)	up [171]	
N5H6S3(3,3,3)	down [171]	
N5H6S3(3,3,6)	down [171]	
N5H6S3(6,6,6)	up [171]	
FIN5H6S3(3,3,3)	down [171]	
FIN5H6S3(3,6,6)	up [171]	
N6H7S3(3,3,6)	down [171]	
N6H7S3(3,6,6)	up [171]	
N6H7S3(6,6,6)	up [171]	
FIN6H7S3(3,3,3)	down [171]	
FIN6H7S3(3,3,6)	up [171]	
FIN6H7S3(3,6,6)	up [171]	
FIN6H7S3(6,6,6)	up [171]	

Glycans	Breast cancer	Liver cancer
N4H5F1S1(6)		up [52]
N4H5S1(6)		up [52]

Note: Nomenclature of glycans: N, N-acetylhexosamine; H, hexoase; F, fucose; and S, sialic acid; (number) denotes the linkage; other numbers denote the number of relative sugar. In N-glycan nomenclature, when F is before N, it is core fucosylation; when F is after N, it is branch fucosylation. e.g. F1N6H7F2S4(3,6,6,6) represents the tetra-antennary, tri-fucosylated N-glycan HexNAc6Hex7DeoxyHex3NeuAc4, which has a core fucose and two branch fucoses, and the sialic acid linkages are α 2,3, α 2,6, α 2,6 and α 2,6, respectively.

Glycan isomer biomarker selection criteria: we only summarized glycan isomers with structural assignments and significant alterations discussed in the references. Isomers without assignments (e.g. isomer peak 1 and peak 2) are not included.