Breast cancer glycan biomarkers: their link to tumour cell metabolism and their perspectives in clinical practice

Tomas Bertok\textsuperscript{a,b}, Veronika Pinkova Gajdosova\textsuperscript{c}, Aniko Bertokova\textsuperscript{a}, Natalia Svecova\textsuperscript{b}, Peter Kasak\textsuperscript{c} and Jan Tkac\textsuperscript{a,b}

\textsuperscript{a}Glycanostics Ltd., Bratislava, Slovak Republic; \textsuperscript{b}Department of Glyciobiotechnology, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic; \textsuperscript{c}Center for Advanced Materials, Qatar University, Doha, Qatar

**ABSTRACT**

**Introduction:** Breast cancer (BCa) is the most common cancer type diagnosed in women and 5\textsuperscript{th} most common cause of deaths among all cancer deaths despite the fact that screening program is at place. This is why novel diagnostics approaches are needed in order to decrease number of BCa cases and disease mortality.

**Areas covered:** In this review paper, we aim to cover some basic aspects regarding cellular metabolism and signalling in BCa behind altered glycosylation. We also discuss novel exciting discoveries regarding glycan-based analysis, which can provide useful information for better understanding of the disease. The final part deals with clinical usefulness of glycan-based biomarkers and the clinical performance of such biomarkers is compared to already approved BCa biomarkers and diagnostic tools based on imaging.

**Expert opinion:** Recent discoveries suggest that glycan-based biomarkers offer high accuracy for possible BCa diagnostics in blood, but also for better monitoring and management of BCa patients. The review article was written using Web of Science search engine to include articles published between 2019 and 2021.

**1. Introduction to breast cancer and glycomics**

The review article was written using Web of Science search engine to include articles published between 2019 and 2021 using key words ‘breast cancer*’; glycomics, glycan*, glycoprotein*, glycopeptide*. The main reason behind choosing last 3 years to cover the update in the field is the fact that in 2019 and 2020 there were published two review papers partly covering the subject of this review. The other reason behind covering last 3 years in this review paper is extensive publication activity related to the topic.

**1.1. Breast cancer**

Breast cancer (BCa) is one of the most common cancer types along with lung, colorectal and prostate (only in men), with peak incidence at ages between 45 and 65 years. In 2020, 2,261,419 new female BCa cases worldwide with 684,996 new deaths (5\textsuperscript{th} most common cause of deaths out of all cancer deaths) \cite{1} were recorded. However, mortality rate can be much lower if routine screening is regularly performed for women aged above 40 years. In 2021, the incidence was predicted to increase even further to 18 women per 100,000 women globally \cite{2}. BCa is the most common cancer type diagnosed in women and is defined as a cancer that develops in breast tissue – most commonly milk ducts (~18%) and lobes (~10%) \cite{3}.

Today, screening and early diagnostics rely on imaging methods, such as digital mammography, hand-held or automated sonography and magnetic resonance imaging \cite{4}. Statistically, 1 woman in 8 is diagnosed with BCa in her lifetime. However, 5-year survival rates might be as high as 99% if BCa is diagnosed at an early stage, \textit{i.e.} for \textit{in situ} non-invasive carcinoma (~63% of all cases). BCa incidence is often highest in more developed countries (also due to awareness and screening programmes), while mortality is highest in developing countries \cite{5,6}. BCa is closely associated with genetic (especially \textit{BRCA 1} and 2 genes mutations) and other (sex, age or race – with higher mortality rate and earlier occurrence in African-Americans) risk-factors \cite{7,8}. After developing invasive (metastatic) BCa (~6% of cases at the time of diagnosis), lymph nodes in the adjacent area are often affected, although a metastasis near a head or a pancreas was also reported \cite{9}.

Breast, in both females and males, is a glandular organ made up of a fatty tissue (an adipose tissue) and a glandular tissue – usually more abundant in females. Breasts contain 12–20 lobes, which further divide into smaller lobules – these are connected \textit{via} milk ducts (Figure 1) \cite{10}. Epidermis of nipple and areola contain more pigments and, by their stimulation, the production of prolactin is enhanced. Based on the location/origin, the BCa is divided primarily into lobular or ductal (non-invasive \textit{in situ} or invasive; ductal and lobular invasive BCa being the most common cancer types) \cite{11}, medullary, mucinous (colloid), tubular and inflammatory BCa/carcinoma. Less common types include Paget’s disease, angiosarcoma or phyllodes tumour \cite{2,10}. Invasive ductal carcinoma (IDC) accounts for 60–75% of all BCa cases \cite{12}. Triple negative BCa (TNBCa,
one of the most aggressive tumours) is described by a deficiency of three surface receptors – progesterone (PR), estrogen (ER) and human epidermal growth factor 2 (HER2) [13]. An efficient drug treatment to increase the overall survival rate for TNBCa patients has long been lacking [14,15].

In the next section, we discuss changes in cell metabolic pathways and their relation to possible alteration of glycan structures as currently known. Categorical combinations of immunohistochemical markers (the three above-mentioned receptors and Ki67) classify patients to five subtypes (Figure 1) [16]. An immunohistochemical marker score in combination with quantitative measures of these four markers provide a better prognostic value for estimation of a 10-year survival rate of BCa patients [17]. It has recently been shown that a tumour-immune microenvironment as well as levels of IgG heavy chain-encoding RNAs can be associated with a poor
prognosis of an early BCa [18,19]. An overview of different BCa subtypes with their characteristics is given in Figure 1.

There are many factors causing the development of BCa, including environmental factors leading to DNA damage and hereditary factors (patients with a family history of breast or ovarian cancers are at higher risk). For instance, defects in RAS/MEK/ERK or PI3K/AKT/mTOR (hyperactivation, modulated by estrogen and HER2 receptors) signalling pathways can lead to a cell’s inability to load its “apoptotic script” [20]. In addition, the malfunction of immune responses towards abnormal cells can lead to tumour development. Due to a lack of specific biomarkers, BCa is quite often over-treated, such as in cases of ductal in situ BCa (DCIS), while it was recently shown that in most cases DCIS in patients with several lesions would never progress to invasive cancer, so to distinguish DCIS patients that may be left untreated is important [21].

Genetic predispositions to BCa significantly affect not only screening but also follow-up recommendations. Patients with a family history very often exhibit a mutation in genes such as BRCA1, BRCA2, PTEN (85% lifetime risk), PT53, CDH1 and STK1 (highly penetrant) or CHEK2, BRIP1, ATM and PALB2 (moderately penetrant) genes [22]. However, magnetic resonance-imaging is still the most sensitive and accessible procedure used for BCa diagnostics. Similarly to prostate’s MRI PI-RADS system, BI-RADS is being used as a tool to make a decision as to whether to proceed further with a breast biopsy (with BI-RADS 3 and above, biopsy is usually considered, with 5 being the highest score). For different results of biopsy concordant/discordant benign/malignant (i.e., depending on whether the lesion is thought to be benign/malignant prior to the core needle biopsy result), different follow-ups (short-term or annual) are usually needed [23]. If biochemical markers are used for the diagnostics/prognostics of BCa, very often a multiplexed format of analysis is required [24], like one recently published, to achieve high assay accuracy [25].

1.2. Glycomics

Carbohydrates are the most common type of biomolecules on Earth and are ubiquitous for the most common enzymatic post-translational modification of proteins called glycosylation [26]. Complex carbohydrates – glycans, are a part of many glycoconjugates, such as glycopolypeptides and glycoproteins, peptidoglycans, glycolipids, lipopolysaccharides, glycosaminoglycans and other glycosides (Figure 2). Most common glycosylation is N- and O-glycosylation, where glycan part is attached to Asn-X-Ser/Thr or Ser/Thr residues, respectively, but there are less abundant glycans such as C-glycans (C-C bond via tryptophan) and the quite unusual S-glycans (C-S bond via cysteine) [27]. Changes in glycan structure and composition caused by different expression of glycan-modifying enzymes, i.e. glycosyltransferases ("writers") and glycosidases ("erasers") [28], are associated with pathological processes – including cancer, promoting cell proliferation, angiogenesis, epithelial-to-mesenchymal transition, migration or extravasation [29,30]. Aberrant glycans are present during development and progression of cancer (metastases) and might lead to chemoresistant cell lines [26]. While aberrant glycans of some membrane-bound glycoconjugates may directly serve as receptors for some viral hemagglutinins (Influenza virus), membrane-associated mucins create a physical barrier and also a "decoy" for the pathogens to protect epithelial cells [26].

Glycosylation is the most common co- and post-translational modification of proteins, while glycans are also present in glycolipids or even in RNA, as shown recently [31]. Glycans mediate a wide range of interactions in living systems and are associated with cancer progression [27]. Glycan analysis lagged far behind the progress in genomics or proteomics due to long-term lack of technology and, even now, glycan analysis largely relies on a release of glycans from the glycoconjugate with subsequent separation and analysis by mass spectrometry (MS) – which is expensive, time-consuming and requires highly qualified professionals to operate [32]. Another
very frequent technique applied in glycan analysis is ultra-performance liquid chromatography, applicable for highly automated and high throughput analysis. 2 F-to obtain high resolution for separation of N-glycans after their release from glycoproteins [33,34]. N-glycans released from glycoproteins can be also detected using highly ultrasensitive method (attomole level) based on combination of a capillary electrophoresis-electrospray ionisation mass spectrometry, allowing linkage specific derivatisation of sialic acid with a small volume of sample required [35]. Since BCa is a highly heterogenous disease, analysis of glycan composition of different glycoconjugates might be more informative when analysing intact glycopeptides obtained prior MS analysis – yielding important information about individual glycosylation sites on multiglycosylated glycoproteins. In case of mannos receptor with 7 glycosylation sites, 26 intact glycopeptides were characterised, whereas 11 of these could be used to distinguish BCa subtype [36]. Pyruvate kinase M2 (PKM2) is also a potential cancer biomarker, upregulated in most cancer cells, playing an important role in tumorigenesis [37]. Furthermore, analysis of intact glycopeptides of BCa cell lines vs. PKM2 knockout cells revealed numerous changes in N-glycopeptide pattern [38].

As for now, accurate intact glycopeptide identification remains a barrier for thorough understanding of the role of glycosylation in pathological processes associated with cancer progression and development. The common state-of-the-art involves protein extraction, denaturation/reduction/alkylation, trypsin digestion, (zwiterionic) HILIC enrichment and analysis using MS techniques [39,40].

For BCa, changes in gene expressions were associated with early onset of the disease and even affected the survival rate, thereby providing a prognostic value, as in the case of glycolipids and B3GNT5 and UGCG genes for ductal in situ carcinoma [41]. Mannosidase-expressing genes were shown to play an important role in correct trafficking of the sodium iodide symporter to the plasma membrane in BCa cell lines [42], while N-glycosylated SGK196 O-mannose kinase was shown to suppress cell migration and invasion [43]. In addition, structures such as β1,6-branched, core fucose [44], glycosaminoglycans and gangliosides may significantly increase the epithelial-to-mesenchymal transition (EMT) and thus promote the formation of metastases, which makes such structures of a high prognostic value and a target for the development of cancer vaccines and monoclonal antibodies-based therapeutics [45]. Glycosylation changes can also be used to diagnose BCa using common plasma or membrane markers; also extracellular vesicles bearing aberrant glycoforms can correlate with cancer progression, as recently described for TNBCa [46].

Glycosylation changes on secreted and membrane proteins, such as under-glycosylation of O-glycans on the mucin-1 (MUC1) protein, and their alternative analysis in situ using various lectins, have long been proposed [47] for BCa detection. A particular aberrant glycan structure might perform different roles in cancer. For example, an overexpressed sTn antigen is correlated with a reduced survival for gastric carcinoma patients, while BCa patients expressing sTn antigen have a dramatically improved prognosis over those BCa patients without the expression of sTn antigen [48]. In most BCa cases, the dominant O-linked glycans are linear, short sialylated glycans based on core 1, whereas in the normal breast, epithelial cells branched core 2 glycans are found exclusively [32,49]. The exception from this statement is the core 2 glycans, which are found in TNBCa patients [50] and are believed to be involved in BCa metastases [32]; such glycan structures can be effectively recognised by Galectin-3 (Gal-3). Synthesis of aberrant O-glycans takes place especially in the absence of the proper function of Cosmc chaperone [51,52]. MUC1 protein with aberrant O-glycans can stabilise EGFR by protecting the receptor from ubiquitination [50].

There is no doubt that a great potential lies in analysing these aberrant glycan structures in order to enhance the accuracy of cancer detection, as well as to provide an insight into early onset of the disease and for further diagnostic/prognostic applications. However, one important limitation in BCa glycan-based research is worth mentioning, i.e. having access to the samples necessary for these kinds of studies. Samples are usually obtained from specialised institutions known as biobanks. According to the OECD, a biobank is "a collection of biological material and the associated data and information stored in an organised system, for a population or a large subset of a population" [53]. The biological material includes fixed or stabilised and properly stored specimens (paraffin-embedded specimens, frozen tissues or bodily fluids, for example). However, as proposed in a recent study by Krieger and Jahn, social data gaps in sample documentation should be taken into account and eliminated in order to address cancer inequities (socioeconomic, sociodemographic and geographic data) [54].

### 1.3. Metabolic pathways leading to aberrant glycosylation in BCa

Since glycosylation is an enzymatic process involving transferases and hydrolytic enzymes, their changed activity and/or substrate enzyme availability, respectively, can significantly affect the final glycan structures present on various glycoconjugates. The simplest case involves the so-called Warburg effect (described by Otto Warburg) – an increased production of lactic acid under anaerobic conditions, which leads to an acidic tumour microenvironment, a chemical signature of tumours [55], including BCa [56]. The cell microenvironment including pH value was previously described as affecting the rates of production of recombinant proteins and their glycosylation, including antibodies [57–59]. In lactogenic cancers, lactate is the molecule involved in angiogenesis, immune escape, cell-migration, metastasis and metabolism, as it provides an important energy source within and among cancer cells. As a main product of glycolysis, it depends on a variety of steps typical of these lactogenic cancers, such as increased glucose uptake, increased expression of glycolytic enzymes and decreased mitochondrial function (the less CO2 produced means a higher intracellular content of carbon fuel) and upregulation of monocarboxylate transporters [60].

The MDA-MB-231 cell line was shown to be more active than MCF-7 when facing an external perturbation [61]. Besides the altered citrate cycle, alanine, aspartate, glutamate, D-glutamine and D-glutamate metabolisms were also affected by acidosis and lactic acidosis, respectively, while different
metabolite concentrations were significantly changed at pH 6.5 (±10 mmol/L lactate), e.g. inositol, (oxo)proline, alanine and some other amino acids, as well as citric acid, palmitate, stearic acid, glycerol-3-P or urea [61]. Tumour acidity, moreover, increases the release of exosomes (extracellular nanoparticles) for breast and other cancer types [62]. In addition, an increased cellular uptake of exosomes in a gastric cancer cell line [20], affecting the tumour’s metastatic potential and presence of various glycoforms of exosomal glycoproteins [63,64], is influenced by low pH. The most important steps of cellular glucose metabolism and their consequences leading to the emergence of tumour molecular fingerprints affecting the tumour microenvironment are summarised in Figure 3.

Another branch of glucose metabolism links the aberrant glycosylation to metabolic pathways, namely hexosamine biosynthesis. The product of this pathway, UDP-GlcNAc is also a donor substrate for some reactions catalysed by glycosyltransferases. As shown by Lucena et al., despite an increased glucose uptake in cancer cells, glucose is shunted through hexosamine biosynthesis rather than by an increased glycolysis and pentose phosphate pathway (PPP) for supporting cells with NADPH for lipid synthesis and elimination of reactive oxygen species (ROS) [65,66]. Wild-type p53, for example, negatively affects PPP as well as mTOR (mammalian target of rapamycin regulating cell growth and proliferation during PI3K cell survival pathway), which regulate fatty acid synthesis and also synthesis of a dolichol carrier – a molecule important in the N-glycan biosynthesis [67]. It is worth noting that glycosylation of pyruvate kinase muscle isoform-2 and phosphofructokinase 1 can reroute the glycolysis more towards PPP [68]. The activity of the rate-limiting enzyme of PPP – glucose-6-phosphate dehydrogenase is dynamically modified by O-linked β-GlcNAc in response to hypoxia, which also provides the precursors for nucleotide synthesis in rapidly dividing cancer cells [69].

The hexosamine biosynthesis shunt referred to above also causes aberrant O-GlcNAcylation for some cell surface glycans to display more α,2,6-bound sialic acid, poly-LacNAc and fucose content (up-regulated FUT8 for instance leads to changed TGF-β core fucosylation and increased migratory abilities of BCA cell lines such as MCF-10, MDA-MB-231, Hs578T and T47D) [70–72]. It was also demonstrated that O-GlcNAc modification of chromatin modifier metastasis-associated protein 1 promotes interaction of this protein with chromatin with subsequent changes in the expression profile of several genes, which contribute to BCA chemoresistance [73]. This evidence with other observations that many chromatic binding proteins contain O-GlcNAc points to the fact that O-GlcNAc modification might be behind modulation of chromatin activity and function [73].

FUT8 also affects BCA cell migration by core fucosylation of E-cadherin (an epithelial marker) [74,75] and core fucosylation of B7 homologue 3 protein, which suppresses the immune response in TNBCa [76]. These changes are, moreover,
important for the cancer cell during EMT [77]. EMT can also be effectively perturbed by hyaluronic acid, which reduces cellular proliferation and invasion in BCA [78].

In contrast, the bisecting GlcNAc clearly inhibited the EMT in hypoxic tumour cells (BCa cell lines MCF7 and MDA-MB-231), where loss of the bisecting GlcNAc correlated with down-regulated expression of the enzyme MGAT3 and its overexpression suppressed cell migration. These combined results showed the importance of the individual monosaccharides present in oligosaccharide glycan structures being linked to the proper positions [79]. A decrease in the bisecting GlcNAc and down-regulation of MGAT3 was also confirmed in BCA in another independent study using three different cell lines (MCF7, MDA-MB-231 and SK-BR-3) [80]. This outcome was achieved using MS-based analysis and a lectin microarray technique and the study concludes that EGFR protein is affected by such a glycosylation change and that the bisecting N-GlcNAc (recognised by Phaseolus vulgaris phytohemagglutinin (PHA-E)) on EGFR inhibits the malignant BCA phenotype via down-regulation of EGFR/Erk signalling [80].

In addition, increased β-1,6-branched N-glycosylation (β-1,6-GlcNAc due to increased activity of N-acetylglucosaminyl-transferase V, GnT-V or MGAT5) affects the biological function of CD147/basigin – a tumour-associated transmembrane immunoglobulin enhancing lactate export in cancer cells [81]. It was noted elsewhere that changes in mucin type O-glycosylation, catalysed by the enzymes from an N-acetylgalactosaminyltransferase family, can promote cancer cell invasiveness since it is greatly overexpressed in BCA [71,82,83]. O-GalNAc glycosylation is highly regulated by the shuttling of GALNTs between Golgi and endoplasmic reticulum (also denoted as the GALA pathway) [84].

BCa is associated with an overexpression of sialyltransferases, like ST3Gal6, which is responsible for an increased synthesis of the selectin ligand sLeα, associated with the severity of BCa [85] and with increased homing to the bone marrow [48]. Sialyltransferases are also involved in BCa metastases [86] and an increased level of α2,6-sialic acid was observed in TNBCa patients [87]. Upregulation of sialyltransferase ST3Gal1 is also associated with a bad prognosis for BCa patients with voncin being affected by such enzymatic activity [82]. In the MCF-7 cell line 80% of voncin O-glycans contain sialyl-T and disialyl-T (with α2,3-linkage) antigens, which reduce its binding to TGFβ1 [82].

Despite N-glycosylation being associated with EMT and BCA metastasis, the glycosylation pathway was not successfully targeted previously. Fluvastatin was shown to reduce the above-mentioned branching of N-glycans via inhibition of the mevalonate pathway. Moreover, induced tumour cell death has been associated with the loss of GlcNAc transferases MGAT1 and MGAT5 [88]. Accordingly, the mevalonate pathway, leading to the production of many important metabolites, including cholesterol and dolichol (important in synthesis of the N-glycan precursor), can be considered as a novel metabolic target in cancer therapy, affecting the glycosylation process and subsequently the glycoprofile of glycoproteins involved in BCA cellular processes [89]. In general, there are some glycan alterations which are considered to exhibit a stimulatory effect on all receptors (such as core fucosylation and β-1,6-branching), while others have the opposite effect. Higher intracellular glucose concentrations, leading to increased Golgi levels of UDP-GlcNAc and MGAT5 activity stimulating β-1,6-branching, cause an increased synthesis of Gal-3 binding sites (galectins are a group of highly conserved lectins of three basic types – prototype, tandem and chimeric). They all bind to LacNAc-based N- and O-glycans and play an important role in various signalling pathways, including apoptosis [90–92], potentiating various signalling downstreams, such as an epidermal growth factor receptor-based one. Mutations of the so-called ERBB family (including HER2) indicate a causative role in some cancer types, thus making them a potential target for immunotherapy, such as a monoclonal antibody Trastuzumab (Herceptin)-based one [93].

The altered expression of different glycan-processing enzymes caused by various factors may also change the response to cancer therapy. A SK-BR-3 BCa cell line was shown to be more sensitive towards the chemotherapeutic agent doxorubicin after the cells were exposed to tunicamycin – an inhibitor of N-glycosylation. Moreover, the binding of Trastuzumab to HER2 is also finely tuned by the level of protein surface glycosylation, as indicated using a quartz crystal microbalance [94]. Among others, core fucosylation in this case seems to be of significance. In TNBCa patients not responding to anti-PD1 (anti-programmed cell death 1) due to modulation of B7H3 glycosylation, a combination of 2F-fucose (a fucosylation inhibitor) and anti-PD1 enhances the therapeutic efficacy of B7H3-positive TNBCa tumours [95]. This is also caused by the fact that the inhibition of core fucosylation (catalysed by FUT8) promotes the anti-tumour response of T-cells due to lower cell surface PD-1 expression [96]. The role of various fucosyltransferases is well summarised in a review paper [97].

An important area for future glycomic research may also include identifying a link between epigenetics and glycosylation in cancer. The DNA-methyltransferase inhibitor 5-AZA-dC (FDA approved) was shown not only to be potentially harmful for patients suffering from a chemo-sensitive cancer (ovarian and TNBCa), but also to be associated with aberrant glycosylation. Greville et al. proposed paying attention specifically to ST3Gal4 and MGAT5 (affected by GATA2 and GATA3 transcription factors), responsible for sialylation and branching, respectively [98]. Moreover, the overexpression of MGAT5, responsible for increased branching of N-glycans, was shown to be associated with mis-/relocation of E-cadherin from the cell membrane to cytoplasm, causing a reduction in cell-cell adhesion and acquiring an invasive cell phenotype [99].

2. Glycans as BCa biomarkers

The human glycome forms a crucial part in cancer research, since targeting cancer-related glycosylation changes has become a potential diagnostic and therapeutic approach [100]. There are two main mechanisms behind the changed glycosylation associated with cancer, i.e. due to incomplete glycan synthesis or due to neo-synthesis [32], and changed glycosylation is a result of several biological events/changes [101]:
- Expression levels of glycosyltransferases (GTs);
- Localisation of GTs in the cellular compartments (i.e. nucleus and mitochondria);
- Expression of chaperones responsible for proper folding of glycoproteins and GTs;
- Site- and protein-specific enzymatic preference of GTs;
- Expression levels of glycosidases during glycoprotein processing;
- Expression levels of hydrolases in lysosomes and cellular secretions;
- Availability of protein substrates;
- Availability, level and activity of activated nucleotide saccharides;
- Activity of monosaccharide transporters;
- Glycoprotein turnover kinetics;
- pH of endoplasmic reticulum (ER) and Golgi apparatus;
- Competition reactions between GTs for similar glycan acceptors.

2.1. Glycan analysis methods

2.1.1. Mass spectrometry (MS)-based glycan analysis methods

In general, in MS-based experiments, glycans are chemically or enzymatically released from purified proteins, cells or from serum, where the most common enzyme for such treatment is a peptide-N-glycosidase F (PNGase F) [102]. After this treatment, glycans are purified by using various types of columns and are derivatised via permethylation or acetylation to obtain a uniform ionisation using mass-spectrometric techniques [103].

MALDI-TOF MS is a powerful analytical platform in clinical proteomics for glycan detection in blood and serum samples. MS in oncology was first used by Petricoin [104] and his group, reporting high sensitivity (100%) and specificity (95%) for the detection of ovarian cancer. This technique is deployed for the discovery of various biomarkers applicable to the diagnostics of several cancer types and to tissue imaging [105]. The majority of the FDA-approved clinically used tumour biomarkers for BCa are glycosylated proteins. For instance, for identification of single glycoprotein cancer biomarkers from depleted serum, 2D differential gel electrophoresis together with MALDI-TOF/TOF MS was used. In this experiment, increased levels of pro-apolipoprotein A1, transferrin, and hemoglobin in BCa were detected, whereas control samples showed decreased levels of apolipoprotein A1, apolipoprotein C-III, and α-2-haptoglobin [106]. The potential of N-glycans as prognostic markers has been confirmed based on the relationship between N-glycan expression and BCa prognosis [107].

2.1.2. Lectin-based glycan analysis methods

A complementary approach to the analysis of potential glycan biomarkers is based on the application of lectins or agglutinins (i.e. glycan-recognising proteins). Lectin microarrays were developed in 2005 when several scientific groups performed multiple glycoprotein analyses [108]. Lectins are (glyco)proteins originating from diverse biological sources including animals, plants, viruses, bacteria and fungi able to recognise and reversibly bind to specific free glycan moieties or glycans attached to proteins or lipids [109,110]. In lectin-based methods, their ability to recognise glycans is used in the identification of aberrant glycosylation patterns, which are then used to study differences in the glycosylation between cancerous and control samples [103]. The glycan-discriminating power of lectins can be integrated with various detection techniques in a form of lectin blots, lectin-based histochemistry/cytochemistry, lectin-antibody sandwich-based assay using an enzyme-linked immunosorbent assay (ELISA), lectin affinity chromatography, lectin microarrays, lectin surface plasmon resonance, etc [111]. The advantage of lectin-based techniques over MS analysis is the luxury of studying multiple lectin-glycan interactions in a single experiment and the fact that there is no need for glycan release and derivatisation prior to the measurement, and lectins can be applied to the glycoprofiling of intact glycoproteins or even cells [112].

2.2. Exciting discoveries related to glycomics in BCa

BCa is a heterogeneous disease associated with several subtypes having different histological patterns and biological characteristics [113]. Furthermore, the response of BCa patients to treatment depends on the BCa subtype, hence different treatment regimens should be applied to BCa patients. Accordingly, it is obvious that, for the BCa biomarker discovery, different subtypes of BCa should be taken into account to identify serological glycan-based biomarkers to discriminate such BCa subtypes. At the same time, we should also aim to find glycan-based BCa biomarkers common to all BCa subtypes, since such biomarkers could be then applied to diagnostics and prognostic purposes or for disease progression monitoring. It is also important to understand the association between glycans and several BCa-related phenomena including chemo-resistance, disease recurrence, brain metastases, hypoxia, production of exosomes, cell cultivation conditions (2D vs. 3D cell cultivation), etc. What is new in these directions? We try to answer this question in the following sub-chapters with some additional information related to the involvement of glycans in BCa.

2.2.1. Glycans in different BCa subtypes

Vreker with his group [113] chemically derivatised enzymatically-released N-glycans to determine the linkage-specificity of sialic acids present in glycans isolated from serum samples. They found three differences between healthy controls and BCa serum samples: a lower level of two triantennary glycans and a higher level of one tetraantennary glycan in BCa patients (Figure 4). Specifically, a lower level of a fucosylated triantennary glycan that carries three α2-3-linked sialic acids (H6N5F1L3) and a non-fucosylated triantennary glycan that carries a combination of α2-3-linked and α2-6-linked sialic acids (H6N5L2E1) was revealed in BCa patients when compared to the control samples. By contrast, a fucosylated tetraantennary glycan that carries a combination of α2-3-linked and α2-6-linked sialic acids (H7N6F1L1E3) was significantly reduced in the samples from BCa patients. The results of the study focused on investigation of glycan changes associated with different BCa subtypes or stages are summarised in Table 1. The study concluded: A distinguishing signature for BCa was not found, although a significant difference between both groups was observed.
An evaluation of literature, together with the results of the current study, does not converge into a general breast cancer N-glycomic signature that distinguishes cases from controls.\textsuperscript{[113]}

Another study also focused on identification of the changes associated with different BCa subtypes. In that case, mannose receptors were enriched from the serum samples using immune-capture \textsuperscript{[114]}. The proteins isolated were then digested on gel and were analysed using LC-MS/MS. The results showed the presence of 7 glycosylation sites and 12 glycan types corresponding to 26 intact glycopeptides in the four BCa subtypes (luminal A, luminal B, HER2+ and TNBCa). Among the glycopeptides, 11 glycopeptides can be used to differentiate BCa subtypes of BCa, which further supported the previous conclusion that a mannose receptor can be used as a potential marker for the identification of BCa subtypes (Figures 5 and 6) \textsuperscript{[114]}.

\textit{N}-glycomic signature analysis was performed using different BCa subtypes taking into account HR, HER2, and N stage. The level of 25 glycan biomarkers were decreased in healthy controls, when compared with the BCa subtypes (Table 2). In particular, glycan (m/z = 1793.636) differentiated HR+/HER2- from the control and glycans with m/
Table 1. Glycans applicable for discrimination of specific BCa subtypes and stages. This is an open access article distributed under the terms of the Creative Commons CC BY license from ref [113].

| Glycans                | Cases vs. HC | Lobular vs. HC | ER+ vs. HC | PR+ vs. HC | Her2- vs. HC | Stage III |
|------------------------|--------------|----------------|------------|------------|--------------|----------|
| H4N5                   | ↑            | ↓              | ↑          | ↑          | ↑            | ↑        |
| H4N4F1E1               | ↑            | ↓              | ↑          | ↑          | ↑            | ↑        |
| H5N5E1                 | ↑            | ↓              | ↑          | ↑          | ↑            | ↑        |
| H5N4F1E1               | ↑            | ↓              | ↑          | ↑          | ↑            | ↑        |
| H6N5L2E1               | ↓            | ↑              | ↓          | ↑          | ↓            | ↓        |
| H6N5F1L3               | ↓            | ↓              | ↑          | ↓          | ↑            | ↓        |
| H6N5F2L2E1             | ↑            | ↓              | ↑          | ↓          | ↑            | ↓        |
| H7N6F1L2E2             | ↑            | ↓              | ↑          | ↓          | ↑            | ↓        |
| H7N6F1L1E2             | ↑            | ↓              | ↑          | ↓          | ↑            | ↓        |
| H7N6F1L1E3             | ↑            | ↓              | ↑          | ↓          | ↑            | ↓        |
| H7N6F2L2E2             | ↑            | ↓              | ↑          | ↓          | ↑            | ↓        |
| H7N6F2L1E2             | ↑            | ↓              | ↑          | ↓          | ↑            | ↓        |
| H7N6F2L3E1             | ↑            | ↓              | ↑          | ↓          | ↑            | ↓        |
| H7N6F2L2E2             | ↑            | ↓              | ↑          | ↓          | ↑            | ↓        |

HC: healthy control.

\[ z = 1622.550 \] (a hybrid glycan H5N3F1), 1704.585 (a complex/hybrid glycan), and 1793.636 (a complex/hybrid glycan) showed significant differences in MALDI-TOF intensity between BCa patients and controls (Table 2) [115].

2.2.2. Glycans in chemoresistance

It can be safely assumed that glycans play a crucial role in processes involved in chemotherapy resistance development of different cancer cell lines via tumour microenvironment modulation, affecting the disease dissemination as well as its invasiveness [52,116]. This statement is supported by studies focused mainly on sialylated glycan epitopes (such as truncated distal sialylated epitopes, decreased α-2,6 or increased α-2,3 expression), such as in case of cholangiocarcinoma (where sialyltransferase inhibitor increased the sensitivity of cancer cells towards 5-fluorouracil) [117], ovary carcinoma [118], colorectal carcinoma [119], pancreatic ductal adenocarcinoma [120] and even in breast cancer mucin type O-glycans [121]. Imbalanced levels of O-GlcNAc in different cancer cell lines was also observed, while this process is quite unique and this epitope present only on cytoplasmic, mitochondrial and nuclear proteins. The donor molecule for this enzymatic process, UDP-GlcNAc, is generated by nutrient-dependent hexosamine biosynthetic pathway, controlled also by circadian signals as shown recently [122,123].

Resistance to chemotherapy is a major problem in BCa, due to disease relapse with subsequent cancer-related deaths. An integrated strategy based on a combination of transcriptomics, proteomics, glycomics and glycoproteomics was used to study the involvement of glycans and glycoproteins in BCa chemoresistance [124]. It was found that paclitaxel (PTX)-resistant MCF7 cells expressed 19 glycoproteins in a different way from a control cell line. Glycan analysis revealed a decrease in multi-antennary branching structures using MALDI-TOF/TOF-MS (Figure 7) and lectin microarray (Figure 8). The study also identified proteins bearing such glycans including members of the ERK signalling pathway [124].

Most of the 21 significantly changed glycans were denoted as multi-antennary branching structures with sialylation and fucosylation. Fifteen structures were down-regulated (i.e. sialylation, fucosylation and branching), and 6 structures were up-regulated (i.e. mannosylation) in chemoresistant cells (Figure 7). Lectin microarray-based experiments revealed significant glycans changes recognised by 7 different lectins (Figure 8). The binding of 4 lectins was stronger to the glycans present in chemoresistant cells including LEL (Lycopersicon esculentum lectin recognising poly-LacNAc), STL (Solanum tuberosum lectin recognising GlcNAc), and two fusoc binding lectins UEA-I (Ulex europaeus agglutinin) and AAL (Aleuria aurantia lectin, recognising fucose). Three lectins exhibited lower binding to glycans of chemoresistant cells including PHA-E (recognising bisecting GlcNAc), VVA (Vicia villosa agglutinin recognising Tn antigen) and DSA (Datura stramonium agglutinin, recognising multi-antennary branching structures) (Figure 8) [124].

Gal-3 is a glycan-binding lectin with a role in BCa progression due to its various functions (i.e. it regulates the availability of glycosaminoglycans [125]) and patterns of expression. The relationship between BCa prognosis and secreted Gal-3 levels was investigated during chemotherapy [126]. The BCa patients with a
Table 2. N-glycans with significantly different MALDI-TOF intensities in BCa patients and healthy controls. This is an open access article distributed under the terms of the Creative Commons Attribution License from ref [115].

| Mass (M + NA) | 1095.379 | 1136.401 | 1282.422 | 1298.441 | 1339.467 | 1542.538 | 1565.514 | 1631.580 | 1745.580 | 1751.608 | 1606.558 | 1668.610 | 1793.636 |
|---------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| BrC Strage    | Stage 1 | Stage 2-4 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 |
| BrC Subtype   | HR+/HER2+HR +/HER2-HR/HER2+HR/-HER2-N(+) N (-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) |
| Mass (M + NA) | 1907.639 | 2067.698 | 2069.711 | 1257.422 | 1419.470 | 1581.520 | 1743.573 | 1905.631 | 1444.499 | 1460.495 | 1622.550 | 1704.585 |
| BrC Strage    | Stage 1 | Stage 2-4 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 | Stage 1 |
| BrC Subtype   | HR+/HER2+HR +/HER2-HR/HER2+HR/-HER2-N(+) N (-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) | HR+/-HER2-HR/-HER2-N(-) |
chemotherapy-induced increase in extracellular Gal-3 level had a longer disease-free interval and significantly lower recurrence rate during follow-up (84 months) than patients with unchanged or decreased Gal-3 levels (Figure 9). These results indicate that plasma Gal-3 could be used as a marker for chemotherapy efficacy, especially in cases where no residual tumour is visible through imaging and as an indicator for predicting long-term patient prognosis [126].

2.2.3. Glycans in BCa recurrence
MALDI-TOF-based N-glycan analysis showed that five N-glycan markers with m/z = 1419 (a glycan terminated in 6
Figure 9. Average plasma Gal-3 levels were calculated at the time of diagnosis (pre), 2 weeks postsurgical tumor removal (Post) and after each chemotherapy cycle (CTX-1-4) in patients receiving adjuvant chemotherapy \((n = 45)\). Notice significant increase in Gal-3 levels with each chemotherapy cycle in adjuvant patients (Pre: CTX2 \(p = 0.008812\); Pre: CTX3 \(p = 0.000717\) compared to initial (Pre) levels). (d) Patients that went in remission and remained disease free over the 84-month follow up period had at least a 2-fold increase (range 2–11-fold) in plasma Gal-3 levels in response to chemotherapy. On the other hand, patients with recurrent disease did not show the same dramatic increase in plasma Gal-3 levels post-chemotherapy \((p = 0.000018)\). Error bars show ±SEM. The figure is from the open access article – ref [126].

Figure 10. Comparison of the level of N-glycans with \(m/z = 1850\) (left) and 2138 (right) for cured BCa patients (open circle) and those with recurred BCa (closed circle). Reprinted by permission from Nature from ref [127], COPYRIGHT 2020.

Figure 11. Comparison of N-glycan expressions that had significant differences \((p < 0.05\), based on nonparametric bootstrap test with pooled resampling method, after a Bejamini-Hochberg correction\) from 231BR vs. 231. The red triangle denotes the upregulation of corresponding N-glycan in 231BR. The green triangle denotes the downregulation of corresponding N-glycan in 231BR. ‘X’ denotes how many times the Y axis is amplified. For example, ‘10X’ means the Y axis of that area is amplified 10 times. Reprinted with permission from ref [129]. Copyright 2020 American Chemical Society.
mannoses), 1663 (a biantennary glycan terminated in galactose), 1688 (a triantennary glycan terminated in GlcNAc with core fucose), 1850 (a biantennary core-fucosylated glycan terminated in one galactose and one GalNAc), and 2138 (a biantennary glycan terminated in one Neu5Ac and one galactose) could distinguish BCa patients from the control group [127]. Moreover, the same five glycans could be used for the monitoring of BCa recurrence during BCa treatment. The results showed that, during monitoring of the disease, in time there were significant differences in the glycan level in the post-treatment phase, as shown for glycan with m/z = 1850 and 2138, respectively (Figure 10).

2.2.4. Glycans in brain metastases
Aberrant protein glycosylation might be a contributing factor behind metastases [128]. Hence, in order to reveal the role that N-glycans play in BCa brain metastasis, membrane cell fraction was enriched using ultracentrifugation with a final glycan analysis by LC-MS/MS [129]. Quantitative glycan analysis from each cell line was compared to MDA-MB-231BR (a brain-seeking cell line). The results indicate a higher sialylation level in MDA-MB-231BR, which indicates that increased sialylation might be behind cell invasion and the crossing of the blood-brain barrier. Highly sialylated N-glycans like H1N5H6F1NeuAc3 and H1N6H7F1NeuAc3 were overexpressed in 231BR, showing their potential as biomarkers for BCa brain metastasis (Figure 11) [129]. The presence of highly sialylated N-glycans in the MDA-MB-231BR cell line was also confirmed in two other studies [130,131].

2.2.5. Glycans in hypoxia
Two TNBCa cell lines MDA-MB-231 and MDA-MB-436 were exposed to hypoxia (0.5–2% O2) and their glycosylation profile was compared to normoxia (21% O2) [132]. The results demonstrated that, under hypoxic conditions, a disialylated triantennary N-glycan was overexpressed in the MB-231 cell line, while in the MB-436 cell line a disialylated di-antennary glycan with core fucose was underexpressed. This is in agreement with the finding that increased branching and sialylation is associated with the metastatic potential of the cells [132].

2.2.6. Glycans in exosomes
TNBCa has a poor clinical outcome and limited treatment options. Chemotherapy, while killing some cancer cells, can result in therapeutically-induced-senescent cells. This type of cells releases a significantly larger number of exosomes than non-senescent cells. A recent study sought to identify N-glycans in exosomes produced by senescent cells (induced by the chemotherapeutic agent paclitaxel) using LC combined with MS [46]. The results showed significant differences in the N-glycan profile and in the composition of glycan-processing enzymes of senescent cells and exosomes released from such cells when compared with non-senescent cells [46].

2.2.7. Glycans in 2D, 3D cell cultures and xenograft
An optimised LC-MS/MS workflow was used for the analysis of N-glycopeptides in 2D- and 3D-cultured BCa cells cultured in vitro and xenografted tumours in mice [133]. The results showed that the BCa cell line MDA-MB-231 expressed different N-glycan types when cultured in different environments. The authors identified 740 N-glycoproteins, 7229 N-glycopeptides, and 269 N-glycan compositions where each of the three groups contained some unique features (N-glycopeptides, N-glycoproteins or N-glycans). Analysis at the N-glycoprotein level revealed 177 unique N-glycopeptides for the 2D-cultured cells, while 26 and 51 unique N-glycoproteins were found for the 3D-cultured cells or tumour xenograft (Figure 12) [133]. This study suggests that the glycan changes observed using cell lines could not be directly extrapolated to glycan changes in the tissues or in the living organisms.

2.2.8. Glycan analysis in living cells
Metabolic glycoengineering is a technique used to modulate glycosylation in the cells by manipulating cellular metabolism with a potential to visualise some glycans directly in living cells (Figure 13) [134]. This approach can then be used for the imaging of particular glycans in living cells [134].

A selective visualisation of different BCa subtypes was accomplished using porous metal organic framework-based nanoparticles [135]. These particles were loaded with a carbocatalyst precursor, i.e. N-azidoacetylglactosamine-tetraacetylated (AcGalNAz) for selective incorporation on the cell membrane (Figure 14). The nanoparticle was fully loaded with the membrane from various cell lines (i.e. ZRM25, particles were covered with the membrane of MCF-7 cells or ZRM231 particles were covered with the membrane of MDA-MB-231 cells (Figure 15). Interestingly, ZRM25 particles were internalised only by MCF-7 (Luminal A subtype) and not by MDA-MB-231 cells (TNBCa cell type), and vice versa (homotypic recognition). Once the nanoparticles were internalised, a glycan precursor was released from the nanoparticles, metabolised and delivered to the membrane. An Azido group was then used for the selective conjugation with a fluorescent dye using click chemistry.

In another approach, the visualisation of a glycan on a specific membrane protein (i.e. MUC1) was made possible using two DNA probes [136]. Azido-modified sialic acid was deposited on the membrane surface by the cell metabolism from its precursor (Ac6GalNAz). A glycan probe (DBC0-GP) was then attached to the azido-modified sialic acid via click chemistry. A protein-recognising DNA aptamer probe (PP probe) was then bound to the MUC1 protein and proximity-induced hybridisation occurred between the glycan probe and the protein probe. In the next steps, gold nanoparticles were selectively deposited only onto such hybridisation sequences. In this way, DNA hybridisation was converted into a photoacoustic signal, which was used for visualisation (Figure 16). This strategy not only facilitated in situ detection of MUC1-specific glycosylation (sialylation) in mice with BCa (MCF-7 cells), but also its dynamic change during tunicamycin treatment [136].

A dual-probe approach for 5S quantification of MUC1-specific glycans (i.e. terminal galactose or N-acetylgalactosamine) was developed [137]. MUC1 was recognised using a protein probe (DNA probe + capture DNA sequence), while terminal Gal or GalNAc was oxidised by galactose oxidase forming an
Figure 12. Comparison of expression of intact (a) N-glycopeptides and (b) N-glycans identified in the 2D-, 3D-cultured BCa cells and xenografted tumors. This is an open access article distributed under the terms of the Creative Commons CC BY license from ref [133].

Figure 13. Diagnostic applications of metabolic glycoengineering, which is based on the incorporation of artificial labels into diseased tissue for bioimaging or biomarker discovery. For in vivo imaging, an animal/cell is first treated with a glycan analogue such as N-azidoacetylmannosamine tetraacetate (Ac₄ManNAz) to selectively install azide groups in tumor-associated glycans, which can be detected through one of several imaging options. Reprinted by permission from Nature from ref [134], COPYRIGHT 2019.
aldehyde group. To this aldehyde group a hydrazide-functionalised glycan probe (DNA sequence complementary to capture DNA sequence) was attached with a final hybridisation between DNA molecules present in the protein and a glycan probe (Figure 17). These assays were used for analysis of three human BCa cell lines and 32 pairs of matched BCa tissue samples. The results showed an increased amount of Gal/GalNAc on MUC1 in BCa tissues over the control tissue samples (Figure 17). BCa patients with a low level of MUC1-specific terminal Gal/GalNAc had a five-year survival rate of 87.5%, while BCa patients with a high level of MUC1-specific terminal Gal/GalNAc had a significantly lower five-year survival rate of 56.3% (Figure 18) [137].

2.2.9. Glycan visualisation of BCa tissues
MS imaging is a new and promising technique which can visualise glycans directly on tissues. This approach was used for the visualisation of HER2+ TNBCa, and metastatic BCa tissues [125] with the conclusion showing that multiple poly (LacNAc) N-glycans are present in HER2+ TNBCa and metastatic BCa. Several N-glycans could be used for the discrimination of BCa sub-types. HER2+ tissues express increased levels of tri-antennary and tetra-antennary N-glycans, while TNBCa tissues express increased levels of highly mannosylated and fucosylated (especially in the N-glycan arms) N-glycans (Figure 19) [125].

The tetra-antennary core fucosylated N-glycan with LacNAc termination was claimed to be associated with poor clinical outcomes in BCa, including lymph node metastasis, recurrent disease, and reduced survival [138]. Another study suggests that especially branched poly(LacNAc) structures are behind BCa progression [139].

An alternative approach to the visualisation of BCa tissues other than MS imaging is based on the use of lectins [140]. The authors synthesised four different laser-cleavable tags, which differed in the m/z ratio. The mass tag was conjugated with an amine reactive moiety (i.e., N-hydroxysuccinimide) through a laser-cleavable site containing sulphur. The amine reactive moiety was used for the immobilisation of a lectin. Using four different mass tags connected to lectins, four different glycan-recognition probes were prepared. These probes were incubated with tissue or cells and, after incubation, the sample was irradiated by an MS laser with released mass tags. The presence of a particular tag in the MS spectra indicated the presence of a specific glycan in the sample, hence multiplexed analysis of four glycans could be performed simultaneously (Figure 20). The novel strategy was used to evaluate glycan alterations on BCa cell line MCF-7 and its drug resistant cell line MCF-7 R to investigate the relationship between drug resistance and glycans at the single-cell level. Alternatively, the assay can be used for analysis of the glycan spatial distribution in fresh human tissues, showing glycan differences between BCa and healthy controls [140].

A novel rapid slide-based MS method for analysing released N-glycans, i.e. MALDI imaging mass spectrometry (MALDI-IMS)
Figure 15. In vitro and in vivo selective glycan labeling of breast cancer subtypes. (a, b) Fluorescence micrographs and analysis of different breast cancer subtypes treated with 50 μM ZGM_{21} or ZGM_{0}. The treated cells were labeled by Cy5-alkyne. The nuclei were stained with Hoechst (blue). Scale bars: 50 μm. Fluorescence intensity of microscopy images was measured by ImageJ software. Data are presented as mean intensity (n = 10). (c) Whole-body fluorescence images of tumor-bearing mice injected with ZGM_{21} (60 mg kg\(^{-1}\)) and ZG (60 mg kg\(^{-1}\)) for 4 days. On the fifth day, DBCO-Cy5 was administrated to the mice. The white arrows mark the position of tumors. Right flank: tumor. Left flank: MCF-7 tumor. (d) Quantification of the fluorescence signal. Error bars were calculated on the basis of three independent experiments. Asterisks indicate significant differences (\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\)). Reprinted by permission from John Wiley and Sons from ref. From ref [135], COPYRIGHT 2021.

Figure 16. Schematic of the photoacoustic imaging system used for the in situ monitoring of MUC1-specific sialic acid (Sia). Reprinted with permission from ref [136]. Copyright 2021, American Chemical Society.

was developed by Drake and his group [141]. The method is based on spraying PNGase F onto a slide surface with directly spotted biological samples. α-cyano-4-hydroxycinnamic acid (CHCA) served as a matrix for analysis of N-glycans by MALDI-IMS. In the study, pooled human serum samples from healthy human donors, control plasma samples, sera from non-obese and sera from obese patients with benign breast lesions or breast cancer were used. Additionally, core and outer fucose locations on the glycan structures were enzymatically analysed with endoglycosidase F3 (Endo F3) sprayed onto the slide. Endo F3 cleaves N-glycans between the first two N-acetylgalactosamine (GlcNAc) residues linked to asparagine, mainly when the first GlcNAc is fucosylated. This approach can be used to distinguish N-glycans containing the core fucose, in contrast to glycans with antennary fucose modifications [141].

2.2.10. Glycan visualisation on necrotic tissues

Glycan analysis can be also useful for discrimination between necrotic and other tissues (cancerous or normal) [142]. An MS-based imaging approach based on evaluation of N-glycans directly in formalin-fixed BCa tissues was used to distinguish tissue regions of tumour, stroma and necrosis. The results indicate that, while the tumour and stromal tissue regions contain especially high-mannose or branched glycans, the glycans found in the necrotic tissue regions displayed limited branching and were sialylated and with decreased fucosylation (Figure 21) [142].
2.2.11. Saliva microarrays

Saliva represents another bodily fluid which can be used for biomarker discovery. In order to investigate glycan changes in the saliva of BCa patients, saliva samples were printed on the microarray slide with an investigation of *Bandeiraea simplicifolia* lectin I (BS-I) binding to it [143]. Four different types of samples were investigated in the study – (66 healthy volunteers (HV), 65 benign breast cyst or tumour patients (BB), 66 patients with BCa stage I (BC-I) and 62 patients with BCa stage II (BC-II). In addition, the glycoproteins present in saliva samples were isolated using BS-I lectin immobilised on magnetic beads with a final glycan analysis by MALDI-TOF/TOF-MS. The expression level of galactosylated glycans recognised by BS-I was significantly increased in patients with BCa vs. HV. In total, 11/10, 10/19, 7/24 and 7/9 galactosylated N-/O-linked glycans were annotated in the samples of HV, BB, BC-I and BC-II, respectively. One galactosylated N-glycan (m/z = 2773.977), and 4 galactosylated O-glycan peaks (m/z = 868.295, 882.243, 884.270 and 1030.348) were found only in BC-I [143].

3. Clinical validation of glycans as BCa biomarkers

Clinical validation of new biomarkers is usually effected via constructing ROC, from which key clinical parameters such as AUC can be extracted and used for comparison of the clinical usefulness/importance of several traditional biomarkers with newly identified biomarkers. At the same time, ROC makes it possible to identify sensitivity, specificity, as well as cut-off values for newly discovered biomarkers. Alternatively novel biomarkers can be identified using statistical analysis such as Kruskal-Wallis or Mann-Whitney U test to evaluate differences in the glycosylation level between samples from BCa or healthy control [144]. Another way how to evaluate if addition of novel biomarker(s) to currently approved/used biomarker(s) can increase discrimination between two cohorts of samples is in a form of heat maps calculating Net reclassification improvement and/or Integrated discrimination improvement [145].

3.1. Traditional BCa diagnostics and biomarkers

Population-based BCa screening, which is performed mainly via mammography, reduces BCa mortality [113]. If BCa is diagnosed at an early stage, survival rate is very high [146]. Mammography has, however, some limitations including low sensitivity (sens.) of 68%, low specificity (spec.) of 75%, high false negative rate (4–34%), underperformance on dense breast tissues and, most importantly, that X-ray imaging might be hazardous to women due to exposure to radiation [113,147]. Moreover, there is quite a high false-
positive rate (61% chance of a false-positive result over a 10-year period [148]), leading to avoidable biopsies [146]. This is why annual mammography is not recommended [149]. Several alternative imaging-based diagnostics tools exist such as ultrasonography (sens.: 83%; spec.: 34%), magnetic resonance imaging (sens.: 94%; spec.: 26%), contrast-enhanced mammography (sens.: 85%; spec.: 66%), computer tomography (sens.: 91%; spec.: 93%), positron emission

Figure 18. Amount of MUC1-specific terminal Gal/GalNAc (a) per cell and (b) per MUC1 protein in MCF-7, T47D, and MCF-10A cells and (c) in 32 pairs of matched breast tissue samples. (d) Kaplan–Meier curves and log-rank tests of breast cancer patients in the high group (n = 16) and low group (n = 16) (log-rank, p < 0.05). Reprinted with permission from ref [137]. Copyright 2020, American Chemical Society.

Figure 19. Comparison of glycan expression with corresponding box plot comparing all area under the peak values for a) H7F1N5 with m/z = 2174.7715, b) H8F1N6 with m/z = 2539.9037, c) H9N2 (Man8) with m/z = 1743.5810, d) H10N2 (Man9) with m/z = 1905.6338, and e) H7F2N5 with m/z = 2320.8294 in representing triple-negative (top) and HER2+ (bottom) breast cancers. Reprinted with permission from John Wiley and Sons from ref [125], COPYRIGHT 2021.
tomography (sens.: 61%; spec.: 80%) and digital breast tomosynthesis (sens.: 91%; spec.: 96%) with some limitations, as summarised in two review papers [150,151]. For example, in the case of digital breast tomosynthesis, exposure to radiation dose is twice as high as in mammography [148] and computer tomography is also associated with radiation risk and costly examination [151].

Blood-based biomarkers such as CEA (carcinoembryonic antigen) and CA 15–3 (cancer antigen 15–3), CA27-29, or CA127 are either approved as BCa biomarkers or clinically used, but they cannot be used for BCa diagnostics due to low specificity and sensitivity [12]. Moreover, some of those biomarkers are not specific to BCa. Accordingly, these serological biomarkers are not recommended for BCa diagnostics, screening or for staging purposes, but rather for disease monitoring or disease recurrence. Hence, much effort is devoted to the discovery of novel serological biomarkers, which can be used for early-stage BCa diagnostics, but such discovery activities have not paid dividends to date [146].

Blood tests represent a new way of diagnostics. The detection of BCa-specific biomarkers in bodily fluids is required for early-stage accurate BCa diagnostics with the aim of achieving high specificity and sensitivity. In order to investigate the glycosylation changes of proteins associated with BCa, sensitive and robust (bio)analytical techniques have been developed, indicating that serum glycan profiles could be applied as potential BCa biomarkers. To date, it has been shown that an increase in sialylation, a difference in fucosylation, an increase in highly mannosylated glycans are typically associated with BCa [12] and these glycosylation changes could be applied as BCa biomarkers.

In this part of the review, we highlight glycan analysis based on the use of lectins and MALDI-TOF MS, which represent future prospects for robust, sensitive, and selective BCa biomarker discovery. These studies will have a huge impact on the improvement of BCa diagnostics and prognosis in the future.

### 3.2. Glycan-based biomarker discovery in tissues/cells

#### 3.2.1. MS-based glycan analysis of cells/tissue

Invasive ductal carcinoma (IDC) as the most common type of BCa was determined by (MALDI-MS)-based glycan analyses using two different derivatisation methods, i.e. 2-aminobenzoic acid (2-AA) labelling and esterification (applied to discriminate sialic acid linkages, i.e. α2,3- vs. α2,6-linked sialic acid) [12]. Forty-seven 2-AA labelled and fifty ethyl-esterified N-glycans were found. The best discrimination power IDC vs. control was revealed for high-mannose N-glycans (H5N2, H6N2, and H7N2 with AUC in the range of 0.942–0.989) and for two fucosylated N-glycan compositions (H3N3F1 and H5N5F1 with AUC in the range of 0.942–0.989). Moreover, high-mannose N-glycans were up-regulated in IDC, while bisecting N-glycans were down-regulated [12]. Another study confirmed over-expression of highly mannosylated N-glycans in BCa tissues compared to control groups with four of them displaying a strong discrimination power represented by AUC in the range from 0.908 to 0.929 [152]. A decreased sialylation could also be applied to achieve strong discrimination of BCa tissues vs. control tissues with AUC in the range of 0.904–0.955 [152].

Herrera et al. [138] also paid attention to fucosylation in BCa tissues, where a single core-fucosylated tetra-antennary N-glycan containing a single N-acetyllactosamine branch F(6)A4G4Lac1 was first confirmed as having a correlation with a poor clinical outcome for BCa patients, as it was expressed more frequently (p = 0.01) in tumours from an early disease stage (stage 1 and 2) patients in comparison with those that survived. To validate this, they performed tissue-based AAL (Aleuria aurantia lectin) lectin microarray together with MS and LC analyses. AUC values for metastatic lymph nodes showed ~2-fold higher expression level of core-fucosylated tri-antennary glycan (F(6)A3G3; p = 0.007) and core-fucosylated tetra-antennary glycan with a single polylactosamine arm (F(6)A4G4Lac1; p = 0.03) when compared to normal lymph nodes [138].

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**Figure 20.** LEFT: (a) The structure of laser cleavable probes. The reactive site containing an amine-specific moiety (α) is connected to the mass tag (γ) by a cleavage site (β). (b) The whole process of analysis of glycans using the laser cleavable probes. RIGHT: Figure 1 LDI-MS spectra of four laser cleavable probes 1–4. With the irradiation of a 355 nm laser, the C–S bond was cleaved, and the mass tags were produced for LDI-MS detection. This article is licensed under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence from ref [140].
3.2.2. Lectin-based glycan analysis of cells/tissue
Molecular and cellular characteristics behind relapse-prone TNBCa are of interest to follow. In one study, the glycoprofiling of tissues from TNBCa-relapsed patients were compared with tissues from TNBCa patients without relapse using lectin microarrays [153]. Out of 45 lectins, four lectins showed enhanced binding to glycoproteins present in the TNBCa-relapsed patients, i.e. TJA-II (Trichosanthes japonica agglutinin), ACA (Amaranthus caudatus agglutinin), WFA (Wisteria floribunda agglutinin) and BPL (Bauhinia purpurea lectin), while three lectins showed the opposite effect, i.e. NPA (Narcissus pseudonarcissus agglutinin), Con A (Concanavalin A) and GNA (Galanthus nivalis agglutinin). The authors predict that the glycan changes recognised by TJA-II, WFA and BPL are associated with O-glycans with β-linked terminal GalNAc residues [153].

3.3. Glycan-based biomarker discovery in serum
3.3.1. MS-based glycan analysis of serum
Kyselova et al. [154] analysed enzymatically released glycans which were permethylated by the capillary-based approach from serum samples from BCa patients and healthy control. The permethylation step reduced degradation of the labile and biologically significant sialylated structures and increased measurement sensitivity. The statistical analysis showed the following N-glycans to have a high diagnostic potential, as may be judged from the AUC values from ROC: N-glycan with m/z = 3864 (AUC = 0.97) and N-glycan with m/z = 2111 did not show any discrimination potential (AUC of 0.49). The study identified a total of eight N-glycans as biomarker candidates based on AUC and P values. In the glycomic profile, additions of fucosyl- and sialyl- residues were found to some
glycoprotein structures. Interestingly, all 8 N-glycans were sialylated (mono-, di-, tri-, and tetrasialylated), whereas 5 of these structures were fucosylated (2 of them di-fucosylated) [154].

Lee et al. [115] studied N-glycan profiles of healthy controls and BCa patients with 4 different BCa stages. N-linked glycans from serum samples were isolated with peptide-N-glycosidase F (PNGase F) and the subsequently released glycans were purified using HyperSep Hypercarb solid-phase extraction (SPE). N-glycan biomarkers were selected and their diagnostic potential was determined using statistical analysis. Results showed an efficient pattern recognition of invasive ductal carcinoma patients, with very high diagnostic performance with AUC of 0.93. Analysis showed promising clinical parameters such as specificity of 82.3%, sensitivity of 84.1%, and 82.8% accuracy for diagnostics of stage 1 BCa and for recognition of hormone receptor-2 and lymph node invasion-based subtypes using N-glycan profiles. This study was of interest due to the fact that the diagnostics of early-stage cancer samples showed a higher AUC than the diagnostics of later stages 2–4. This can be explained by a higher prevalence of some N-glycosylated signs found in the early BCa stages than in the terminal BCa stages.

The focus on peaks, one complex/hybrid glycan (m/z = 1444.499) and four hybrid glycans (m/z = 1460.495, 1606.558, 1622.550, and 1768.610) were detected as biomarkers for discriminating BCa stages from control sera. Apart from that, a hybrid glycan (m/z = 1622.550) and a complex/hybrid glycan (m/z = 1704.585) were used for differentiating HR-/HER2+ from healthy controls, as well as a complex/hybrid glycan (m/z = 1793.636) being applied to distinguishing HR+/HER2- from the healthy group [115].

The approach with an enzymatic release of N-glycans present in serum with subsequent glycan purification using columns packed with porous graphite carbon (PGC) was used as a novel MALDI-TOF MS platform for glycan analysis [127]. Differentiation between control and BCa samples was effected using three N-glycan markers (m/z = 1419, 1663, and 2138) identified as positive biomarkers, whose MS intensities increased in BCa, and two glycans (m/z = 1688 and 1850) as negative biomarkers. The AUC attained was 0.91 and accuracy 88.6%. Furthermore, a high-mannose-containing N-glycan (M6N2) with m/z = 1419 was found in increased levels in BCa [127].

In BCa serum analysis, a higher level of core fucosylated, non-galactosylated N-glycan (m/z = 1485.5337) was observed in the cancerous obese serum, as well as a lower level of bisected N-glycan (m/z = 1866.6608) in benign obese serum in the control [141]. A higher level of monogalactosylated N-glycan (m/z = 1501.5286) was observed in both non-obese samples, and the non-obese and obese benign samples also had a high level of the abundant fucosylated biantennary N-glycan (m/z = 1809.6393) [141].

Another approach to BCa N-glycan analysis present in serum was performed via a linear ion-trap mass spectrometry, where N-glycans were enzymatically released, purified on PGC columns and permethylated [155]. Student’s t-test revealed significant differences in 11 N-glycan structures, in which the expression was significantly (P < 0.05) higher in BCa cases than the controls, i.e. corresponding to high-mannose (m/z = 809, 1013, 1218, 1320) and fucosylated N-glycans (m/z = 815, 1040, 1127, 1228, 1352, 1409, 1453). Moreover, di-fucosylated N-glycans were revealed with peaks at m/z = 1127, 1228, 1352, 1409 and 1453 with significantly higher levels in BCa patients (P < 0.001). AUC values for the following glycans with m/z = 1127, 1228, 1352, 1409 and 1453 were as follows: 0.789, 0.854, 0.844, 0.826 and 0.764, respectively. From these results, we can conclude that a di-fucosylation degree can be used as a diagnostic marker for distinguishing between BCa and a control group. In order to increase the diagnostic potential, the comparison of BCa biomarker CEA and di-fucosylation degree was performed with ROC curve analysis. CEA level combined with glycans showed an AUC value of 0.963 in comparison to CEA alone (AUC = 0.794), which means that the diagnostic capacity of CEA could be improved by measuring the di-fucosylation level [155].

Besides total serum N-glycome analysis, immunoglobulin G (IgG) has shown the association of altered glycosylation patterns with the tumour progression in BCa also [156]. IgG as the major humoral component influences an immune response and has the potential to serve as a BCa biomarker. Kawaguchi-Sakita et al. [156] analysed serological N-glycosylation of IgG’s Fc region in BCa patients. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to gain the heavy chain fraction from the isolated IgGs. N-glycans were subsequently enzymatically released from IgGs. Seven selected N-glycans (m/z = 2471.9, 2208.8, 1863.6, 1847.6, 2516.9, 2212.7, and 2050.7) proved the AUC of 0.874 (P < 0.0001). Serum IgG Fc region of N-glycan profiling can serve as an advanced and highly robust model for distinguishing healthy controls from BCa patients [156].

A subsequent study, focused on the profiling of purified IgG and whole serum of BCa patients in Ethiopian women, was performed by Gebrehiwot et al. [157]. Affinity chromatography was used for IgG purification from serum. Both whole serum and isolated IgGs were purified and enzymatically pre-treated. Released N-glycans were captured onto hydrazide-functionalised BlotGlycoH beads via reversible hydrazone bonds. The beads with captured N-glycans were exposed to the trans-iminisation reaction with O-benzylloxycinehydrochloride with subsequent mild acid hydrolysis of a hydrazone bond (Figure 22). The released and tagged N-glycans were measured. According to the t-test, 35 N-glycans were significantly up-regulated in the whole serum samples of BCa patients relative to the controls. With AUC of 0.8–1.0, seventeen complex type N-glycans showed the diagnostic potential for the early stage (I and II) BCa. The selected glycans were mainly core-fucosylated, branched and sialylated structures (4 bisecting, 8 core fucosylated, and 12 sialylated) correlated with an invasive and a metastatic potential of cancer. Samples from stage III BCa patients contained only 2 glycans with bisected and fucosylated structures (m/z = 2261 and 2264) and samples from stage IV BCa patients showed 3 mono-sialylated glycans (m/z = 2261, 2439, 3007), all differentiating BCa patients from the controls. On the other hand, two core-fucosylated and agalactosylated N-glycans from IgG (m/z = 1591, 1794) could be useful for differentiating BCa stage II patients and healthy controls (AUC = 0.944 and 0.921, p ≤ 0.001) [157].

### 3.3.2. Lectin-based glycan analysis of serum

Fry et al. [108] worked on a glycomic analysis of metastatic BCa, based on a microarray technique consisting of 45 lectins with defined binding preferences. Serum samples with
fluorescently labelled glycoproteins were applied to the lectin arrays. In comparison with non-metastatic sera, metastatic sera proved statistically significant (P < 0.01) differences in binding to four lectins, elevated binding was observed to AOL (Aspergillus oryzae lectin recognising core fucose as well as α1-2, α1-3 and α1-4 linked fucose) and GNA (Galanthus nivalis agglutinin, recognising high-mannose N-glycans). The authors observed a decreased signal using RCA120 (Ricinus communis agglutinin), and PHA-E (Phaseolus vulgaris erythraeagglutinin) lectins for BCa samples compared to control samples. PHAE with preferred binding to galactosylated biantennary N-linked glycans with a bisecting GlcNAc with a P-value of <0.0001 showed the best discrimination between metastatic and non-metastatic sera [108].

Sera from metastatic BCa were also analysed by using galectins which specifically bind carbohydrate chains containing β-galactosides. The binding ratio (galectin1/galectin8) provided a significantly improved discrimination with an average value of 2.33 (range 0.7–4.5) and with AUC of 0.98 [158]. Another study confirmed the significant role of galectin8 in TNBCa promoting cell adhesion and the migration of MDA-MB-231 cells [159].

Another galectin-based study was performed by Jouseti with his group. They determined human Gal-3 in healthy and BCa serum samples by the ELISA method [160]. A significant increase in Gal-3 (P < 0.001) was observed in cases of BCa over healthy sera. ROC analysis revealed sensitivity of 85%, diagnostic specificity of 79.5% with the best cut-off level of Gal-3 of 2.44 ng/ml [160].

3.4. Glycan-based biomarker discovery in saliva
The combination of both techniques, lectin microarray and MALDI-TOF/TOF-MS, was used for validation of the salivary protein of BCa patients (healthy volunteers, HV), benign breast cyst or tumour patients (BB), patients with breast cancer in stage I (BCa-I) and in stage II (BCa-II) [143]. A microarray platform was performed with Cy5-labelled BS-I (Bandeiraea simplicifolia) lectin to target galactosylated structures in pooled samples from each group. For MALDI-TOF/TOF-MS, glycoproteins from pooled saliva were isolated by BS-I-magnetic particle conjugates and N-glycans were enzymatically released by PNGase F, whereas O-glycans were released by using NaClO. Purification and desalting steps were performed by Sepharose 4B microtubes. In a microarray experiment, significant differences were observed in the group of HVs vs. BCa-I (mean rank difference = 2.00, p = 0.008), HVs vs. BCa-II (mean rank difference = 2.21, p < 0.001), BB vs. BCa-I (mean rank difference = 1.51, p = 0.044) and BBs vs. BCa-II (mean rank difference = 1.67, p = 0.008). In MALDI-TOF/TOF-MS, 11/10, 10/19, 7/24 and 7/9 galactosylated N-/O-linked glycans were identified from the pooled saliva samples of HV, BB, BCa-I and BCa-II. Unique glycans present only in BCa-I were the following – one galactosylated N-glycan peak (m/z = 2773.977), and 4 galactosylated O-glycan peaks (m/z = 868.295, 882.243, 884.270 and 1030.348). These findings can be used for early-stage BCa diagnostics based on analysis of saliva samples [143].
3.5. Specific protein glycoprofiling as biomarkers

All the results presented above were based on analysis of a whole glycome either in tissues/cells, serum or saliva. Although such approaches are able to identify the glycan changes associated with BCa, usually the target proteins bearing aberrant glycans are not known. In our opinion, in order to render glycan-based biomarkers really robust, it is more important to glycoprofile specific proteins than to determine the whole glycome in samples. A good example is specific glycoprofiling of PSA applied as a prostate cancer biomarker [101,161–163] to prostate cancer diagnostics and prognosis.

3.5.1. Glycoprofiling of cancer antigen 15-3 (CA 15-3)

With a focus on BCa biomarker determination, an antibody-lectin sandwich assay was applied to detection of CA15-3 glycosylation in sera with BCa (Figure 23) [144]. In an ELISA assay format, anti-CA15-3 monoclonal antibody capturing CA15-3 in serum was immobilised, oxidised to de-activate glycans in the Fc domain and subsequently various biotinated lectins were transferred into the wells. Afterwards, the binding between lectin and glycan was detected by using poly-HRP-conjugated streptavidin. Con A lectin with the highest affinity to mannosylated N-glycans was selected due to the best signal using this platform. The sensitivity and specificity achieved for BCa stages discrimination from benign stage are as follows – BCa stage I (AUC: 0.64; sens.: 63%, spec.: 69%), IIA (AUC: 0.78; sens.: 77%, spec.: 75%), IIB (AUC: 0.79; sens.: 69%, spec.: 86%) and III (AUC: 0.75; sens.: 80%, spec.: 65%). The antibody-lectin sandwich assay platform exhibits a diagnostic potential, as this system made possible serum dilution of 1:2,000 in comparison to the current ELISA with serum dilution of 1:50–1:100 [144].

An improved approach to the detection of CA15-3 glycosylation in sera with BCa was performed via europium (III)-doped nanoparticles (Eu³⁺-NPs) coated with lectins [164]. Two antibodies recognising protein core and expressed siaalted carbohydrate epitope were tested in the study. Biotinylated antibodies were immobilised on streptavidin-coated yellow low-fluorescence microtiter wells and subsequently CA15-3 standard/samples were added and detected by using lectin-Eu³⁺-NPs as a tracer in time-resolved fluorescence. Out of a panel of 28 lectins, WGA (wheat germ agglutinin recognising terminal GlcNAc) and MGL (macrophage galactose-type lectin recognising terminal GalNAc) lectins, performed satisfactory binding. In comparison with the conventional CA15-3 immunoassay with CA15-3<sup>MGL</sup> and CA15-3<sup>WGA</sup>, CA15-3<sup>MGL</sup> and CA15-3<sup>WGA</sup> afforded the highest AUC (0.942), then CA15-3<sup>MGL</sup> (0.852) and the conventional CA15-3 immunoassay (0.828) for discrimination of metastatic BCa patients from control (Figure 24). At 90% specificity, the clinical sensitivity of the assays was 66.0%, 67.9% and 81.1% for the conventional CA15-3, CA15-3<sup>MGL</sup> and CA15-3<sup>WGA</sup> assays, respectively. The newly developed CA15-3<sup>WGA</sup> assay was capable of correctly identifying 81% of BCa patients

![Figure 23](https://example.com/figure23.png) Schematic diagram of the antibody-lectin sandwich assay. Immobilized anti-CA15-3 antibody captures CA15-3 in serum, and glycosylation of CA15-3 is detected with a biotinylated lectin followed by HRP-conjugated streptavidin. Blues boxes represent glycans of the coated anti-CA15-3 monoclonal antibody, and of FBS used as a blocking agent. The red boxes represent glycans of CA15-3. This is an open access article distributed under the terms of the Creative Commons Attribution License from ref [144].

![Figure 24](https://example.com/figure24.png) ROC plot displaying the AUC of conventional CA15-3 (green), CA15-3<sup>MGL</sup> (purple) and CA15-3<sup>WGA</sup> (red) from metastatic breast cancer patients (n = 53) and healthy control (n = 20). This is an open access article distributed under the terms of the Creative Commons Attribution License from ref [164].
with metastases, compared to 66% using a conventional CA15-3 assay, when only 10% of controls were misdiagnosed with both assays. The CA15-3MG and CA 15-3 biomarkers were used to monitor the chemotherapy success in BCa patients (analysis of samples taken before chemotherapy and after 6 weeks). The CA 15-3-based method identified 10 BCa patients (40% of studied), CA15-3MGA method 12 BCa patients (48%) and CA15-3MG 18 patients (78%) with a response to chemotherapy treatment. Hence, new CA15-3bectin assays can distinguish BCa patients better than the conventional CA15-3 analysis [164].

3.5.2. Glycoprofiling of α-1-acid glycoprotein (AGP)
Specific glycoprofiling of α-1-acid glycoprotein (AGP) was used to distinguish between different BCa stages, i.e. benign (BN) and BCa stages (BCa I), BCa IIA, BCa IIB, and BCa III using an enzyme-linked lectin assay (ELLA) [165]. AGP was extracted from serum samples using SDS PAGE and then applied to ELLA for glycan analysis. Three different types of glycosylation (fucosylation, high-mannose-type and sialylation) of AGP were associated with BCa. Terminal fucosylation and high-mannose-type glycans appeared to be the lowest in BCa I and such glycosylation changes provide high sensitivity and specificity that make BCa I clearly distinguishable from other BCa stages and BN. Hence, a decrease in AAL lectin and jacalin binding to AGP could be useful for early-stage BCa diagnostics with AUC of 0.8 (except for jacalin-based discrimination BCa I vs. BCa III) (Table 3). Moreover, using both lectins, it was possible to distinguish BCa I stage from other BCa stages [165].

3.5.3. Glycoprofiling of immunoglobulin A1 (IgA1)
Specific glycoprofiling of IgA1 as a BCa biomarker was also evaluated using serum samples [166]. The results obtained using MS analysis indicate an increase in disialo-biantennary N-glycans on IgA1 from BCa patients and increased asialo-Thomsen–Friedenreich antigen (TF) and disialo-TF antigens in O-glycans compared to control individuals. Lectin-based ELLA assays in a sandwich configuration indicated an increase in Sambucus nigra agglutinin (SNA) binding to IgA1 from BCa patients showing the presence of α2,6-sialic acid. Increased Helix pomatia agglutinin (HPA, recognising mainly GalNAc) binding to IgA1, on the other hand, could be used to predict distant metastases and tumour size [166].

3.5.4. Glycoprofiling of HER2
A disposable, electrochemical biosensor based on the conductive interface modified by a hydrogel layer was used for covalent attachment of antibodies for a specific interaction with HER2 [167]. Once the HER2 protein was bioaffinity-captured on the biosensor interface, HER2 glycosylation was analysed in-situ using lectins. The bioanalytical device was then used for analysis of HER2 glycosylation in two serum samples (one from a healthy, high BCa-risk woman and the other from a woman with a 2nd stage BCa). The results obtained using PHA-E lectin indicate that serum of the BCa patient contained a significantly higher amount of HER2 recognised by PHA-E lectin than the serum of the healthy woman. The results obtained by the biosensor device were successfully validated by ELISA-based analysis. Although this approach is very promising for possible BCa diagnostics, unfortunately only a minor fraction of serum samples contain HER2 protein [167].

4. Conclusions
The review shows that analysis of glycans can provide useful information to better understand and study a heterogeneous disease such as BCa. In the review, we provided basic information about metabolism behind BCa with consequences related to the synthesis of aberrant glycans, which could then be used as BCa biomarkers. We showed that glycan-based biomarkers could be used for the discrimination of BCa subtypes and stages and for better understanding of cellular phenomena including chemoresistance, disease recurrence, brain metastases, hypoxia, production of exosomes, cell cultivation conditions (2D vs. 3D cell cultivation), etc. Glycan determination can also be used for their direct visualisation in cells/tissues or even in living cells or tissues. Glycan-based analysis performed using lectins (Table 4) or instrumental-based approaches (Table 5) indicate the true potential of glycan-based biomarkers for diagnostics, prognosis and for monitoring of BCa.

5. Expert opinion
Glycan analysis performed by mass spectrometry requires highly sophisticated instrumentation and data evaluation, which currently limits its use in routine clinical practice. We think that lectin-based glycan analysis is more compatible with clinical practice, especially in cases where it is performed in an ELISA format of analysis, since this assay format should be then transferable onto automatic machines developed by industry leaders.

Early-stage diagnostics based on glycan analysis in bodily fluids is possible, as documented by the determination of glycans in serum or saliva. It is quite interesting that we have not as yet identified any study detecting glycans in urine, since this would be a completely non-invasive treatment. It is, however, necessary to validate whether glycoproteins which could be connected to BCa are really released into urine for such assays. Most of the glycan-based analysis was performed by whole glycome analysis using cells/tissues or in serum, but we think that the specific glycoprofiling of proteins

Table 3. Sensitivity, specificity, NPV and PPV for discriminating BCa I from BN, BCa II, BCa IIB and BCa III using percentage of AAL- or jacalin-reactive AGP. This is the article published under the Creative Commons Attribution 4.0 license (CC BY) from ref [165].

| Assay type            | Comparison | Sensitivity | Specificity | PPV | NPV | AUC |
|-----------------------|------------|-------------|-------------|-----|-----|-----|
| % of AAL-reactive AGP | BC I vs. BN | 77          | 68          | 71  | 74  | 0.8 |
|                       | BC I vs. BC II | 65         | 68          | 86  | 80  | 0.9 |
|                       | A           |             |             |     |     |     |
|                       | BC I vs. BC II | 92         | 77          | 50  | 97  | 0.9 |
|                       | II B        |             |             |     |     |     |
|                       | BC I vs. BC III | 64        | 77          | 59  | 80  | 0.8 |
|                       | III         |             |             |     |     |     |
| % of jacalin-reactive AGP | BC I vs. BN | 60          | 85          | 81  | 68  | 0.8 |
|                       | BC I vs. BC II | 82         | 82          | 88  | 88  | 0.9 |
|                       | A           |             |             |     |     |     |
|                       | BC I vs. BC II | 75         | 80          | 60  | 93  | 0.9 |
|                       | II B        |             |             |     |     |     |
|                       | BC I vs. BC III | 56        | 60          | 42  | 73  | 0.6 |


It should be stated that glycan-based biomarkers need to be validated using large clinical validation studies to make such results statistically significant and robust, i.e. by analysing 500–1,000 samples from BCa patients with the pool of BCa patient samples including various BCa subtypes and stages. To correctly evaluate the diagnostic value of a biomarker in clinical practice, one needs to carefully consider what the clinically relevant environment is and to design the study to simulate these parameters to a highest extend possible. The results should be compared to current gold standard applied for cancer screening/diagnostics, being a histological evaluation of a biopsied tissue to calculate sensitivity and specificity. However, BCa biopsies might have a false negative rate as high as 13.3% for core needle biopsies without any imaging guidance [170], being significantly lower for ultrasound-guided procedures [171]. For highly cancer-specific methods, all the specimens in a control cohort should be confirmed with a primary diagnosis in a subsequent follow-up (rebiopsy, imaging methods). To properly set the parameters for control cohorts in diagnostic (validation) studies, all the specifics should be carefully considered. Glycan alterations are known to occur with aging as well, so the mean/median age for both observed cohorts should be set properly – this might be an issue especially for age-related diagnoses [172]. As for BCa, other comorbidities and lifestyle should be carefully considered for each individual involved in the study, e.g. alcohol intake was shown to increase the risk of BCa of 20–60% for heavy drinkers. Hormonal factors, lifestyle habits and family history should also be evaluated [173], however, carefully designed diagnostic studies should be driven primarily by the desired sensitivity/specificity of the proposed laboratory method. Since histological evaluation of a tissue has a false positive rate of 0 (e.g. 100% specificity), glycan-based diagnostics as a cancer-specific blood-based (liquid) biopsy should aim for the highest possible sensitivity at a fixed specificity close to 100% as well [174].
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**Declaration of interests**

Tomas Bertok, Aniko Bertokova and Jan Tkac are employees of Glycanostics Ltd. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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