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8. General Discussion

Glycan modifications of proteins and lipids add a high degree of complexity to the cellular phenotype. While this complexity is an analytical challenge, it also offers a wide range of opportunities for biomarkers and treatment targets. Glycans form an integral part of the cell’s outermost layer and an array of ligands for other cells or pathogens, and are therefore involved in many biological processes including malignant transformations [1-3]. Aberrant glycosylation has been recognized as a hallmark of cancer, but it remains subject of debate whether this transformed glycan-phenotype is a cause or consequence of the disease [4]. By analyzing the glycosylation changes and associated consequences, we can hopefully better understand the biology of cancer and use this knowledge for the development of novel clinical assays.

This thesis deals with the analysis of cancer-associated glycomic changes with the main focus on colorectal cancer (CRC). Current knowledge on CRC-associated glycan changes and their biological role have been reviewed in Chapter 1 of this thesis. In Chapters 2, 3, and 6, we developed novel, high-end methodologies for the glycomic analysis of tissues and cell lines to be able to expand our knowledge on cancer glycomics and to overcome some limitations of current techniques. By applying these new methods, this thesis also covers the characterization of changes in glycosylation in CRC tissues as well as cell lines, thereby contributing to the understanding of CRC biology while identifying cancer-specific signatures underlying CRC development. These signatures can be further explored as potential markers to improve patient care. Additionally, in Chapter 5, we extended our research to pancreatic duct adenocarcinoma (PDAC) and characterized the N-glycome of PDAC cells with different metastatic potential and of a normal pancreatic duct cell line.

Glycosylation in the Medical Context: History and Progress

While the history of glycans dates back to the middle of the 19th century, the recognition of their functional role and importance beyond energy-storage and structural support (e.g. chitin) developed only slowly [5,6]. Major limitations arose from the available analytical methods which could not cope with the glycans’ complexity and structural diversity [6]. In 1900, Landsteiner made the discovery of three different blood types allowing for the first successful blood transfusion, but their structural identification as glycans only happened 60 years later [7, 8]. Following the ABO blood groups, several other clinically relevant glycans and glycoconjugates were discovered such as heparin for treatment of thrombosis or bacterial polysaccharides, which were used to develop vaccines [5]. Hudak and Bertozzi give a nice overview over the history of glycans in the medical context [5]. In the late 20th century, glycans became more and more prominent in clinical applications. Technological advances facilitated the characterization of glycosylation and allowed the chemical synthesis of carbohydrates as well as the production of glycoconjugates in non-human expression systems and the specific engineering of glycans with distinct properties [1, 9, 10]. Until 2013, eleven out of the top 20 best-selling biopharmaceutical drugs were glycoproteins, and many currently used protein-based (serum) biomarkers are glycoconjugates (e.g. carcinoembryonic antigen (CEA), cancer antigen (CA)19-9, prostate specific antigen (PSA)) – demonstrating their prominent role in clinics [11]. Although glycoconjugates are broadly present in clinical assays, hitherto little attention has been paid to their glycosylation itself – a topic which is now gaining more interest with, for example, the approval of a specific fucosylated variant of alpha-fetoprotein (AFP-L3) for the improved early detection of hepatocellular carcinoma [12].
ANALYTICAL CHALLENGES AND ADVANCES

The detailed structural characterization of the cancer-associated glycome is an initial step to increase our knowledge of cancer glycobiology and hence resulting in the identification of biological mechanisms, potential biomarkers, and treatment targets. Along with the challenge of identifying markers and understanding disease settings comes the need for robust, sensitive analytical methods and the often underestimated importance of good data analysis tools which both are applicable for basic research, large clinical studies, and daily clinical practices. At the same time, developed protocols need to be suitable for the available sample materials, while meeting the requirements with regards to efficacy and costs.

Our initial study on glycosphingolipid (GSL)-glycans from CRC tumor and control tissues, as described in Chapter 2, employed 2D-LC-MS/MS on tissue homogenates which is a suitable method to characterize complex mixtures and which remains a good approach for in-depth analysis of glycosylation. The applied tissue homogenization is part of conventional protocols to extract the analytes, but has the disadvantage of losing spatial information. MALDI-mass spectrometry imaging (MSI) offers a new possibility for tumor tissue analysis at molecular level. The technique combines the characteristics of mass spectrometry with microscopy/immuno-histochemical staining which allows the characterization of molecules in a spatial manner. Thereby it provides new means to identify biomarkers for patient stratification, or therapy targets, but also to resolve tumor heterogeneity for a better understanding of the tumor biology [13, 14].

Only recently the analysis of N-glycans in situ has been pioneered [15, 16] and has since been applied by different research groups [17, 18]. We developed this method further by transferring and improving a sialic acid linkage-specific derivatization for glycopeptides developed by our group [19] to formalin-fixed paraffin embedded (FFPE) CRC and leiomyosarcoma tissues for N-glycan MSI analysis as described in Chapter 6. The stabilization and neutralization of sialic acids overcomes biases in the glycan profile, especially in MALDI-TOF-MS, while the linkage differentiation reveals additional biological information and may contribute to a better understanding of the role of glycans in cancer progression. Employing this workflow of linkage-specific derivatization of N-glycans by double amidation for MALDI-MSI analysis, we observed distinctive localizations of glycans containing α2,3- or α2,6-sialylation in various tissues, which provided novel information on tumor glycobiology that could not be obtained from native N-glycan analysis (Chapter 6). However, the implications of the specific glycan isomers in certain tumor regions or their potential in clinical assays need further evaluation. Therefore, we now initiated a CRC study applying the MALDI-MSI workflow developed in Chapter 6 on a small cohort to compare tumor glycomic spatial fingerprints of well differentiated versus poorly differentiated CRC tumors, and to correlate this with disease outcome. Our N-glycan MALDI-MSI method also forms the basis for other future applications including the identification of more defined markers which can be applied in image-guided surgery as well as identification of targets for specific drug delivery systems, but also for patient diagnostic and stratification. With regard to therapy, α2,6-sialylation has been previously shown to influence treatment outcomes [20, 21]. Consequently, the in situ derivatization offers a new tool to determine the abundance of this epitope in tumor tissues which could be explored for prediction of treatment responses, but also for understanding of the underlying mechanisms of treatment resistance/sensitivity when attributed to a certain tumor area or cell type.

The linkage-specific sialic acid derivatization is a great improvement for MS-based glycomics as it also permits to characterize α2,3-linked sialic acid-containing sialyl Lewis antigens more reliably – an epitope which has frequently been associated with various cancers [22, 23]. Additionally, no tandem MS or application of orthogonal analytical methods is needed for the linkage distinction, which is not
Evaluating the glycosylation of tumor cell lines

Mass spectrometry imaging forms a powerful tool which has advanced glycomic tissue profiling and characterization as well as biomarker discovery directly from tumor material, all of which are the basics to understand the underlying disease mechanism and cancer biology. However, functional studies are required to validate the generated hypotheses. Therefore, cell lines such as cancer cell lines are often used as in vitro model systems to study disease-related processes [24]. Strikingly, only few studies have shown the suitability of cell lines as model systems at genomic level and have compared them to tissues [25-27], while the glycomic characterization of cell lines is hitherto even more limited [28-30]. The linkage-specific sialic acid derivatization technique for released N-glycans from human plasma [31] was therefore adapted for cell lines and applied to the characterization of CRC and PDAC cell lines in Chapters 3, 4, and 5. With our CRC cell line study described in Chapter 3, we contributed to the characterization of model cell lines, which is an important aspect in order to increase the comparability and reliability as well as for interpreting results correctly. As a second step, we further compared the profiles with a previous study on N-glycans in CRC tissues [32] as well as other literature [33] (Chapter 3) to evaluate the potential of these cell lines as glycobiological model systems. Our results showed that the expressed complex type N-glycans in cell lines were to a large part also present in CRC tumor tissues, but also demonstrated the broad diversity between different cell line N-glycomes. The latter shows the importance of larger scale functional studies instead of generalizing interpretations of results from one or two cell lines. A more elaborate study, mapping cell lines to specific tissue regions and generation of a database as suggested in Chapter 3 would greatly improve in vitro experiments by enabling a choice for the best representative cell line for a certain tissue type or malignancy phenotype. As a next step in that direction, we are planning a glycomic comparison between primary CRC cell cultures and corresponding tumor tissues from the same patient. Furthermore, 3D-cultures could be a valuable option to better mimic the tumor tissue, but robustness, handling, and throughput are still limiting their application [34-36] and thorough characterization is essential.

Another aspect of technological developments is the sample amount and downscaling. Most of current protocols for investigating cellular glycosylation require several million cells and involve multiple preparation steps, which can introduce loss of material or biases in the remaining sample. Chapter 3 portrays a PVDF-membrane based 96-well format N-glycan release protocol for small amounts of cells (250,000), which was used to characterize the above-mentioned CRC cell lines. The applicability and robustness was further evaluated by analyzing PDAC cell lines and primary PDAC cell cultures (Chapter 5) as well as other cell types such as melanoma cell lines (unpublished work), and monocyte THP cell lines [37]. In the future, this protocol could also be exploited for samples obtained via laser micro dissection or small amounts of tissue homogenates. A further downscaling of this method is highly desirable and the ultimate goal is single-cell analysis, which demands major analytical improvements. The latter
becomes important when recognizing that cell cultures and tissues represent very heterogeneous mixtures of cells and often only part of those cells are responsible for an e.g. invasive phenotype [38, 39]. The heterogeneity of cells is also affecting MALDI-MSI-based studies and higher resolutions are needed to make the analysis of only few cells possible.

Another point of interest for the clinical applicability of methods is the overall analysis time, which was also addressed during the method development in Chapter 3. In the described protocol, the PNGase F release time could be reduced to 3 h to realize a sample preparation within one day. Evaluation of the efficiency of the release method showed that the N-glycan profile remained stable after 3 h digestion compared to overnight digestion for most cell lines, whereas signal intensity was increased after overnight digestion [40]. Therefore, exploring new, recombinant PNGase F enzymes with higher efficiency may help to further shorten analysis time and increase sensitivity, but also robustness. Other methods to decrease N-glycan release durations utilize high pressure or microwaves [41], but these were not explored in this thesis.

Interestingly, during the N-glycomic characterization many CRC cell lines, but also PDAC cell lines, revealed high levels of N-glycans with terminal N-acetylhexosamine (HexNAc) residues (HexNAc≥Hex; Hex = hexose), which could be promising novel candidates as therapy targets or biomarkers. Fragmentation analysis by MALDI-TOF/TOF-MS/MS was sufficient to confirm compositions but did not permit the distinction of bisecting N-acetylgalactosamine (GlcNAc), terminal GlcNAc, LacdiNAc/LDN (GalNAcβ1–4GlcNAc), blood type A (GalNAcα1–3Galβ1–3/4[Fucα1–2]-GlcNAc-R), or SdA (NeuAcα2–3GalNAcβ1–4Galβ1–4GlcNAc-R) epitopes. Therefore, we additionally performed RP-LC-ESI-MS/MS experiments, which allowed the identification of LacdiNAc structures in CRC and PDAC cell lines as well as the SdA antigen in the CRC cell line CaCo2 (Chapters 3 and 5). Information concerning branching and detailed structures is, however, still lacking.

Discriminating these structures would add valuable information and contribute to a better understanding of the cancer glycobiology. LacdiNAc structures, for example, were found to be mainly associated with parasites [42], while in mammalians the expression of this epitope was relatively low [43]. In some cancers, such as CRC, however, elevated levels of LacdiNAc epitopes were found [44]. Transcript levels of the corresponding glycosyltransferase, β4GalNAcT3, were found to be upregulated in CRC and, accordingly, overexpression of the enzyme in the CRC cell line HCT116 increased the invasive phenotype significantly [45]. A mechanism involving the integrin-mediated signaling pathway promoting a malignant phenotype in CRC cells has been proposed, in which focal adhesion kinase and extracellular signal-regulated kinase are being activated by β4GalNAcT3 gene expression [45]. Another study identified LacdiNAc epitopes on epidermal growth factor receptor (EGFR), which regulated stemness in colon cancer cells and was accompanied by sphere formation, migration, and invasion [46]. It has further been shown that LacdiNAc epitopes could be recognized by different glycan-binding proteins involved in pathogen recognition, for example Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), macrophage galactose-type C-type lectin (MGL) as well as galectin-3 [43, 47]. Several studies have collected evidence that (fucosylated) LacdiNAc (LDNF) structures together with Lewis X and truncated O-glycans (T, Tn antigen) can skew the immune response to Th2 profiles through binding to host lectins such as MGL and DC-SIGN [48]. The skewing to Th2 has been suggested to be a main factor in immune evasion of the tumor which contributes to immunotherapy resistance [48], offering a new target for intervention. Galectin-3 was associated with tumor progression and metastasis [49, 50], also in CRC (cell lines) [51, 52]. Rather controversial reports describe galectin-3 as anti-apoptotic and/or pro-apoptotic factor [53, 54]. Likewise, terminal GlcNAc epitopes were found to be highly expressed in tumor tissues and were linked to tumor development and high metastatic potential [48]. Experiments with siRNA in CRC cell lines showed that terminal GlcNAc
Clinical use of protein glycosylation

With a more accurate structural characterization of glycans and a better understanding of their interactions, especially with glycan-binding proteins, a broad spectrum of targets for intervention has become available. For instance, our MALDI-MSI analysis on CRC FFPE tissues revealed co-localization of the N-glycan Hex5HexNAc4(α2,3)NeuAc1(α2,6)NeuAc1dHex1 with stroma-containing tumor areas, while the isomers containing either two α2,3-linked sialic acids or two α2,6-linked sialic acids were associated with collagen-rich, muscle, and/or necrotic areas (Chapter 6). The proportion of tumor-stroma is subject of clinical trials for the improved prognosis of CRC [64] and the N-glycomic signatures associated with the tumor-stroma tissue could provide additional information and specificity.

Elevated levels of especially large (more than eight monosaccharides), mono-sialylated gangliosides with two or three fucose residues were identified in GSL-glycans from CRC tumor tissues as compared to control tissues from the same patients (Chapter 2). Interestingly, several glycan-based-vaccines for immunotherapy rather than prophylaxis have been developed based on glycolipids and are investigated in clinical trials (reviewed by Franco et al. [65]), like the glycolipid-based Globo H (Fucx1-2Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc) vaccine for breast cancer [66]. Globo H and related globo-type epitopes are now also evaluated for vaccination in colon and other cancers [67]. Recently, therapeutic glycolipid-derived anti-glycan antibodies against Lewis B epitopes on glycolipids and glycoproteins and others against various gangliosides have been developed and have shown antibody-induced cell death in different cancer tissues and cells [68]. Studies on immunization with fucosyl GM1 (alone or in combination with other glycolipid glycans) have shown encouraging results for regressions inhibit NK cell response towards tumors [55]. In contrast, bisecting GlcNAc was attributed to a tumor-suppressing role [1, 56, 57], and similarly the SdA antigen was mainly found to be expressed in normal colon tissues [58, 59]. In general, knowledge on the role of terminal GlcNAc residues as well as LacdiNAc in cancer and potential clinical applications is still limited and needs further investigations.

To unravel these different structures and the mechanisms involved, more effort needs to be invested in improved fragmentation analysis. While different separation techniques such as LC or CE coupled to ESI-MS or ion mobility MS offer great tools to obtain structural details [41, 57], MALDI-TOF-MS-based techniques are also desirable to maintain high-throughput and simpler data evaluation/handling. Exploring high-energy fragmentation to obtain informative cross-ring fragments (A or X ions) is an attractive option which can aid in resolving linkages and branching not only when performed on permethylated glycans, but also on native glycans [60-62]. Moreover, negative ion mode MALDI-TOF/TOF-MS/MS has been suggested to improve fragmentation, especially for nitrate adducts [41]. These approaches could also be useful to further characterize the fragment ion at m/z 615.2 [M+Na]+ which was described in Chapter 2. This fragment ion was repeatedly observed in RP-LC-ESI-MS/MS spectra of globo-type GSL-glycans and assigned as an N-acetylneuraminic acid linked to the AA-labeled hexose – for which a mass spectrometric artifact or rearrangement product can hitherto not be excluded.

Finally, N-glycans represent only part of the cellular glycome and inclusion of other glycan classes such as O-glycans and GSL-glycans is needed for a comprehensive assessment of cellular glycosylation, preferably in a sequential approach to save precious biological material. Recently, the oxidative release of natural glycans (ORN) in large scale was described, which uses sodium hypochlorite as basis to release N-, O-, and GSL-glycans from cells within minutes [63]. This method offers a great tool to investigate the three main glycan classes from one sample without restrictions or biases due to enzyme selectivity. However, the applicability on small amounts of samples or even in situ for MALDI-MSI needs to be explored.

**Clinical use of protein glycosylation**

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melanoma and small cell lung cancer treatment and induced a higher immune response as compared to smaller epitopes [69, 70]. Thus, larger structures, as also found highly expressed in our CRC study on GSL-glycans, would merit exploration as cancer vaccines. One potential candidate is the GSL-glycan Hex7HexNAc5NeuAc1dHex2, which showed more than 3-fold higher expression in CRC tumors as compared to control tissues, where it was only low abundant. The latter is especially important and addresses the challenging aspect in vaccination and other targeted strategies that the cancer-associated antigens are often only elevated in the tumors but are to a certain extent also expressed on normal cells. Vaccines therefore need to overcome the immune self-tolerance to induce a sufficient immune response [4, 71]. A more detailed structural elucidation could further improve the selection of potential targets and biomarkers since different isomers may be present in tumors and controls.

To increase the efficiency of vaccines, non-human epitopes and thereby also not present on healthy cells have been explored. Promising results have been achieved in clinical phase I and III trials with antibodies against the non-human N-glycolylneuraminic acid-containing gangliosides for vaccination studies in melanoma, breast cancer, and lung cancer [72]. Glyco-vaccines for immunotherapy of infectious diseases as well as in cancer have further benefited greatly from new improvements in synthetic chemistry [73, 74] and another approach to trigger immune responses is the chemical modification of natural occurring glycans by exchanging functional groups or covalently binding the glycan part to a protein [75]. For example, structurally modified GM3 gangliosides led to higher antibody titers in immunized mice as compared to the natural occurring GM3, but was not tested in a cancer setting [75]. Newer approaches of vaccines for immune therapy have utilized multivalent molecules which included – next to the glycan epitope – the addition of helper T cell epitopes and/or toxins and have shown promising results regarding improved immune recognition of MUC1 and others [76, 77].

Sialyl Lewis antigens may be other promising targets for cancer therapy as they were identified on glycolipids and glycoproteins in CRC tissues (Chapters 2 and 6) as well as in CRC cell lines (Chapters 3 and 4), but no distinction of sialyl Lewis A or X could be made. Likewise, primary pancreatic cancer cells with high metastatic potential in zebrafish showed an increased expression of sialyl Lewis A epitopes as compared to a normal pancreatic duct cell line (Chapter 5). Sialic acid (and other monosaccharide) binding proteins like SIGLECs, selectins, and galectins have recently been used as successful targets for chemical inhibition of cancer-related signaling by glycan-based mimetics [78]. Glycan-based (small) molecules serving as glycomimetic inhibitors of lectins or glycosidases are more and more being explored and have been successfully applied as e.g. the anti-viral compound known as Tamiflu [79]. While initially glycomimetics were monovalent structures, more advanced glycan-based drugs present multivalent structures to increase interaction with glycan-binding proteins. Major progress for glycan-based drugs has been made with anti-viral and anti-toxin drugs, but the developed techniques can aid to detect new molecules which can be explored for targeted anticancer therapies [48]. First clinical trials in different disease settings including hepatocellular carcinoma have revealed promising results with the inhibition of the binding between selectins and sialyl Lewis antigens, which prevents inflammation as well as hijacking of the leukocyte homing mechanism by tumor cells for extravasation [80-82]. Intervention in the selectin binding can therefore offer a broad application in many cancers, but careful investigations are needed to determine to which degree ‘healthy’ leukocyte homing is affected. Furthermore, sialyl Lewis antigens are not the only ligands for E-selectin and gangliosides have, for example, been recently identified as ligands on human myeloid cells [83].

Whereas sialyl Lewis antigens were linked to a mechanism of tumor cells for extravasation using E-selectin [84, 85], the role of other blood group antigens in CRC (and others) has received little attention in recent years and reports are rather confusing. The latter may be caused by the often-ignored location in the colon when analyzing such tissues. It has, for example, been shown that the
In Chapter 4 we further postulated a hypothesis on the role of CDX1 and other transcription factors in the regulation of fucosyltransferases involved in antennae fucosylation. Several studies have shown the influence of transcription factors and epigenetic changes involved in the regulation of glycosylation [94, 95]. Hepatocyte Nuclear Factor (HNF) 1α and 4α, for example, have been identified to regulate plasma protein fucosylation [96] and were associated with CDX1 [97], and differentiation [98], respectively. Moreover, expression of fucosyltransferases FUT1 and FUT2, for example, was suppressed by hypoxia inducible factor (HIF) 1α in two PDAC cell lines [99]. In line, we observed a general decrease of multi-fucosylation in PDAC cells with higher metastatic potential, whereas the primary cell culture PDAC2 – which showed most aggressive behavior in zebrafish – revealed unexpectedly extremely high levels of multi-fucosylation, suggesting that different antigens and pathways are involved (Chapter 5).

Metabolic changes can also influence glycosylation via differential expression of transcription factors or other mechanisms. The long known Warburg effect, in which cancer cells switch their metabolism to glycolysis, has been recognized as a hallmark of cancer and implications on the cellular glycosylation have been shown [100, 101]. Recently, the hexosamine biosynthetic pathway (HBP) and its role in the cancer-related epithelial–mesenchymal transition (EMT) have been discussed [102]. One downstream metabolite of HBP is uridine diphosphate (UDP)-GlcNAc, which is needed for N-glycan branching and has been associated with cancer [102]. In light of the metabolic influences on the cellular (glycan) phenotype, the robustness of the glycomic profiles of a cell line with regard to culture conditions, confluence, passage number, and others is of high importance. In addition, the genomic instability during long culturing is often stated as critical point for the use of cell lines, making the comparability of one cell line obtained from different laboratories questionable [103]. We demonstrated not only that each cell line has a unique/different N-glycan profile (Chapter 3), which has implications for the interpretation of in vitro and in vivo studies, but we also observed differences between cell lines from the two participating laboratories using different culture media (ongoing study). While biopharmaceutical companies have realized for a long time that the standardization of culturing conditions is essential to guarantee a homogeneous end product that is monitored through regulatory authorities [104], such a standardization for model systems in academic research has not been achieved yet. It has been shown that controlling the glycosylation by medium design is possible which, in addition, can give valuable insight in metabolic pathways, especially when glycomic, genomic, and metabolomics approaches

**Factors Modulating Protein Glycosylation**

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are combined with systems biology [105]. Effects of the medium on the glycosylation profiles have been elegantly reviewed by Hossler et al. [104]. In order to assess the robustness of N-glycan profiles during cell culture, we plan to study the glycosylation of several CRC cell lines in different culture media (DMEM, RPMI, serum-free). Likewise, we started a pilot study combining N-glycan analysis with intracellular metabolomics in thyroid cancer cell lines. However, from various studies it has become clear that the effects on the glycosylation are highly cell line- and protein-specific, and varied even within a cell population or between different clones, making a case-to-case study even more important [106]. In the biopharmaceutical industry the definition and monitoring of critical quality criteria with regard to glycosylation is gaining importance and similar approaches should be incorporated in academic research to standardize and thereby improve the comparability of results obtained with cell line model systems.

**CONCLUSION**

Despite recent advances, the use of glycan biomarkers, vaccines, or therapeutics is still rather limited, especially for CRC and pancreatic cancer. New, reliable, non-invasive, and low-cost biomarkers for diagnosis, monitoring, and patient stratification as well as targets for treatment/intervention are urgently needed since cancer remains a major cause of death and still we know too little about its (glycan-involving) mechanism. While for many cancers the mortality has slowly declined due to new population screening approaches, an opposite trend is expected for the coming years, in which we encounter a rapidly growing, but also aging population, as well as an increased adoption of a cancer-risking lifestyle [107-109]. This does not only present a burden for patients and their family, but has also major implications for healthcare costs.

In summary, the investigations/results of this thesis contribute to the understanding of CRC-associated glycan changes by developing essential novel methods to study these changes as well as the application of these methods to find specific cancer signatures which can be explored for clinical assays. Another aspect was the evaluation of the potential and quality of cell lines as model systems, which are the basis for functional studies, and last to resolve tumor heterogeneity for diagnostics, prognosis, and treatment targets by MSI.

Characterization of glycosylation can offer valuable information regarding the glyco-phenotype involved in various cancer-related processes and thereby is a valuable source for novel biomarkers, while providing new insights into the understanding of mechanisms and biology of the cancer which plays an important role in the development of new therapies. Glycans are especially suited for clinical applications since their biosynthesis is largely affected by the disease state and reflected as a certain glyco-phenotype [110]. Furthermore, it has been shown that the glycan profile shows inter-individual differences, which affects population studies, but offers great advantage in personalized medicine [111]. Additionally, the glycan profile of a healthy individual was shown to be stable over time and changes were associated with physiological and environmental factors, suggesting a strong diagnostic potential of glycans [111]. Ruhaak et al. suggested a system-wide compositional glycan screening using plasma or serum which can provide information on the health condition of a person and is suited for large-scale clinical studies and daily routines as an initial step in diagnosis [112]. Following this, a more in-depth characterization can be performed for patients at risk with a disease-related glycan phenotype to unravel structural information and carrier proteins or lipids for more specific diagnosis and individual treatment [112]. Furthermore, combining different omics-platforms could bring major advances in cancer biology and technological improvements for absolute quantification as well as exploration of biomarker panels instead of single markers is likely to improve their sensitivity and specificity. While
the challenge is the discovery of biomarkers, an even greater hurdle is the validation of potential candidates, understanding their role in the disease setting to identify new treatment targets, and finally the translation of these findings into clinical applications. Therefore, close collaboration between basic researchers, laboratory professionals, clinicians, and policy-makers is essential in order to overcome current limitations and contribute to better (personalized) patient care.

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