Glycosylation profiling with mass spectrometry: method development and application to cancer biomarker studies

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CHAPTER 1

Introduction

General introduction

Cancer is one of the leading causes of death worldwide. Breast cancer is the most common cancer in females. Pancreatic cancer has a very poor prognosis, with almost as many deaths as incidences.[1] The detection of cancer at early stage is challenging, but highly important as this often comes with a better prognosis and more treatment options. For breast cancer population screening and pancreatic cancer high-risk screening imaging techniques are used for detection. As is true for almost any diagnostic test, current screening methods are not optimal in terms of sensitivity and specificity, resulting in false positives and false negatives, which could have far-reaching consequences for patients. The need for new biomarkers and the development of new highly sensitive and specific methods is high, without compromising on low invasiveness and risk for the patients. To this end blood-based screening has great potential and as a result multiple blood based markers have been reported from omics-discovery studies, although none of these have provided the desired sensitivity and specificity that are needed for clinical implementation for population/high-risk screening.[2] In addition, blood-based biomarkers could add to the process of clinical decision making to improve treatment. In cancer, the expression of glycosyltransferases and glycosidases is altered, which leads to changes in glycosylation of proteins and lipids.[3,4] Qualitative and quantitative analysis of the glycosylation of proteins might provide a better understanding of these changes. Moreover, recent advances in glycoanalytical strategies have allowed glyco(proteo)mics to be implemented in cancer biomarker studies for exploring glycans as a potential source of biomarkers.

The research described in this thesis was focused on development of released *N*-glycosylation analysis methods for dried blood spots as well as for serum, of which the latter was applied to a pancreatic cancer case-control study. Furthermore a breast cancer case-control study was performed. In this introduction a clinical background of breast- and pancreatic cancer is provided, the basics of protein glycosylation are described and the analysis of glycosylation from blood-derived samples with mass spectrometry is explained.

Cancer

For 2018 it was estimated that 18.1 million new cancer cases would be diagnosed worldwide and that 9.6 million people would die from the disease.[1] Of these new cancer cases it was estimated that breast cancer would account for over 2 million incidences and 626 thousand deaths. For pancreatic cancer the number of incidences was estimated to be much lower (459 thousand), however, because of its poor prognosis the number of deaths (432 thousand) is almost equal to the number of incidences.[1] It is predicted that the number of pancreatic cancer related deaths will in the future be even higher than the deaths related to breast cancer, while the incidence of breast cancer is more than four times higher.[1] As cancer is the first or second cause of death in 91 countries, the number of studies related to cancer is vast.

Much attention is paid to study the biology of cancer and its pathophysiology. Cancer cells often develop from normal tissues, caused by mutated genes. These genes can be inherited, and thus be present at birth, but often these are acquired through exposure by environmental factors (e.g. radiation, chemicals, asbestos, bacteria/viruses) or lifestyle-related factors (e.g. cigarette smoke, alcohol, sunlight, food-related factors). The result of the mutated genes is not presented from the onset. It takes time for the cancer cells to develop and even more time before symptoms will appear. Some cancers, such as pancreatic cancer, are often diagnosed when these have already reached an advanced stage, decreasing the possibility for curative treatment and patient survival chances.[5]

Pancreatic cancer

Pancreatic cancer is the seventh cause of cancer deaths, which is not due to a high incidence, but rather a result of aggressiveness of the cancer, leading to a poor prognosis for pancreatic cancer patients.[1] The pancreas is located in the abdomen, where it is surrounded by other organs and large blood vessels (i.e. aorta and inferior vena cava), the pancreas is therefore a relatively inaccessible organ.[6] It contains a duct system through which produced enzymes are transported to the digestive system. These digestive enzymes are produced in lobules located throughout the pancreas being part of the exocrine system.[6] The pancreas also has an endocrine function: it produces hormones to regulate the blood sugar level.[7] Most pancreatic cancers (85-90%) are pancreatic ductal adenocarcinomas (PDAC) which are originating from the exocrine system. Neuroendocrine tumors (NET) are only found in 1-2% of the pancreatic cancer patients.[5]

In most patients that are diagnosed with pancreatic cancer the tumor is at an advanced stage and resection is in many cases not possible. Pancreatic cancer is at an advanced stage when the celiac axis or superior mesenteric artery are affected

or in case of metastasis.[8] The symptoms of pancreatic cancer are often aspecific, including malaise, weight loss and pain in the abdomen. Most pancreatic cancers have metastasized ahead of manifestation of the disease and in cases the initial diagnosis was a localized cancer, the patients still die from metastatic or recurrent cancer.[9] Notably, patients are also diagnosed with pancreatic cancer as a side-finding in scans for unrelated matters (e.g. trauma).[9] To increase the percentage of patients where resection is still possible, screening modalities for genetic high risk groups are being developed to diagnose pancreatic cancer at an earlier stage.[10]

As the incidence of pancreatic cancer is relatively low, the benefit of a general population screening does not outweigh the downsides such as false-positives and high costs. Therefore currently only persons at high risk are tested. Besides genetic risk factors, also clinical risk factors for pancreatic cancer were found: chronic pancreatitis, diabetes and a family history of pancreatic cancer, but also age, smoking and obesity, which are nonspecific.[9]

Some serum biomarkers are available for pancreatic cancer, such as CA19-9. This marker has a low sensitivity and specificity which prevents its use in screening. Instead, CA19-9 is used for monitoring of patients during treatment.[9] Promising results have been reported from protein biomarker discovery studies on pancreatic juice, but this liquid is difficult to obtain with inherent risks for the patient.[9,11] Alternatively, imaging techniques are used for screening, however with ultrasonography the sensitivity is relatively low because of the location of the pancreas in the abdomen.[12] Contrast-enhanced computerized tomography is also used for diagnostic purposes and can show relatively high sensitivity and specificity, but this technique is more invasive.[9] A third imaging technique that is used for pancreatic cancer diagnosis is magnetic resonance imaging (MRI), but this technique is expensive and less convenient for the patients as they have to remain motionless during the scan.[13] Nevertheless, annual MRI screening of high risk patients was shown to be successful to detect pancreatic cancer at a resectable stage.[14]

The development of a screening test for pancreatic cancer is challenging, but not limited to the above mentioned difficulties. False-positive cases are highly undesired in pancreatic cancer as the follow-up evaluations are relatively invasive[9], besides the influence on the mental wellbeing of the patient. Moreover, if a screening test would diagnose patients at an earlier stage, it does not necessarily mean that the natural development of the cancer can be altered in order to increase survival time.[9] This should be taken into account when evaluating a screening method, as implementing a screening test is only worth it when it improves the prognosis or well-being of the patient. For pancreatic cancer the higher resection rate and better survival of screened patients (often diagnosed at earlier stage) compared to sporadic pancreatic cancer cases suggests that it meets the requirement.[14]

Breast cancer

Breast cancer is the leading cancer diagnosed in women. Of all cancer incidences in women, more than 24% is breast cancer. Cancer mortality in women is in 15% of the cases the result of breast cancer.[1] It is predicted that within thirty years breast cancer incidence will reach more than 3 million new cases annually due to population aging and westernization of the developing countries.[15]

The breast is located on the outside of the chest muscles, under the skin. The nipple is in the center of the skin and is under the skin surrounded by 15-20 lobes. These lobes consist of 20-40 lobules, which produce the milk that is in the end excreted by the nipple. The breast also consists of fatty tissue, of which the amount determines the size of the breast. Additionally, blood vessels and connective tissue are present.[16,17] Most breast cancers start in the milk-producing and -transport system of the breast (e.g. the lobules, milk-ducts), but can be caused by the expression of multiple different genes.[18] Breast cancer is genetically and clinically very heterogeneous, and multiple different classification systems exist (e.g. receptor status, DNA classification).[19] In addition, prognostic staging (TNM classification) of breast cancer is based on tumor size (T), lymph nodes involvement (N) and metastasis (M) of the disease.

Population-based breast cancer screening programs are carried out to reduce mortality, and for example in the Netherlands consist of mammography every two years for women aged between 50 and 75 years.[20] This technique consists of X-ray photos taken from the breasts in search for abnormalities. Unfortunately, the percentage of cases that were not detected by mammography lies around 25%. In addition, around 75% of the women that are send to the hospital for additional tests, turn out to be a false-positive of the mammography test.[21] From these results it is no surprise that there is an ongoing debate on the use of mammography for breast cancer screening.[22] Although there is clear evidence that the use of mammography for screening of breast cancer reduces mortality,[21,22] there are also multiple downsides to this method. To begin with the false-positive results, which lead to unnecessary biopsies and also feelings of anxiety and stress for the patients. Additionally the risk of overdiagnosis is high, resulting in demanding treatments for patients where the cancer will not result in death.[22]

For breast cancer, risk factors are genetic mutations (e.g. BRCA1, BRCA2) and a family history with breast cancer, but amongst others also menarche at younger age, late first pregnancy (>30 years) or nulliparity, late menopause, oral contraceptives and a history of chest irradiation increase the risk. The average life-time risk for breast cancer is less than 15%, however, the above mentioned factors can increase this number for the individual patients. The BRCA1 and BRCA2 mutations increase the lifetime risk (LTR) of breast cancer up to 82%, however only

15% of the familial breast cancers is explained by these genes.[23] Other known, but rare, mutations connected to increased breast cancer risk are PTEN (LTR 85%), TP53 (LTR 25%), CDH1 (LTR 39%) and STK11 (LTR 32%).[23] Additionally, multiple DNA repair genes interacting with BRCA mutations or -pathways which double the risk of breast cancer have been identified (CHEK2, BRIP1, ATM, PALB2).[23]

Breast cancer testing in women carrying the BRCA genes starts at younger age, as these mutations can lead to the develop of cancers at younger age. However, mammography has some disadvantages in these cases, as younger women often have denser breast tissue which negatively influences the sensitivity of mammography. Another technique which is often used for the detection of breast cancer is MRI. Compared to mammography, MRI is relatively expensive. However, MRI has a high sensitivity in breast cancer, although the specificity is relatively low (more false positives). Notably, for young high risk patients the combination of mammography and MRI increased sensitivity.[24]

As breast cancer is a very heterogeneous disease[19] and screening challenges such as overdiagnosis and interval breast cancer (developing within 12 months after a mammography test considered normal) are present, additional research on screening methods and early cancer detection are urgently needed. New high sensitivity and specificity tests need to be developed to be able to detect all different breast cancer subtypes and to prevent overdiagnosis.

Glycosylation

The human genome consists of approximately 30,000 genes that each code for a specific protein. For each gene various molecular protein species are found, which are called proteoforms[25], resulting in more than one million different proteins presented in the human body.[26] Various types of proteoforms result from splicing upon human deoxyribonucleic acid (DNA) transcription, where introns are removed from the messenger ribonucleic acid (RNA), or from post-translational modifications (PTMs), in which structural changes occur after translation of the RNA to proteins.[27] These PTMs have influence on for example the structure and solubility, leading to changes in function and activity of the proteins.[28] Many different types of PTMs are known, all with various functions and each with a very specific presence and abundance on proteins. Most of these modifications are enzyme-catalyzed and several are reversible.[27,28]

A frequently observed PTM is glycosylation, which is defined as "the enzyme-catalyzed covalent attachment of carbohydrate to a polypeptide, lipid, polynucleotide, carbohydrate, or other organic compound, generally catalyzed by glycosyltransferases, utilizing specific sugar nucleotide donor substrates".[29] Glycosylation is commonly found on secretory as well as membrane proteins in single cell- and multicellular organisms.

Monosaccharides

Glycans are built from monosaccharides, which in solution exist in an equilibrium of the cyclic and linear form. Upon cyclization of a linear monosaccharide an additional chiral center is formed at C-1, resulting in a so-called α- and β-anomer (exemplified for glucose in Figure 1). The anomeric center of a monosaccharide can interconvert between both isomers via the linear structure. However, in a oligo- or polysaccharide, only one monosaccharide can interconvert at the anomic center, namely the one that is not bound to another monosaccharide and this is called the reducing end. This name originates from the redox reaction that may be performed on the aldehyde in the cyclization (Figure 1) resulting in an oxidized carbonyl group.

Glycans are complex oligosaccharides which, in contrast to linear polysaccharides such as hyaluronan and heparin, often are branched structures. This branching increases the complexity of the glycans and results in many isomeric structures, which subsequently might influence the function. Moreover, the variety of monosaccharide building blocks that are commonly present in glycan structures further increases complexity. In addition, monosaccharides are linked via glycosidic bonds, which in theory can be formed between all hydroxyl-groups present on the monosaccharides.

The great variety of possible glycans makes the study of their function challenging. Glycans are influencing the structure and conformation of proteins, which leads to changes in protein interactions and protein activity.[30,31] In addition, glycans are involved in many biological processes, such as cell recognition, cell signaling, inflammation, immune defense and regulation processes including cell survival and growth.[32]

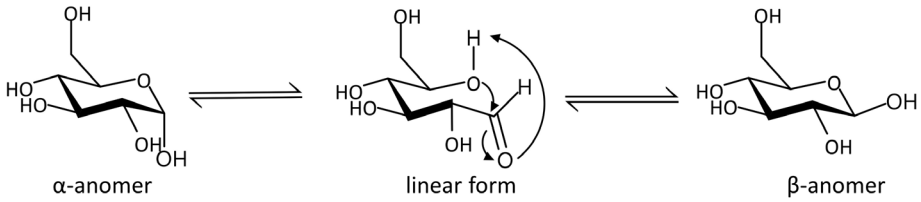

Figure 1. Equilibrium of cyclic and linear glucose.

Protein glycosylation: structures and biosynthesis

Glycosylation is found on many different compounds in human cells, for example on proteins. The most common glycan structures found on proteins are *N*- and *O*-glycans. Enzymes couple monosaccharides to form branched and often complex polysaccharides. The substrate specificity of these glycosyltransferases determines the primary structure of a glycan.[33] *N*-linked glycans are linked to an asparagine (Asn) via the nitrogen atom from the Asn amine group. Not all asparagines in a protein are potential glycosylation sites. The specific amino acid sequence Asn-X-Ser/Thr, where X cannot be a proline, is referred to as a structural motif for potential *N*-glycan attachment.

N-glycans all share the same core structure consisting of three mannoses (Man) and two *N*-acetylglucosamines (GlcNAc): Manα1–6(Manα1–3)Manβ1–4GlcNAcβ1–4GlcNAcβ1-Asn-X-Ser/Thr. Three different classes of *N*-glycans are defined: oligomannose-type, complex-type and hybrid-type *N*-glycans. The oligomannose glycans consist of mannoses only in their core structure; complex glycans have on both outer mannoses of the core so-called 'antennae' attached, which start with GlcNAc and are followed by a variety of different monosaccharides; the hybrid type *N*-glycans contain on the Manα1-6 arm only mannoses, while on the Manα1-3 arm a complex antenna is attached (Figure 2a). The most common monosaccharides found in human *N*-glycans are besides GlcNAc and Man: fucose (Fuc), galactose (Gal) and *N*-acetylneuraminic acid (Neu5Ac), the latter is also referred to as "sialic acid".

Roughly, the biosynthesis of *N*-glycans is a two-step process, which takes place in the endoplasmic reticulum (ER) and the Golgi. The build-up of the glycan starts in the ER with a dolichol phosphate to which monosaccharides are one by one

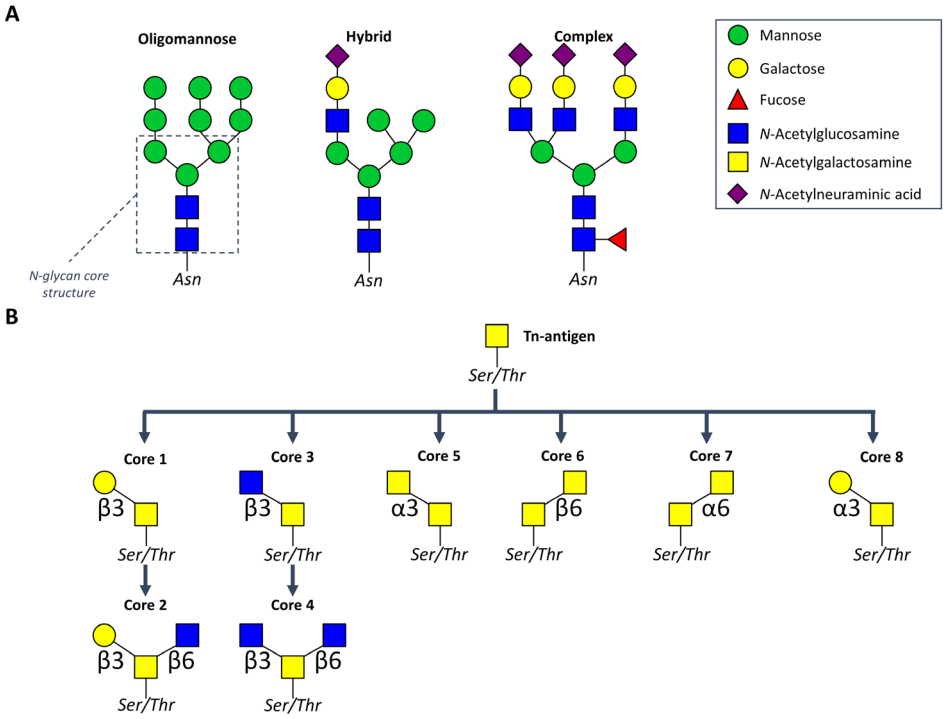

Figure 2. (a) *N*-glycan types (oligomannose, hybrid and complex) and general *N*-glycan core. (b) Tn-antigen and *O*-glycan cores.

coupled by enzymes. An oligomannose glycan, consisting of nine mannoses and extended with three glucoses on the α1-3 arm, is formed. This glycan is in its entirety transferred to the asparagine of the glycosylation site of the protein and all other glycans are derived from this single glycan structure. Subsequently the glycan is trimmed and rebuilt in the Golgi, which gives the protein its specific function.[34] Although this pathway might seem a devious process, the glycan intermediates serve as a quality control for the protein.[34]

The other most commonly found glycan structures on proteins are *N*-acetylgalactosamine (GalNAc) *O*-glycans. These glycans are linked to Ser or Thr via an oxygen atom (GalNAcα-Ser/Thr). Unlike *N*-glycans, *O*-glycans do not share a single core structure: eight different core structures are known, of which core 1 and 2 are most often found (Figure 2b).[35,36] All *O*-glycan cores contain an *N*-acetyl galactosamine and besides this monosaccharide *O*-glycans also can contain galactose, *N*-acetyl glucosamine, fucose and sialic acid sugars.[35] In contrast to *N*-glycans, *O*-glycans are directly synthesized onto the protein (per monosaccharide) after *N*-glycosylation, folding and protein oligomerization.[36]

Glycosylation and cancer

In tumor cells alterations in glycosylation are often observed and it has been suggested that these play specific roles in the progression of the disease.[3,37,38] In cells, major changes of *N*- and *O*- glycosylation are associated with transformation of the cell in early stage as well as in progression of the disease and metastasis.[39] Furthermore, an increase in specific glycosyltransferases in cancer influences the production of glycans and alters the 'normal' glycosylation, which subsequently may influence the structure and function of the glycoprotein.[39] The conformation of the glycoproteins, as well as the interaction with other molecules and oligomerization can be changed, which may contribute to the progression of the tumor. Also, the interaction between the tumor and its microenvironment might be altered.[3,39,40] On tumor cells specific glycan structure are often found to be highly expressed, of which the Tn-antigen (Figure 2b) is an example.[39]

Altered *N*-glycosylation features which are often reported are increased branching and fucosylation (upregulation of FuT8) in cancer patients.[41] The latter has been associated with the regulation of cell proliferation[39], but also decreased cell adhesion and cancer development.[3,42] The increased branching of glycans in cancer cells has been associated with the upregulation of MGAT5 and is involved in tumor growth and metastasis.[42–45] Additionally, expression of sialyl Lewis antigens (sLe) is upregulated.[39,41] The glycosylation epitope sLeX is found on the cell surface, but also on glycoproteins in serum of cancer patients.[46] Notably, it was reported that the most lethal cancers, which are pancreatic, lung and gastric cancer, show the highest serum sLeX levels.[47]

Analytical methodology

To understand the role of glycosylation in cancer and other diseases, information on glycosylation changes, in terms of structures and/or amounts present, is collected. This data can be gathered from different human sample types, such as whole blood samples, serum/plasma samples, tissue, saliva and urine, depending on the research question and goal.[48–52]

Glycosylation analysis can be performed on different levels. It can be analyzed from intact glycoproteins, from glycopeptides or from solely the glycans themselves. Each of the levels provide different information on the glycosylation. With intact analysis all modification on a glycoprotein are presented in the system in which they function. The number of PTM's per protein can be analyzed and also the information on combinations of PTM's can provide insights in the function of the protein. However, the analysis of intact proteins can be challenging as a good separation of all different proteoforms is difficult to establish, particularly when proteins contain many glycosylation sites or are part of a complex mixture. Alternatively glycopeptide analysis can link PTM's to the exact location on the protein, giving insight in the occupation and type of glycans on a specific glycosylation site. This however is still challenging with complex mixtures of many different glycoproteins.

The focus of this thesis is on the analysis of released glycans from glycoproteins in complex samples (e.g. serum and blood). Although the obtained glycan information cannot be linked to glycosylation sites or specific proteins on this level of analysis, highly informative data on overall glycan heterogeneity can be obtained from very complex samples. Changes in certain types of glycosylation, such as levels of different antennarities, fucosylation or sialylation, might give an indication on processes that are related to the disease investigated. Moreover, these up- and down-regulation of certain types of glycans might still be linked to certain glycoproteins, as information on the glycosylation of these specific proteins is available.[53]

Over the years, various analytical methods have been described for the structural, qualitative or (semi-)quantitative analysis of glycans. Nuclear magnetic resonance (NMR) has for example been used to analyze the structure of glycans.[54–56] The advantage of NMR is that the exact structure of the glycan can be determined and thus can differentiate between isomers. However, the sample needs to be nearly pure, because mixtures of glycans can cause great difficulties in interpreting the data. In addition, the interpretation of NMR spectra from large structures is also challenging, due to the similar environments many of the protons encounter. Another limiting aspect of NMR is the amount of sample required for analysis, which is extremely

high in comparison with a sensitive technique such as fluorescence detection, UV detection or mass spectrometry (MS).[57]

Fluorescence detection and UV detection are often used after liquid chromatography (LC) separation of labeled glycans. Also electrospray ionization (ESI)-MS is generally used after LC separation of glycans with and without prior labeling. LC separations of glycans have been extensively reviewed for various stationary phases, specifically hydrophilic interaction liquid chromatography (HILIC), porous graphitized carbon (PGC) chromatography and reversed phase (RP) chromatography.[58–62] The different stationary phases are used for different research questions and purposes. HILIC chromatography is often used for complex samples as it has a high peak capacity. PGC however, has the great advantage of separating glycan isomers, including linkage isomers. On the other hand, RP-LC is usually highly reproducible and also widely available among research laboratories.[62,63] Additionally, glycan separation with capillary electrophoresis (CE) is also a possibility and is often performed in combination with fluorescence or MS detection.[64–7]2 A more in-depth analysis of glycan structures can be done using endo-glycosidases. A sequential use of these enzymes in combination with the techniques described above, can reveal the monosaccharide structure of glycans.

In this thesis matrix-assisted laser desorption/ionization (MALDI)-MS is used as an analytical technique for released glycans. Both MALDI-time of flight (TOF)-MS and MALDI-Fourier-transform ion cyclotron resonance (FTICR)-MS are evaluated and applied to released glycan analysis.

Serum and blood sampling

In the research described this thesis, glycosylation analysis is performed on serum, plasma, whole blood and dried blood. In general, the quality of preanalytical processing of blood samples is extremely important for the quality of the outcomes of the research. Inaccurate results might be obtained when the preanalytical processing is not thoroughly thought out prior to cohort collection. Technical variables such as variation in collection tubes, sampling protocols and storage temperature might lead to biases in the outcome of the research, but also biological variables such as age, gender, ethnicity and lifestyle factors might play a significant role in this.[73] For proteomics studies it was shown that the standardization of preanalytical processing is of great importance[73], while it appears that glycans are less influenced by the preanalytical processing conditions.[74]

Research has indicated that glycosylation profiles are very stable compared to other serum or plasma analytes such as proteins.[74] For plasma samples no effect of storage conditions before centrifugation as well as longterm storage at -20 versus -80 °C has been found on MALDI-TOF-MS *N*-glycosylation profiles. Also using different

collection tube additives and overnight shaking of the sample did not introduce any difference in the profiles.[74]

The stability of glycans in DBS is investigated in this thesis. In the past stability of glycans in DBS has already been evaluated at room temperature and -80 °C, concluding that also in DBS the glycome is stable.[49] Proteins and peptides however, degrade relatively fast in DBS.[75] The stability of the glycome in DBS makes the use of DBS samples for glycan analysis very attractive for sample collection. Especially because it is less invasive compared to venipuncture and might even be performed by patients themselves.

Glycan release

Intact glycan analysis is an upcoming field, but before that glycosylation was mainly analyzed on released glycan level.[76] Released glycan analysis is still widely applied and also in this thesis this approach is used. An important criterium for a release method is that it should not have a bias towards certain glycans, and thus should only be selective for the cleavage site. In addition, it should not modify the glycan except for the cleavage of the target bond. Moreover, the reaction has to be efficient enough that a sufficient amount of released glycan material can be recovered. It should also be mentioned that it is highly desirable that a free reducing end is available for labeling after glycan release.[76]

Both chemical and enzymatic releases of glycans are possible. Chemical release of glycans is performed via for example β-elimination at alkaline conditions. With this method *N*-glycans as well as *O*-glycans can be released, dependent on the alkaline conditions used: with harsh alkaline conditions the *N*-glycans are released from the proteins, whereas with dilute alkaline conditions only *O*-glycans are released. Notably, with this reaction the protein backbone is heavily affected and the GlcNAcs are deacetylated.[77]

N-glycans are often cleaved using peptide-*N*-glycosidase F (PNGase F). This enzyme breaks the bond between the Asn and GlcNAc, which results in an aspartic acid residue and a glycosylamine.[78] The stability of the amine is not very high at a neutral pH, resulting in a hydrolysis of the amine to a hydroxide group. The reaction takes place under mild conditions, leaving the protein backbone intact. In addition, it is specific for *N*-glycans, as PNGase F only cleaves glycans that are attached to an asparagine.[79] Unfortunately no enzyme has been found yet that cleaves all types of *O*-glycans.[78]

Glycan derivatization and purification

Derivatization reactions are performed to improve the molecular properties of an analyte for analysis. On glycans, often derivatized sites are the reducing end,

the carboxylic acid of the sialic acids and all hydroxyl groups in the molecule. Reducing end modifications are usually labels which can be used to analyze glycans with fluorescence or UV detection.[80–83] Also for MS detection labels are used, which might enhance sensitivity or provide semi-quantitative information upon fragmentation.[84–86] Additionally, stable isotope labelling can be used for the direct comparison of samples or as an internal standard.[87–89] In this thesis the reducing end of the glycans is not modified other than the reduction of the reducing end in the case of the *O*-glycans which happens during the release reaction.

Sialic acid modifications are essential for the analysis of sialylated glycans with MALDI-MS. In-source/post-source fragmentation, resulting in the loss of sialic acids, is often observed with MALDI-MS.[90,91] Previous studies have reported that this is due to the carboxylic acid that is located next to the glycosidic bond. With an internal reaction the glycosidic bond is broken, resulting in the loss of the complete sialic acid.[90,92] This type of cleavage has also been observed for peptides.[93,94] To stabilize the sialic acids they can be methyl- or ethyl esterified.[50,90,91,95,96] In addition the carboxylic acid can be amidated and/or permethylated.[97,98]

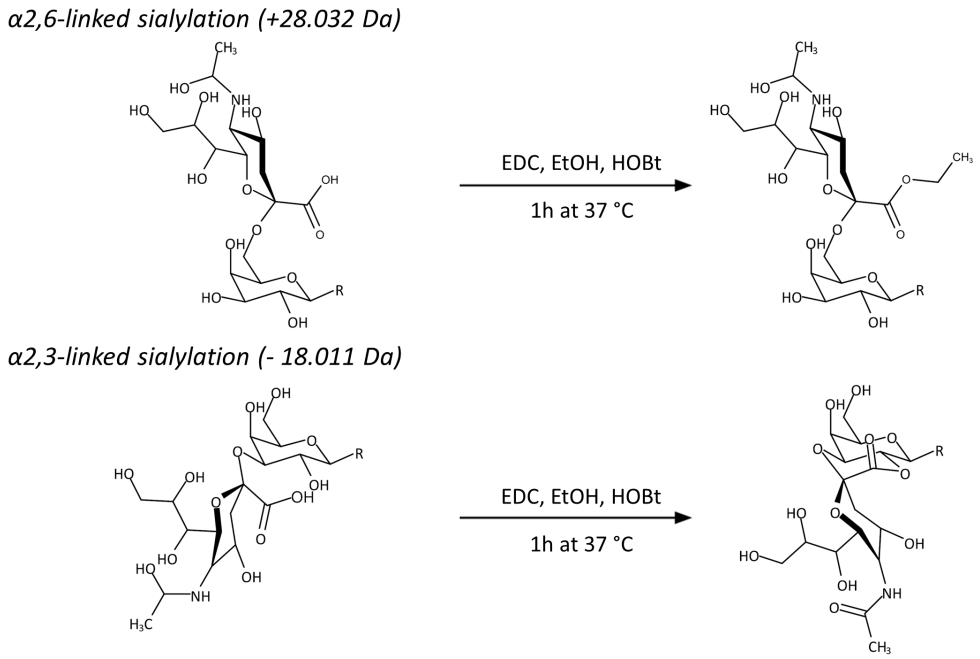

Figure 3. Ethyl esterification reaction of α2,6-linked sialic acids, and lactonization reaction of α2,3-linked sialic acids.

In this thesis ethyl esterification (*N*-glycans) and permethylation (*O*-glycans) are performed to stabilize the sialic acids. It should be noted that many of the above

mentioned derivatization reactions are linkage-specific. Sialic acids are linked to their neighboring galactose (in the case of *N*-glycans) with an α2,3- or α2,6-linkage. Conformationally the carboxylic acid of the α2,3-linked sialic acid is in proximity in space to the galactose, which leads to an internal reaction forming a lactonized structure (Figure 3). On the contrary, the carboxylic acid of the α2,6-linked sialic acid has a significant distance in space from the galactose and forms an ester at the carboxylic acid instead of the internal ring (Figure 3). In the case of ethyl esterification, the mass differences obtained in comparison with nonderivatized are -18.011 Da and +28.031 Da for the α2,3- and α2,6-linked sialic acids respectively. In MALDI-MS analysis this mass difference between the glycans containing α2,3- or α2,6-linked sialic acids, can be used to gather linkage-specific information on the glycosylation in a sample.[91]

In permethylation of the *O*-glycans not only the carboxylic acid is modified but all hydrogens of the hydroxyl and amine groups are replaced by methyl groups. This modification decreases the hydrophilicity of the molecule, which is beneficial in for example RP separations, but also for sensitivity in ESI-MS.[62,99,100]

MALDI-MS

Biomarker research is often performed on large sample cohorts. To minimize batch effects, it is preferred that samples are measured in a short time period. A high-throughput analysis method such as MALDI-MS can fulfill this need as the general measurement time per sample is 10 to 20 seconds, depending on laser frequency and analyte concentration.

MALDI is a soft ionization method which was introduced in 1985. It is called 'soft' as MALDI ionizes analytes without breaking the chemical bonds in the analyte molecule.[101] This allows direct mass measurement of biomolecules, such as glycans. Nevertheless, for certain glycan structures in-source fragmentation occurs in small amounts upon MALDI ionization.[92,102] For example, the loss of sialic acids as mentioned earlier in this Introduction and consequently this requires a different strategy (such as derivatization).

In general, MALDI analysis starts with applying a small drop (few microliters) of the sample onto a MALDI target together with a matrix. The matrix consists of small organic molecules which have a strong absorption at the wavelength of the laser.[103] The sample and matrix then dry together to form analyte containing matrix crystals on the MALDI target plate. Under vacuum conditions in the source these crystals are ablated with shots of an ultraviolet laser, resulting in analyte ions. The ions are then transferred to the mass analyzer, which can be a TOF or FTICR instrument, and mass-to-charge (m/z) ratios are determined.[104]

There are also some challenges generally encountered in MALDI-MS. First,

the dried sample/matrix spots on the MALDI target are often not homogeneous. For glycan analysis generally 2,5-dihydrobenzoic acid (DHB) matrix is used, which forms various large crystals thus resulting in heterogeneous spots. A rather large shot-to-shot variation is therefore frequently observed in MALDI-MS analysis.[105] To compensate for this effect and to improve repeatability of measurements multiple shots on a spot can be averaged. When these are recorded in an unbiased random pattern the average spectrum obtained from a spot is relatively stable.

Second, in MALDI-MS chemical noise is often present in the spectra. Chemical noise can arise from impurities in the sample, but more challenging is the chemical noise caused by matrix ions.[106] Especially with low analyte concentrations and in the lower mass region (below *m*/*z* 800) many signals originating from matrix clusters are visible. Analysis of small glycans at low concentrations can thus become challenging.

Mass analyzers

Ions are evacuated from the MALDI-source and accelerated by an electric field towards the mass analyzer, in TOF this is the flight tube. This tube is field-free and the ions are separated here based on their velocities. From the time it takes the ions to move through the flight tube to the detector the *m*/*z* ratios are determined. A TOF analyzer can analyze ions over a very broad mass range, making it very suitable to combine with a MALDI-source. These measurements can be performed in linear mode, as well as reflectron mode. A reflectron deflects the ions back into the flight tube to a second detector. Here, the kinetic energy dispersion of ions with the same *m*/*z* is corrected, which improves mass resolution. Ions with a higher kinetic energy will travel deeper into the reflectron, giving them a slightly longer path than ions with a lower kinetic energy, which results in the ions reaching the detector at the same time. However, using reflectron mode results in a mass range limitation and a loss of sensitivity.[103]

Another analyzer which can be combined with a MALDI-source is FTICR. The ions are transported to an ion trap cell within a magnetic field. For detection purposes, the ions are excited and the corresponding cyclotron frequencies are measured from an induced image current. In Fourier transform MS all ions are excited simultaneously, resulting in a complex wave, which can be transformed into individual frequencies. The obtained frequency spectra is Fourier transformed into a mass spectrum that displays *m*/*z*-values for each species with a corresponding intensity.[103]

Scope

The aim of this thesis is to develop robust and high-throughput methods for the analysis of *N*-glycosylation from serum proteins, to facilitate biomarker discovery focused at early detection of cancer. For this purpose a glycan profiling method with a MALDI-FTICR-MS platform was established and applied to a pancreatic cancer cohort.

In Chapter 1 of this thesis an overview was provided on cancer, with the emphasis on pancreatic and breast cancer from a clinical point of view. In addition a general introduction to glycosylation and glycan analysis was given.

The second part of this thesis, consisting of Chapters 2, 3 and 4, focuses on method development for glycosylation analysis. A high-throughput method including MALDI-TOF-MS for the analysis of *N*-glycans from serum was reported earlier and this method was used as a starting point for the development of the method described in Chapter 2. Points of improvement are the prevention of side reactions and the automation of cotton-HILIC SPE. Moreover, the glycans are analyzed with ultrahigh resolution MALDI-FTICR-MS, resulting in resolved instead of overlapping peaks and in highly confident glycan assignments. In Chapter 3 the method from the previous chapter is extended to the analysis of DBS instead of serum. For clinical applications the sampling of a DBS is desired as it is less invasive and cumbersome than the collection of venous blood specimens. A method for the analysis of released glycans from DBS was developed and evaluated for different storage conditions. Chapter 4 elaborates on the MALDI-FTICR-MS measurements of glycans from complex samples and explores the use of ultrahigh-resolution approaches for the comprehensive detection of *N*- and *O*-glycan signals over a vast *m*/*z* range.

Chapter 5 describes the application of the method from Chapter 2 to a pancreatic cancer biomarker study. Here, case and control samples from two independent cohorts are compared for their *N*-glycosylation profiles. This allows to study the glycosylation differences between pancreatic cancer patients and healthy controls and might in future be used for the improved diagnosis of pancreatic cancer. In Chapter 6 a similar method is applied to a breast cancer case-control biomarker study. Here, the focus is on the comparison of the results with findings described in literature.

In Chapter 7 a general discussion is given on the work described in the previous chapters. Finally, a perspective is provided on method development and translational research.

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