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

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CHAPTER 7

Discussion and future perspectives

From method development to a standardized protocol

In this thesis, automated analysis of released *N*-glycans from serum and plasma proteins is described (Chapter 2). This analytical method was extended with a protocol for the analysis of released glycans from dried blood spots (DBS) (Chapter 3) and the MS-part of the protocol was further elaborated on, with increased resolving power of the measurements that elucidated overlapping signals of isobaric *N*-glycans and small *O*-glycans in the MALDI-matrix region of the mass spectrum (Chapter 4).

Sample preparation, automation and MS measurements

Method development involves a stepwise process in which an existing protocol is further optimized or alternatively a method is established from scratch. Both approaches require a combination of new knowledge with the latest innovations. For the automated *N*-glycosylation profiling method that was developed in this study, a previously established protocol was used as a starting point.[1] The initial protocol was improved through multiple steps. To begin with, the pH in the enzymatic release reaction was lowered to prevent side reactions in the subsequent derivatization reaction. Second, a manual solid phase extraction (SPE) protocol based on cotton hydrophilic interaction liquid chromatography (HILIC) that is generally used in our research group[2,3] was converted into an automated version. And last, MALDI-FTICR-MS was introduced for glycan profiling instead of MALDI-TOF-MS to improve resolving power and accuracies of the mass measurements.

In a previous study multiple byproducts and fragmentations of glycans were reported after detailed analysis of the glycan mass spectra, which complicate the interpretation (peak assignments) of the spectrum.[4] Although application of MALDI-FTICR-MS allowed resolving overlapping signals, still additional signals resulting from unwanted side-reactions were present in the spectra that potentially bias the (relative) quantitation part. To prevent side reactions at the reducing end of the glycan, the pH of the release solution was lowered to enhance hydrolysis of the enzymatic release product, which contains an amine group at the reducing end (Chapter 2). This had to be carefully balanced, because a low pH can lead to protein precipitation, including the precipitation of the release enzyme. Moreover, the activity of protein *N*-glycosidase F (PNGase F) can be affected by a lower pH, or turn inactive at pH<4.0.[5]

The inclusion and automation of a cotton HILIC-based purification instead of the previous filter-plate method was important for the glycan detectability: from experience it was known that in MALDI-TOF-MS analysis the glycans in the *m*/*z* range larger than 3100 were lower in intensity when purified with the previous

method[1] compared to manual cotton HILIC methods.[2,3] This difference can be explained by either a lower recovery of larger glycans or a suppressing effect from other compounds present after filter-plate purification. The automation of a manual protocol is often not a one-to-one transferable process and in the case of cotton HILIC purification adjustments were needed. In manual pipetting processes of the samples the liquid level and SPE material can be carefully controlled, whereas this is standardized in an automated procedure. Especially for cotton HILIC SPE this was challenging since the cotton strand should remain at the end of the tip at all time during the pipetting procedure. Manipulation of the liquid can loosen the cotton strand and visually checking the potential movement of this strand is necessary. To solve this for the automated pipetting platform, porous frits were added to the pipetting tips, forcing the cotton strand to stay at the end of the tip, while letting the liquid pass. Control of the purification was ensured without the need of human involvement. This change of purification method resulted in improved detection of glycans in the higher mass range (m/z>3100), which especially in combination with MALDI-FTICR-MS resulted into the inclusion of more *N*-glycan species in relative quantification analysis.

Glycosylation profiling with MALDI-FTICR-MS allowed the detection of 112 different glycan compositions, with MALDI-TOF-MS at 86 different compositions (Chapter 2).[6] Although the FTICR-MS spectra exhibit a high resolving power, not all signals were fully resolved with the initial measurement method. Importantly, these glycan signals could be identified upon implementation of absorption mode data processing instead of the initially used magnitude mode (Chapter 4).[7] It is noted that absorption mode spectra improve identifications in profiling, but are not suited for relative quantification due to "negative" peaks near the analyte signals that originate from phasing artefacts. In future, absorption mode measurements might be used for relative quantification, when phasing software is able to prevent this baseline distortion or extraction software is able to cope with these peaks. Still, for the detection and confident identification of overlapping species this measurement mode is a valuable addition to the magnitude mode measurements.

Sample collection and the importance of standardization

The developed released *N*-glycan protocol can be widely applied to any serum or plasma cohort for which *N*-glycosylation profiles are the anticipated data (Chapter 2). This was exemplified in a pancreatic cancer case-control cohort (Chapter 5). In addition, the released *N*-glycan protocol is suited for DBS samples with a specific extension (Chapter 3), which would be an easier way of collecting samples from patients, as it even can be collected by the patient self.

Most biomarker studies have been carried out on retrospective cohorts

of clinical blood samples, that is material previously collected. The pre-analytical variables are therefore not necessarily optimal for the analytes and/or research question. Moreover, the primary concern of the clinician lies on the health and well-being of the patient and as a consequence the procedure and execution of sample collection has lower priority.[8] Research efforts and anticipated clinical diagnostics increasingly shift from a wide range of generally applied biomarkers to specific biomarkers analyzed in high-end assays to aim for personalized medicine and study the biology of an individual. As the outcomes of these type of assays may be affected by the quality of the collected sample, analytical scientists should bring their quality requirements into the clinical field.[8]

A critical aspect of sample collection is that it requires standardization both when a patient sample is acquired in the clinic, but also in case the patient performs self-sampling. Without a clear and unambiguous protocol for sample collection and -storage, variation may be introduced, resulting in biases or random variation in the results. The standardization of the pre-analytical procedures is therefore as important as the standardization of the analytical method itself. The samples in the present studies (Chapter 5 and 6) were collected according to a standardized protocol and the pre-analytical procedures were performed in a specialized laboratory. However, not every sample collection center has this type of facility and resources, but also in a standard lab facility high levels of standardization should be urged. Moreover, there should be aimed for a national, European or even global type of standardization in sample collection for different types of analytes. To get this done, it might take a long time and much effort, but the great advantage of this would be the ability to collect large sample cohorts, also for relatively rare diseases, which will improve biomarker research.

In the standardization of a sampling procedure, it should be considered that patient lifestyle and also care decisions influence the sample and that the analytical profiles obtained from a patient may not represent the actual health status of a patient.[8] Additionally, when blood is in a tube, biological (enzymes), chemical (hydrolysis, oxidation) and physical (aggregation) degradation can occur in the sample, which might lead to alterations of the biomarkers in the sample.[8,9] However, MALDI-TOF-MS glycosylation profiles have shown robustness with regard to fluctuations in time before centrifugation of blood specimens, serum and plasma storage times, storage temperatures and tube additives (such as EDTA).[10]

Besides the sampling protocol, other aspects need to be considered, such as the type of sample vials used and the storage conditions. The type of matrix in the sample container or the material of the container itself can influence the analytes and therefore the outcome of the analysis.[8] Additionally, the storage temperature can influence the degradation of the sample. It is, for example, known that in case of

protein analysis storage temperatures have significant effects on protein quantities.[11] Notably, glycosylation profiles did not differ for different storage temperatures. In addition, for DBS it was shown that even storage at 37 °C did not alter the glycosylation profile (Chapter 3).

To implement DBS collection into the clinic for patient sample collection spot quality needs to be further investigated. As the samples will be collected by different people (preferably the patients) information on 'good' and 'bad' spot quality for glycosylation analysis needs to be available. In addition, the stability of glycosylation profiles over a longer time period should be studied, as spots might need to be preserved as reference sample for new measurements in case of longitudinal sample collection.

Longitudinal samples can be measured at various time points without inclusion of the previous sample as a reference, provided the performance of the analytical protocol and instruments is kept stable over time. The technical variability over time should be minimized and a short-term version of this was already shown (Chapter 2). The technical variation between sample-batches and measurement days was determined, resulting in a less than 10% average variation of the ten most abundant glycans in the spectra.[6] We see this as an acceptable variation in our method, however lowering this number would make our method more sensitive for biological variation, which is in cohort analysis of course the preferred variation found. With the current technical variation probably subtle differences between cases and controls are missed, while these could provide essential information about glycosylation changes in disease. To decrease technical variation, attention should be paid to the last part of the protocol: MALDI-spotting and MALDI-MS analysis. It is known that sDHB-matrix does not dry as a homogeneous crystal layer, but very inhomogeneous with large crystals and partially empty spots. Averaging multiple shots at the spots helps to obtain a relatively stable spectrum, but still variation is introduced. The development of a homogeneously drying matrix suitable for released glycan measurement might therefore be necessary to decrease the total technical variation of measurements in the future.

The use of software for data-processing and interpretation

Multiple software tools were used for high-throughput data processing and -analysis of the obtained MS spectra. One of the software tools used throughout this thesis is in-house developed MassyTools (Chapter 2, 3, 5 and 6).[12] Additionally, specific scripts in R software tool were available for the calculation of derived traits, which enables us to analyze shared features of glycans, such as the branching, fucosylation, sialylation and bisection (Chapter 5 and 6).

These tools are useful and essential for fast processing of mass spectrometry

data from for example released glycans. In addition, the automated workflow minimizes the human intervention, for which also the risk of human mistakes is decreased. However, often human involvement is required for interpretation and to monitor the critical steps in a procedure. The software can still output processed data, without necessarily showing that there was an irregularity in the processing. This was for example the case in our MassyTools sample processing, where peak extraction is performed directly after calibration. An overview of the quality of the calibration of all spectra is not displayed (it is outputted per spectrum), although this is information should be evaluated before the start of the extraction. A bad calibration can highly influence the data obtained after extraction and therefore also the results of a study. For this reason, in the described studies the parts-per-million (PPM)-errors of the calibrants in all spectra were evaluated manually before peak extraction. The summarized output of crucial information about the quality of each processing step would be a valuable and important addition to data processing software tools.

Understanding of the software and the acquired data is essential for the right interpretation of the data and to answer the research questions with the right information. Therefore, software should not be treated as a black box, but be evaluated if it meets the purpose of the research and fits the type of data. To illustrate this, software can be used to find an elemental composition for an observed m/z[13], however, these type of software tools often do not differentiate between molecular compositions that are possible according to chemistry and random compositions coincidently matching the m/z.

Also, in glycosylation and MS analysis software tools should be used with caution as a tool to help human work but not to replace it completely. In glycosylation analysis the biosynthetic pathway should be taken into account when identifying MS-signals, as the inclusion of compositions that are not in accordance with the biosynthesis can complicate the interpretation of the data or spread 'fake' results into the field. Additionally, user-defined settings should be re-evaluated when changing systems. An example for this is the extraction width (in the mass window) of signals in MALDI-TOF-MS and MALDI-FTICR-MS spectra. For MALDI-TOF-MS spectra a single extraction width was sufficient, whereas for MALDI-FTICR-MS every analyte required a specific extraction window (Chapter 2).

A certain understanding of glycobiology is important for interpretation of results, as is demonstrated in this thesis (Chapter 5). For example, in the pancreatic cancer study, glycan traits A3F0S and A4F0S showed a decrease in patients while A3FS and A4FS were observed at elevated levels. When interpreting each trait individually it could be concluded that sialylation levels are changing in patients. However, by interpretation of all traits together it is far more likely that the effect

is caused by the change in fucosylation. In the case of A4F0E this might also be the case, as generally the α2,6-linked sialylation in increasing, but the non-fucosylated fraction of glycans is decreasing and appears to be the stronger effect in these glycans. Additionally, derived traits only make sense when most of the glycans in one trait show the same effect, and the resulting change described in this derived trait is not the result of for example one single glycan. Without human interpretation of the data the conclusions on this data could have been significantly different.

Limitations of total plasma *N*-glycome analysis

MALDI-FTICR-MS analysis of released *N*-glycans is a useful method for screening changes in the *N*-glycome of blood-based samples. The method is rapid, almost fully automated and exhibits a good repeatability. However, as every method, this method also has its limitations.

The most important one is that the glycan information represents a summary of all glycoproteins in the blood. The released glycans are obtained from many different glycoproteins and end up in the same mixture, so the glycans cannot be traced back to their originating glycoprotein or to the glycosylation site on that protein. Although for some glycoproteins it is known what type of glycans they carry[14], it can only be speculated from a total serum *N*-glycome profile which glycoproteins are involved in causing specific signatures and glycosylation differences. Nonetheless, global serum glycosylation analysis can still indicate what the potentially interesting glycoproteins for a follow-up study would be and give insights in major changes occurring in disease.

A second limitation is found in the MALDI-MS readout that cannot distinguish all potential isomers, although sialic acids are detected with linkage-specificity, due to the derivatization method applied. For other isomers complementary methods are required, for example separation methods (e.g. liquid chromatography or capillary electrophoresis). In cancer analysis (Chapter 5 and 6), it would for example be beneficial to know the location of the fucose on a glycan, as it turns out that fucosylation is strongly increased in pancreatic cancer (Chapter 5), but that from the current data it cannot be derived whether this increase solely involves antenna-fucoses and may be linked to the formation of sialyl-Lewis X (sLeX) moieties or that also core fucosylation is changing.

Clinical translation

Many studies on biomarker research have been performed in the past and although a great potential was foreseen, only a very limited number of biomarkers have successfully been translated into the clinic.[15,16] Multiple reasons for this translation gap have been discussed, such as the complexity of diseases and lack of knowledge about the specific diseases. In addition, technical challenges and the use of inappropriate samples and study design for the research question or validation have been mentioned.[17] The latter is also related to the collection of the samples, which has been discussed in this thesis. Here, close collaboration between clinicians, the clinical laboratory and research scientists is important.

Translational research

A screening test should meet specific requirements, of which one of the most important is that it should identify a disease when it is present (sensitivity) and rule out the disease when it is not present (specificity). In many cancer biomarker studies this criterion is not met sufficiently, leading to false positives and false negatives.[18] To bring a test into the clinic, this clinical validity is highly important as it should perform better than the currently used diagnostic method, which is often called the 'gold standard'.[19] A new clinical test should also generally provide benefits as compared to the previous one. For example, it should not include disproportionate increase of invasiveness or risk for patients. In addition, the analytical validity of a test, as was already discussed above, is highly important for a method to be transferred into the clinic.

As mentioned earlier, a limited number of biomarkers were successfully translated into a clinical test and multiple reasons for this where mentioned.[15,17] For this reason, an international initiative suggested that for the diagnosis of diseases improvements in biomarker selection should be made and that clinical development should focus more on unmet clinical needs.[20] The focus of biomarker research is not always directed towards shortcomings in clinical practice, but might also be influenced by non-clinical factors such as technological innovation (not necessarily for healthcare purposes) and financial pressure.[20] To stimulate the translation of biomarkers into the clinic in future, efforts should be made to deal with these aspects of research.

Cancer biology and blood-based cancer detection

Tumors are complex tissues, consisting of multiple cell types with various types of interactions.[21] Not only the tumor cells themselves, but also the surrounding tissues are involved in the development of the cancer.[21] Studies of human cancers

have shown that development of tumors is a result of subsequent genetic changes which provide an advantage for tumor cell growth which ultimately leads to the transformation of normal cells into malignant tumor cells.[22] In the year 2000, Hanahan and Weinberg suggested six essential changes in cell physiology that together are the basis of cancer growth, namely self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis.[22] Later, it was proposed to also add immune hallmarks of cancer to this list.[23] In 2011 the concept of cancer hallmarks was reviewed and additional changes were added, namely genome instability and mutation, tumor promoting inflammation and also deregulating cellular energetics and avoiding immune destruction.[21] The involvement of glycosylation in these various hallmarks has been overviewed by Pearce et al., showing that different types of glycan groups play a role in cancer development.[24] It should be noted that the hallmarks of cancer are not exclusively true for cancer, but many of them can for example also apply to benign tumors which are unlikely to become malignant.[25]

In biomarker research the overlap of physiological processes in different diseases is often a challenge as it can highly influence the performance of a potential clinical test. In this thesis (Chapter 5) it was already discussed that inflammatory glycosylation changes were found in pancreatic cancer patients, which was explained by cancer related inflammation of the tumor microenvironment.[26] Here, it was also noted that the specificity of the analysis should be further evaluated in a cohort setting containing other cancer types and benign diseases, as this would better mimic a clinical setting. In other words, care has to be taken when drawing conclusions on specificity from a pure case-control study. In addition, as blood travels through the whole body and the sample is thus not locally derived at the tumor, it might be challenging to pinpoint where the alterations of blood-derived analytes are originating from.

In 2018, a prime example was presented that combined multiple types of blood-derived biomarkers (genetic- and protein markers) to identify the presence of cancer and the organ it originated from.[27] Unfortunately, this test appeared to be very sensitive for some cancers (ovary and liver), but for example for breast cancer the sensitivity of the test was poor.[27] Nevertheless, the concept of combining multiple analytes to increase sensitivity and specificity of blood-tests for cancer diagnosis was shown to be effective and this might be the way to go in the future. To this end, glycosylation is a valuable add-on to the genetic- and protein markers as was exemplified (Chapter 5) with profiles from pancreatic cancer patients that significantly differed from those of healthy controls.

Besides the use of these glycosylation changes as biomarkers, attention should also be paid to the origin of these changes. Understanding should be gained

on why the glycosylation of proteins in blood is changing and if this change serves a function in the progression of the disease. From cell-surface glycosylation it is known that it has a function in tumor development, but for secreted proteins this is not well understood.[28–30]

Concluding remarks

In summary, this thesis provides a high-throughput analysis method for released *N*-glycans including enzymatic release and automated sialic acid linkage-specific derivatization, cotton HILIC purification and MALDI-target spotting, for analysis of samples with high resolution MALDI-FTICR-MS (Chapter 2). It also describes the extension of this method for dried blood spots (Chapter 3) and approaches to further increase resolution to resolve overlapping species (Chapter 4). The automated method was applied to a pancreatic cancer study (Chapter 5), where glycosylation profiles were obtained from total serum and classification analysis was performed. A similar method was used for the total serum *N*-glycosylation analysis of breast cancer cases and controls (Chapter 6).

To translate biomarkers into the clinic, the analytical- and clinical validity of a test should always be critically evaluated. Samples should be collected in a standardized manner and (pre-)analytical protocols should be unambiguous in use. In addition, automation of methods and data analysis software can be very useful in terms of fast processing and repeatability, but critical steps of the procedures should always be critically monitored to ensure the quality of the conclusions drawn from the data. General glycosylation analysis from blood-derived samples can give a relatively simple and fast overview of the glycosylation changes with disease, but has its limitations in terms of origin of the glycans and isomer analysis. Therefore, the use of complementary analytical techniques is recommended for studies following total plasma/serum *N*-glycome analysis.

In the past, only limited number of biomarkers were successfully translated into the clinic, which might be the result of the complexity of the diseases, technical challenges and factors distracting the focus of the clinical needs. In future, much progress on overcoming these challenges should be made. In addition, overlapping physiological changes between diseases challenge the specificity of tests. Here, combining multiple types of analytes in one test might enable us in the future to better distinguish between different diseases and might ultimately lead to a blood test for the diagnosis of different types of cancer.

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