Glyco(proteo)mic workflows for cancer biomarker discovery

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Chapter 6

Discussion and Perspectives

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Glycosylation is highly amenable to surrounding influences in both healthy and disease microenvironments. As a result, this protein modification has great potential for the discovery and development of biomarkers. Thus, this thesis aimed to uncover unique glyco(proteo)mic signatures in prostate (PCa) and colorectal (CRC) cancer via the respective development of intact protein and released glycan MS workflows. Several advancements were made with regard to the methodology and new insights were gained regarding the respective samples. Nevertheless, several technical challenges were encountered for the developed platforms. In this chapter, potential improvements are discussed as well as future directions and necessary methodological developments for the described biomarkers in order to facilitate translation of the results beyond the discovery phase.

Sialylated and Fucosylated Isomeric Glycans

Sialylation and fucosylation are important contributors to the development of cancer[1] and isomers of these glycosylation features play different roles during normal and metastatic cellular function. In **Chapter 4**, we demonstrated a method that allowed efficient isomer separation (sialylation and fucosylation) as well as mass differentiation (sialylation) using a C18 column on a RPLC-MS system. In this regard, linkage-specific derivatization of sialic acids was performed whereby α2,3- and α2,6-sialic acids underwent amidation (–0.98 Da) and ethyl esterification (+28.02 Da), respectively. Interestingly, it was observed that structures with an ethyl esterified α2,6-sialic acid showed a longer retention time than their amidated α2,3-sialic acid counterpart. This is likely due to the greater capacity of the amide group to form hydrogen bonds with water molecules[2] than the ester group, thus increasing its hydrophilicity.[3–5] In addition, the ester group increases the hydrophobicity of the molecule due to the propensity of its nonpolar carbonyl group to form nonpolar interactions. Additionally, fucoses are relatively apolar in comparison with other monosaccharides. Thus, a core fucose, which is located more closely to the fluorescent label than the antennary fucose, may affect the interaction of the glycan with the stationary phase, resulting in a longer retention time.[6] Importantly, the clinical relevance of isomer differentiation was highlighted in **Chapter 5** whereby significant differences in the relative abundances of

sialic acid-linkage and fucosylated isomers were observed between pre- and post-operative CRC patients.

There have been continuous technological developments which have also demonstrated efficient differentiation of sialylated and fucosylated isomeric species. For example, ion-mobility MS (IMS) has been applied to analyze released *N*-glycans.[7–10] In the study by Manz *et al.*, it was demonstrated that a fragment ion corresponding to the sialylated antenna from two chromatographic peaks containing H5N4S2 showed different drift times.[10] Consequently, the ratio of α2,3- to α2,6-linkage could be determined based on the extracted ion mobilograms. Furthermore, in addition to the MS/MS spectra, antennary fucosylated fragment ions were also confirmed using ion-mobility.[10] Those results were illustrated using HILIC yet, undoubtedly, it would be interesting to hyphenate IMS with the RPLC platform presented in **Chapter 4** in order to assess isomers with different branching positions. Notably, sialic acid derivatization is still required on this platform as we demonstrated that the derivatization procedure is important in order enable retention of these sugar residues using a C18 column. In addition, sialic acids are known to be very labile monosaccharides and during ionization sialic acid loss can be observed.[11] In this case, derivatization has been shown to have an important stabilizing effect on sialic acids.[12,13]

Another interesting approach is the investigation of ion ratios in order to identify isomers containing antenna with specifically linked sialic acids.[14] Wagt *et al.* built on the work of previous studies[8,15] with their analysis of H5N4$S_{2,3}$1$S_{2,6}$1 isomers in the total plasma *N*-glycome and fetuin. They observed that the ratio of specific Y-ions indicated a more prominent loss of the 3′-linked antenna compared to the 6′-linked antenna following fragmentation, regardless of the sialic acid linkage. Similarly, recent work by Maliepaard and co-workers reported that sialic acid linkages as well as 3′- and 6′-linked antennae could be determined based on the relative intensities of glycan oxonium ions in isomerically defined glycopeptide standards. In this case, the authors also noticed that 3'-linked saccharides already fragmented at lower collision energies than their 6′-linked counterpart.[16] Interestingly, we also observed different fragment Y-ion ratios of sialylated tri-antennary glycans in **Chapter 4**. Although this phenomenon was not explored by our study, the aforementioned findings suggest that the fragment ion ratios could be further investigated in order to examine antenna occupation by specifically-linked sialic acids.[14,16]

Biomarker Discovery: Technical Challenges

To define the requirements for each stage of disease biomarker research and development, a set of guidelines have been developed for proteomic methods.[17] These tiers range from discovery (3) via validation (2) to clinical application (1). As a result, the field of glycomics can make use of such guidelines for the development of glycosylation-focused biomarkers. With regard to biomarker discovery, the requirements for tier 3 methods are less stringent and important parameters include the specificity of analyte identification as well as the repeatability of the measurement.[17] Naturally, development towards a tier 2 method requires even greater performance in these two areas as well as others, such as measurement precision. Finally, tier 1 methods require the highest degree of analytical validation, as well as reference standards, precision, and quantitative results. In relation to this, labeled internal standards are suggested for both tier 1 and tier 2 assays. However, it should also be noted that glycomics faces specific challenges, which hinders the field from meeting some of these criteria. For example, the development of isotopically-labeled internal standards for glycomics is problematic owing to the non-template driven biosynthesis of carbohydrates.[18] In this case, it may only be possible to produce specific isotope-labeled glycan standards which would present challenges in the case of a multipanel biomarker test. In addition, the sensitivity of the measurement may also affect meeting the aforementioned requirements. In this case, sufficient sensitivity must be achieved during an analytical measurement in order to reach an adequate signal-to-noise (S/N) ratio for quantification.[19] This ensures that the analyte signal may be differentiated from the surrounding noise which often results in more confident identifications via improved mass accuracy and isotopic pattern, as well as a more reliable quantification.

Sensitivity

Sensitivity during denaturing intact protein MS analysis may be compromised as proteins become multiply charged during ESI, resulting in an analyte signal that is diluted over the protein charge envelope. Any inhibition to sensitivity can be problematic particularly in cases whereby the proteoform of interest has a low abundance. This was observed in **Chapter 2** as low abundant cleaved PSA proteoforms were not identified in some patients, possibly due to a lower amount of

captured prostate-specific antigen (PSA). This created difficulties in the comparisons between patients as the relative abundances were skewed when total area normalization was applied (data not shown). In order to overcome these challenges, there are several approaches that could be followed. As mentioned in **Chapter 2**, the analysis of urine following digital rectal examination is expected to contain a higher PSA concentration.[20] Furthermore, online concentration techniques in the field of CE could also be applied, including sample stacking and transient isotachophoresis.[21] Despite this, the distortion of the relative abundances highlights an important limitation with regard to performing relative quantification that could also present further challenges during subsequent development and translation of the method.[22] We also investigated normalization based upon the most abundant peak (data not shown), however this resulted in greater relative standard deviations. In a clinical context, absolute quantification using an internal standard is usually the preferred approach.[18,23] This was achieved by Jian *et al.* when performing intact protein analysis of a recombinant human monoclonal antibody (mAb) by using a stable-isotope labeled analogue.[24] In this case, the analogue protein contains amino acids (arginine and lysine) that are heavy isotope labeled ($^{13}C_6$ and $^{15}N_4$). The analogue protein was spiked at known concentrations into the non-labeled sample prior to sample preparation and the resulting deconvoluted spectra shows the masses of both the non-labeled mAb and the internal standard. Thus, by comparing the peak intensity ratio of the most abundant peak from the non-labeled mAb and its analogue, absolute quantification was performed using the calibration curve that was constructed across the concentration range of the spiked internal standard. Importantly, the authors described that the variability during sample preparation and MS measurement is compensated by the internal standard. In order to apply this approach to our workflow (**Chapters 2** and **3**), a PSA standard could be cultured and extracted from an isotope label-enriched medium. Ultimately, the choice of quantification approach must be fit-for-purpose according to the stage of research that is being conducted and, most importantly, must be suitable for analyzing patient samples when conducting clinical research.

The released *N*-glycan RPLC-FD-MS workflow demonstrated in **Chapters 4** and **5** showed a lower number of *N*-glycan assignments and, subsequently, poorer sensitivity in comparison with CE-ESI-[25] and MALDI-MS[26] approaches. In this sense, coupling

the RPLC system with a high-performing MS would likely result in a greater number of *N*-glycan identifications. Furthermore, one of the advantages of RPLC is the potential to use nanoflow columns which would also increase the sensitivity. However, it should be noted that current fluorometers are not capable of performing detection at such low flow rates, thus this would greatly inhibit the advancements that were achieved with regard to the FD-MS quantification approach. A straightforward approach to further improve the sensitivity of this method would be by increasing the amount of *N*-glycans injected onto the column. For example, the developed workflow utilizes only 0.6 µL of plasma for sialic acid derivatization and 57 nL of plasma released *N*-glycans are injected onto the column, equivalent to 1% of the starting material. Despite this, it is challenging to use a volume more than 3 µL, containing 0.6 µL of plasma, for the derivatization reaction as an increase in the sample volume requires a proportional increase in the other reaction components, including subsequent CH_3CN addition. Importantly, CH_3CN must reach a suitable concentration so that the glycans may bind to the HILIC plate during clean-up. In **Chapter 4**, this limit was already reached as the reaction volume (75 µL) was quenched with the addition of CH_3CN (225 µL, 87.5%), resulting in a final volume (300 µL) that was at the maximum of the well capacity.

The use of glycan-specific beads during sample preparation would be an interesting approach to overcome volume constraints as well as multiple vacuum centrifuge steps. In addition, this method would theoretically allow the entire amount of plasma-released *N*-glycans to undergo fluorescent labeling and sialic acid derivatization. For example, Váradi *et al.* demonstrated a carboxyl coated magnetic beads protocol for efficient clean-up following PNGase F release and fluorescent labeling.[27] However, complications may arise during the sialic acid derivatization reaction due to the reactivity of the carboxyl groups on the beads with EDC and HOBt, similar to the derivatization reaction itself.[28] Another study reported by Nishikaze *et al.* illustrated a linkage-specific sialic acid derivatization approach using hydrazide beads which are chemoselective for aldehyde groups on the glycan reducing-end.[29] Interestingly, sialic acid derivatized *N*-glycans were eluted in water and reduced to dryness using a vacuum centrifuge which was followed by reducing-end labeling with 2-aminobenzoic acid (2-AA). Other studies reported direct labeling of the glycan reducing-end following release from the beads.[30,31] Therefore, it would be interesting to evaluate a procedure that allows on-bead linkage-specific sialic acid derivatization followed directly by

fluorescent labeling in order to achieve a true “one-pot” protocol. Overall, these modifications to the protocol would improve both the sensitivity and throughput, both of which are important parameters for further clinical translation.

Future Directions for Glyco(proteo)mic Biomarkers

Prostate-Specific Antigen

The PSA test measures the concentration of total PSA (tPSA) in serum, however tPSA consists of various proteoforms, each of which may be associated with different prostate diseases, including PCa.[32] With regard to other biological matrices, such as urine, less is known regarding the correlation of PSA proteoforms with PCa and other prostate conditions. For example, cleaved PSA proteoforms have not been described in previous reviews regarding urinary markers for PCa.[33–36] In addition, reports on urinary PSA mainly focus on variations of tPSA concentrations in urine and serum,[37] rather than assessing specific proteoforms of this protein. As previously mentioned, PSA glycosylation traits have been cited as a potential target for improving PCa diagnosis. However, a recent study assessing PSA glycosylation in urine did not show any differentiation between PCa and non-PCa groups.[38] As a result, the validity of urinary PSA proteoforms for patient stratification must still be investigated. For example, benignPSA (bPSA) proteoforms are associated with the development of benign prostate hyperplasia (BPH)[39] and, in this case, **Chapters 2** and **3** showed promising results as bPSA was detected in the urinary PSA profile. Interestingly, the clinical utility of bPSA was examined previously in sera using an immunoassay containing antibodies specific for cleaved bPSA (single Lys_{206} or double Lys_{169}, Lys_{206} cleavage) whereby it was reported that bPSA was significantly increased in BPH.[40,41] However, a small sample size was used in **Chapter 2** and, therefore, further investigations on urinary PSA proteoforms should be performed as part of a larger study. The analysis of a larger cohort may then be combined with the data processing workflow presented in **Chapter 3** in order to derive clinically significant results.

Despite this, it is expected that BPH exists frequently in older men regardless of whether cancer is present[40] and, therefore, bPSA should be measured alongside other cancer-specific forms of PSA, such as proPSA.[42] It should be noted that PSA proteoforms such as complexed PSA and proPSA were not detected with the setup

used in **Chapter 2**. A possible explanation could be that complexed PSA is found in serum when mainly protease inhibitor alpha 1-antichymotrypsin forms a complex with intact active PSA.[43] Alpha 1-antichymotrypsin is a serum glycoprotein[44] and thus it may be less likely that it is present in urine in order to form complexes with PSA. With regard to proPSA proteoforms, it is possible that the sensitivity of the developed method in **Chapter 2** is currently not high enough in order to detect intact proPSA. Kitata *et al.* demonstrated that proPSA is present in urine, albeit at approximately 1% of the active PSA concentration.[45] Interestingly, that study also analyzed a small cohort of PCa patients and showed that a panel consisting of a combination of proPSA forms could distinguish PCa patients from healthy controls.[45] In addition, the authors reported that Lys-C digestion resulted in longer proPSA peptides (9 – 16 amino acids) which is more suitable for MS analysis using selected-reaction monitoring.[45] Thus, this digestion strategy could also be applied to our workflow (**Chapter 2**) in order to investigate whether the antibodies used in the immunocapture protocol are also specific for proPSA.

Total Serum *N*-glycosylation

The potential of the total plasma/serum *N*-glycome as a cancer biomarker has been evaluated across multiple conditions, including investigations into lung,[46] breast,[47,48] stomach,[49,50] pancreatic,[51] prostate,[52] and colorectal[26] cancer.[53] Furthermore, there are several reports which perform characterization of the plasma/serum *N*-glycome, thus providing further in-depth information in relation to the sample.[14,54,55] Despite these promising results, it has been described that serum *N*-glycome profiles display large inter-individual differences[56] and are influenced by several factors including age,[57] sex,[58] and lifestyle.[59] For example, in **Chapter 5** we observed significant differences in the pre-operative relative abundances of specific *N*-glycans amongst different histological types in CRC. However, it could be argued which differences attribute to the progression of the disease in comparison with those differences that are already present between individuals in the general population. Thus, it is not yet clear to what extent inter-individual differences may confound the results and how such measurements can be integrated into current clinical pathways. In order to overcome this challenge, Hennig and co-workers describe that longitudinal analysis of the human plasma/serum *N*-glycome may be the best course to follow.[53] In this regard, Hennig *et al.* demonstrated that a significant difference in an individuals' plasma *N*-

glycome in relation to external events such as injury or illness could be observed over time.[53] Furthermore, the authors also showed that significant changes in one individuals' profile over time due to such events were not observed when compared with the profile of all healthy volunteers,[53] suggesting that the specificity of *N*-glycan biomarkers found in plasma and serum may be hindered in studies that measure only a single timepoint. Interestingly, our study in **Chapter 5** illustrated that pre-surgery differences in the relative abundances of specific *N*-glycans between histological types were eradicated when surgery was performed. In this sense, it would be interesting to further evaluate the change over time of the serum *N*-glycome as this could reveal the relative impact of events such as disease or surgery, which might otherwise be hidden due to already prevalent inter-individual differences. In this manner, a personal serum *N*-glycome "fingerprint" may be developed and used to monitor an individuals' response to internal or external events, thus further developing a personalized approach to medicine.

Perspectives on Biomarker and Method Translation

A disparity exists between the large number of potential biomarkers that have been reported by "omics" techniques and the small proportion that find their way into clinical implementation.[60] This is partially due to poor study design which is reflected by the lack of reproducibility of results between studies.[60,61] Furthermore, with regard to the development of MS-based assays, the aforementioned guidelines[17] do not encompass some critical aspects of clinical research such as clinical validity and effectiveness.[62] In this sense, it is imperative to define the unmet clinical need early on in the development process so that outcomes related to clinical validity and effectiveness may also be defined.[63] In comparison, non-MS based tests have had relatively greater success with regard to clinical implementation. Immunoassays, such as enzyme-linked immunosorbent assays (ELISA), are the preferred technique in clinical laboratories[64–66] mainly due to their high sensitivity, throughput, speed, and they allow absolute quantification of the proteins of interest, provided an antibody is available. With regard to glycomics, a plate-based assay approach may also facilitate easier implementation within the clinics and various techniques including immunoassays,[67] lectin-[68] and glycosidase-based[69] assays are being investigated.

Although plate-based assays such as immunoassays are more established in the clinics, there are also significant challenges associated with the technique. For example, there is large variability between platforms, selectivity may be hampered by autoantibodies, and sensitivity is susceptible to any change that may affect protein-antibody binding.[18] Moreover, proteoform heterogeneity is generally not distinguished by antibodies.[70] As a result, method transfer between immunoassay and MS techniques can take considerable time and effort, possibly due to the lack of specificity in which the former detects proteoforms.[70] Such challenges may be overcome via the application of techniques with greater specificity that are capable of detection without antibodies. In this regard, online separation techniques, such as CE and LC, with optical detection show promise in relation to biomarker translation. Importantly, the biomarker profile should be previously characterized using MS. In any case, some advantages offered by optical detection techniques includes the generation of readouts that are easier to interpret than mass spectra, absolute quantification in combination with a standard curve, and lower training requirements for operators. These technologies have already been implemented in order to measure hemoglobin proteins in diabetic[71,72] as well as thalassaemic[73] disorders, and glycomic applications are also under development.[74,75] In this sense, an approach incorporating optical detection could be taken in order to further translate the methods and results presented in this thesis.

As previously mentioned, the feasibility of patient differentiation using urinary PSA proteoforms (**Chapter 2**) still needs to be assessed. Based on the advancement of the data processing workflow that was achieved in **Chapter 3**, the method is now set for investigating a larger cohort of PCa patients. In this case, MS detection and quantification is still recommended as specificity with regard to proteoform detection and their association with various PCa groups must be examined. However, further translation of the method could be considered by transferring the technique onto a capillary zone electrophoresis-ultraviolet light (CZE-UV) platform, as previously mentioned. Farina-Gomez *et al.* analyzed standard seminal PSA and demonstrated the separation of ten electrophoretic peaks using CZE-UV in normal polarity mode with an average RSD of 3.7%.[76] However, in that study the authors did not report the analysis of any patient samples nor directly characterize the proteoforms under each peak. Thus, cross-validation between the results of the two platforms would be

required in order to ensure that electrophoretic peaks detected by UV are the same as the proteoforms determined by MS. Interestingly, a similar approach was followed in **Chapter 5** whereby online LC separation was followed by MS detection and it was observed that the FD-MS quantification of *N*-glycans closely resembled the FD quantification of chromatographic peaks. Thus, the chromatographic peaks could be used as a surrogate signal for the *N*-glycan structures during further translation of the method. Similarly, fluorescent *N*-glycan signals have been used to determine the association between serum *N*-glycans and inflammation and biological age[77] as well as treatment escalation in inflammatory bowel disease.[78] Notably, the findings from those studies have been translated into commercially available products.[79,80]

Nevertheless, MS remains the 'holy grail' of biomarker detection and quantification, owing to the unparalleled levels of sensitivity and specificity that may be achieved with this technique. In addition, MS allows multiplexed quantification of various proteins and isoform differentiation.[70,81] In this regard, multiple reaction monitoring (MRM) approaches on triple quadrupole MS-systems are the most well-known examples of MS clinical implementation.[18,81–84] Furthermore, the application of intact transferrin glycoprofiling for the detection and differentiation of congenital disorders of glycosylation has also been shown.[18,85] Despite these examples, MS techniques have not generally entered into routine application within clinical practice.[86] To summarize, there are still significant challenges to be expected when implementing glycomics as well as MS in the clinical laboratory. These challenges, as outlined above, include the absence of internal standards for glycomics, poor study design, and a lack of demonstrable clinical validity and effectiveness. In relation to glycomics standards, a possible solution could be the synthesis of standards where a panel of only a few biomarkers are being used. Although in the case of the total serum *N*-glycome, which reports on global features of glycosylation, this would hardly be conceivable. Furthermore, other hurdles include test result standardization,[83,87] as well as maintaining overall instrument performance.[83] However, in regard to the latter, newly released instruments are focusing more on instrument calibration strategies, user-friendly interfaces, and automatable solutions for data processing and reporting whilst maintaining a high level of sensitivity and resolution.[88] These developments should improve consistent instrument performance and uptake amongst non-specialist operators.

Overall, the full potential of MS in clinical laboratories has yet to be fully realized. To achieve this, continued communication and collaboration among key stakeholders, such as clinical and analytical chemists, clinicians, as well as instrument vendors, is necessary. Ultimately the aim must be the translation of the biomarker and not necessarily the analytical method itself. Thus, the method that was involved in the discovery of the biomarker may not be the same method that is implemented for routine clinical measurement. Nevertheless, transfer between different techniques can take considerable effort and the results may not always be replicated. Accordingly, we must continuously strive to improve our methodologies to meet the needs for both biomarker discovery and translation to the clinical laboratory.

Concluding Remarks and Prospects

The workflows developed in this thesis followed an approach of demonstrating new insights regarding the biomarker profile of interest by applying newly improved methodologies. In both cases, the analytical performance was validated and the clinical validity of the analytical setup was also illustrated. Nevertheless, the presented approaches are at different stages of their respective lifecycles. For example, in relation to PCa and PSA, the approach described in **Chapters 2** and **3** must still undergo a true proof-of-concept study with a larger number of patient samples in order to determine whether urinary PSA proteoforms hold any association with the development of the disease. Furthermore, important questions must also still be addressed, including: *can proPSA be found in urine and, if so, why is it not detected using the CE-ESI-MS assay?* For example, the profile of seminal and serum PSA is already more established, yet the question remains: *What new insights could be gained from applying the in-depth analytical approach to study PSA from other biological fluids and what differences related to different disease conditions can be observed?* Following this, the workflow presented in **Chapters 4** and **5** is already at an advanced proof-of-concept stage and several serum *N*-glycomic signatures in CRC were corroborated. Despite this, further developments regarding the throughput of the sample preparation and sensitivity of the measurement are still required. Furthermore, the approach should be applied to a large cohort of cancer patients, primarily within a longitudinal study setup. It would be interesting to evaluate other types of samples with the method to further explore the structural and clinical relevance of various species of isomeric *N*-glycans. Finally, although this thesis adds important insights regarding the biomarkers of interest as well as methodological advancements, biomarker translation beyond the discovery phase is the main bottleneck with regard to biomarker implementation with the clinics. Thus, steps must continuously be taken in order to carry out well designed studies that address clearly defined unmet clinical needs which demonstrate obvious patient benefits.

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