Glycoproteomics assays for prostate cancer biomarker discovery

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Appendices

English summary

Prostate cancer (PCa) is the second most prevalent cancer among men worldwide when assessing age-standardized incidence rates. The primary method for early PCa diagnosis involves measuring the serum concentration of prostate-specific antigen (PSA), with elevated levels (> 3 ng/mL in the Netherlands; > 4 ng/mL in the US) indicating the potential presence of PCa. However, the conventional PSA test exhibits a low specificity. Thus, clinical challenges persist, including the differentiation between PCa and benign prostatic hyperplasia (BPH) and distinguishing indolent PCa from aggressive forms. This underscores the need for a more specific biomarker for early PCa detection and stratification. Previous studies have reported altered glycosylation features in two prostate-secreted glycoproteins, PSA and prostatic acid phosphatase (PAP) in PCa patients, e.g. variation in α-2,3 sialylation, fucosylation and the level of LacdiNAc[1]. The aim of the research described in this thesis was to identify PCa biomarkers for early detection and to improve patient stratification, focusing specifically on the glycomic profiles of PSA and PAP. In addition, as PSA plays an important role with regard to fertility, its glycosylation -in relation to male infertility- was also touched upon in this thesis. For this purpose, mass spectrometry (MS) based glycoproteomic methods were established to map the glycoprofiles of PSA and PAP derived from various biofluids.

Chapter 1 provides an overview of the background and unmet clinical needs in PCa and male infertility, providing insights into glycosylation, general analytical approaches in glycoproteomic studies, and the glycosylation of PAP and PSA in relation to PCa. In **Chapter 2**, an in-depth glycoproteomic assay was developed to investigate the glycosylation of PAP with capillary electrophoresis (CE)–MS. The straightforward assay was successfully applied on a pooled urine from patients that underwent a digital rectal examination (DRE). Three PAP *N*-linked glycosylation sites were identified and characterized, showing very diverse glycosylation features per site, including differences in sialic acid (SA) linkage-isomers. The developed assay possesses great potential for evaluating the biomarker value of PAP glycosylation in a PCa urine cohort. In **Chapter 3**, the relationship between PSA glycosylation and male infertility was explored. A high-throughput analytical method was established with matrix-assisted laser desorption/ionization (MALDI)-MS, implementing a two-step SA linkage-specific derivatization. By its application on a seminal plasma cohort, PSA glycosylation was found to be not indicative for male fertility conditions. Other seminal proteins should be investigated for their contribution to altered glycosylation of seminal plasma in male infertility. Interestingly, a novel feature – ketodeoxynononic acid (Kdn, an uncommon SA in mammals) – was observed on seminal PSA *N*-glycans for the first time (**Chapter 4**). Subsequently, it was also found in urinary PSA, and

1 Antenna structure where *N*-acetylgalactosamine is β1,4-linked to *N*-acetylglucosamine.

seminal and urinary PAP. The presence of Kdn-containing *N*-glycans on PSA and its exclusive α-2,3 linkage were confirmed with four complementary MS based analytical approaches. This inspired us to further explore the presence of Kdn on other, non-prostate originated, human glycoproteins by assessing publicly available datasets (**Chapter 6**). Intriguingly, Kdn-containing *N*-glycans was also observed on α1-acid glycoprotein from human plasma. More research is needed to study the occurrence and biological roles of Kdn on human glycoproteins. Whether it also plays a role in diseases, e.g. PCa, should be investigated. In this thesis, it is hypothesized that plasma PSA is largely produced by cancer cells and carries cancer-type glycosylation features, while urine PSA is largely produced by normal prostate cells and bears health-type glycosylation features thus can be used as a baseline for personalized diagnosis and prognosis of PCa. To verify this, the PSA glycoproteomic assay presented in **Chapter 3** was further expanded for plasma PSA analysis in PCa in **Chapter 5**. Using plasma comes with some challenges especially higher sensitivity is needed, therefore, reversed-phase liquid chromatography (RP-LC)-MS was employed. TMT multiplex labeling was introduced prior to SA derivatization which increased the sample measurement throughput, enhanced the ionization of PSA glycopeptides and allowed the simultaneous analysis of paired plasma and urine samples. The assay showed high sensitivity and allowed to reliably quantify 20 target analytes from 24 ng PSA derived from 4 mL plasma (6 ng/mL). A pilot study is ongoing analyzing a small cohort consisting paired plasma and urine samples from nine individuals (PCa and non-PCa). This should provide insights on the glycosylation differences between plasma and urine-derived PSA, the glycosylation differences between PCa and non-PCa in both biofluids and biomarker candidates of PCa. The thesis concludes with a discussion on the multiple bottom-up glycoproteomic analytical methods showing diverse properties (in-depth analysis, high throughput, and high sensitivity), emphasizing their advantages and limitations (**Chapter 6**). A general discussion was made on their future applications in case-control studies for biomarker discovery of PCa and male infertility. The work presented in this thesis provides a stepping stone towards improved PCa diagnosis and stratification.