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Unravelling the sugar-coating of prostate-specific antigen : method development and its application to prostate cancer research

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ENGLISH SUMMARY

With more than 10,000 new patients diagnosed on a yearly basis in the Netherlands, prostate cancer (PCa) is the second leading cancer in men. Currently, the serum concentration of the glycoprotein prostate-specific antigen (PSA) is being used as an indicator for PCa. While, this test is clinically applied worldwide, the PSA test lacks specificity, exhibits low sensitivity and more importantly it has a poor predictive value, resulting in a high number of unnecessary biopsies. Therefore, a marker is needed that can stratify the patient in a more accurate manner (*e.g.,* the differentiation between aggressive and non-aggressive prostate cancer). The aim of this thesis was to explore and gain a better insight in the glycosylation of PSA in urine, its correlation with PCa and the potential of using glycan biomarkers for the prediction or reflection for the progression of the disease. For this purpose the powerful analytical platform capillary electrophoresis hyphenated with mass spectrometry via electrospray ionization (CE-ESI-MS(/MS)) was explored with a special focus on the analysis of glycan and glycopeptides, by employing a porous nano-sprayer for the MS coupling.

To achieve this goal, first a better insight is needed of the current clinical pathway, the unmet clinical need of PCa and its relation to PSA. Therefore, the first part of this thesis provides an in-depth overview on these topics (**Chapter 1**). Moreover, this chapter describes the biosynthetic pathway of glycosylation as well as the biological role of glycosylation and if glycan biomarkers have the potential of overcoming unmet clinical needs, which, for PCa is the need for a better marker in the early diagnosis of PCa and, most importantly, the differentiation between indolent from aggressive tumors. In addition, to understand why CE-ESI-MS was chosen as analytical platform, a basic overview is provided in this chapter.

The second part is focused on the developments that have been made and implemented for the analysis of PSA with CE-ESI-MS (**Chapters 2** and **3**). As indicated by literature, a specific glycosylation feature of PSA, namely sialylation, could be a potential glycan biomarker for PCa. In particular the elevation of the α2,3-linked sialylated species seems to be a promising marker. Since differentially sialylated species cannot be readily distinguished by mass spectrometry, it was investigated if the different isoforms of the sialic acids (α2,3 *versus* α2,6) could be separated on a high-resolution separation platform (CE-ESI-MS; **Chapter 2**). Due to a difference in electrophoretic mobilities, a baseline separation of the α2,3- and α2,6-sialylated glycopeptides was achieved by CE. Interestingly, while the isomeric sialylated glycopeptides have similar physicochemical properties it was found that there was a relative small difference in pK_a units of $3.4 \cdot 10^{-2}$, providing a possible explanation for the difference in electrophoretic mobility. Notably, no extra sample treatment was needed besides the common procedures for a bottom-up approach (reduction, alkylation and digestion of the glycoprotein). In addition, this study revealed the micro-heterogeneity of the single *N*-linked glycosylation site of PSA. The sensitivity of the CE-ESI-MS platform was assessed in **Chapter 3**. Previous studies demonstrated that, when acetonitrile is used as a dopant gas around the ESI emitter, using

nanoLC-ESI-MS as a platform an overall higher sensitivity could be achieved for glycopeptides. In **Chapter 3**, we introduced for the first time a dopant enriched nitrogen (DEN) gas between the CE and MS instrument. Compared to conventional CE-ESI-MS, 25-fold higher sensitivities for model glycopeptides were obtained, allowing for limits of detection unreached by state-of-the-art nanoLC-ESI-MS. Interestingly, the DEN-gas appeared to positively affect the repeatability and intermediate precision compared to the conventional CE-ESI-MS platform. This development opened up new avenues for analyzing the heterogeneous glycosylation of PSA even more in-depth due to the improved sensitivity and precision when compared to the obtained results in **Chapter 2**. **Chapter 4**, the last chapter of the method development section investigates the usage of the CE-ESI-MS platform equipped with DEN-gas for the analysis of released *N*-glycans. This is especially important when minor abundant glycoforms need to be investigated in complex mixtures (< 0.01 % relative abundance). In addition, the development could also be applied on samples that contain low abundant proteins or when limited sample amounts are available. As *N*-glycans can be either negatively charged (carrying one or several sialic acids) or neutral (non-sialylated) the separation of the CE can be hampered, as well as the detection with MS could be biased. For this purpose an easy workflow was developed for the neutralization of sialic acids, which simultaneously enabled the differentiation between α2,3- and α2,6-linked sialic acids by MS (based on the difference in their derivatized mass). With the introduction of a cationic hydrazide tag (Girard's reagent P) at the reducing end, a uniform charge was attributed to all *N*-glycans. An in-depth study of the total human plasma protein *N*-glycome (TPNG) allowed the identification of 167 glycoforms, including sialic acid linkage-isomers, which is hitherto the highest identified number of glycoforms for TPNG thereby establishing CE-ESI-MS as a powerful tool in the field of *N*-glycan analysis.

In the third part of this thesis, the findings and improvements from **Chapters 2** and **3** were combined and used to study the correlation of PSA glycosylation with PCa. Here, for the first time a high-performance PSA Glycomics Assay (PGA) was presented (**Chapter 5**). PSA was captured and purified from patients' urine followed by tryptic digestion and was analyzed with CE-ESI-MS equipped with a DEN-gas. In total, 67 *N*-glycopeptides were identified from the PSA that was pooled from patients' urine. Moreover, based on positive controls (PSA standard spiked to a female urine pool) an average interday relative standard deviation of 14% was found for 41 *N*-glycopeptides. While we established a powerful tool for the in-depth relative quantitation of different PSA glycoforms in patients' urine, these data did not allow differentiating PCa patients from non-PCa patients.

Finally, the last part of this thesis (**Chapter 6**) offers a general discussion about future developments as well as the potential use of a PSA glycosylation assay in the clinical setting, showing the relevance of the results and how these may contribute to further clinical applications towards personalized medicine.