REPORT
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CANCER

NON-INVASIVE BIOMARKERS FOR STRATIFICATION OF THE RISK OF THE PROSTATE CANCER: GLYCOFORMS OF THE PSA AND MULTIPARAMETRIC MAGNETIC RESONANCE

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1. Project Summary

Prostate cancer (PCa) is the most common cancer among men in Europe. Serum levels of prostate specific antigen (PSA) are the most used biomarker for PCa detection. Total PSA (tPSA) levels are usually increased in PCa, however they also increase in other prostate benign pathologies as benign prostate hyperplasia (BPH). Thus, this biomarker is neither specific enough to distinguish BPH from PCa, nor non-clinically significant PCa (non-csPCa) from csPCa, especially in men with tPSA levels ≤10 ng/mL. This lack of specificity prompted our project, which aims to develop new non-invasive biomarkers to improve PCa diagnosis and to reduce PCa overdiagnosis and overtreatment. Several blood, urine and tissue tumour markers have been described and they outperformed tPSA for PCa and csPCa diagnosis, but at present only prostate health antigen (PHI) and prostate cancer antigen 3 (PCA3) are approved by the Food and Drug Administration (FDA). In the last decades, the implementation of multiparametric magnetic resonance imaging (mpMRI) has reduced 27% the number of unnecessarily biopsies and ameliorate the identification of csPCa with higher sensitivity than tPSA, however there is no consensus with the established cut-off, which can compromise its specificity.

Altered glycosylation pattern of glycoproteins have been extensively reported in cancer. In PCa, changes in PSA sialylation, core fucosylation or GalNAcβ1-4GlcNAc (LacdiNAc) have been described. Previous studies of our group using Sambucus nigra lectin (SNA) affinity chromatography showed a significant increase of α2,3-sialylated PSA glycoforms (over 30%) in serum from aggressive PCa patients compared with low/intermediate-risk PCa and BPH, highlighting the potential of %α2,3-sialic acid (SA) PSA as a high-risk PCa biomarker. Nonetheless, the potential use of %α2,3-SA PSA for PCa risk stratification had not been studied in a large cohort of serum samples neither characterised the specific PSA sialoforms that are altered in aggressive PCa. Despite the potential of SNA affinity chromatography for analysing %α2,3-SA, it is a time-consuming methodology that needs expert staffing, and therefore it cannot be easily translated into clinics. Thus, it is of utmost importance to develop new high-throughput methodologies to be translated into clinical practice.

Under these premises, in this project we aimed to identify the PSA glycoforms associated with aggressive PCa and explore the feasibility of using a lateral flow assay

(LFA) to facilitate the detection and quantification of sialylated PSA. In addition, the analysis of %α2,3-SA PSA in a large cohort of patients has been performed together with PHI and mpMRI values, and their combinations, to evaluate their potential to improve the sensitivity and specificity in PCa detection and risk stratification.

2. Project Outcomes / Obtained Results

In a first study (Gratacós-Mulleras, A et al., Scientific Reports, 2020), the identification of the main PSA glycan structures from aggressive PCa patients was performed from six aggressive PCa serum samples with high levels of tPSA (>300 ng/mL). Standard PSA, purified from healthy individuals' seminal plasma, was used as a control. PSA was purified and separated by SNA-lectin affinity chromatography. All PCa samples presented α2,3-SA PSA glycoforms >30% as previously described by our group. The PSA collected fractions were immunoprecipitated, resolved on SDS-PAGE, and PSA bands were excised and digested with PNGaseF to perform the N-glycan sequencing. The structural characterisation of fluorescently labelled PSA N-glycans was performed by high performance liquid chromatography (HPLC) combined with exoglycosidase digestions. The results revealed that PSA sialylated glycoforms with LacdiNac residues were increased in aggressive PCa, whereas the disialylated core fucosylated structures with α2,6-SA, which are the major PSA glycoforms in healthy individuals, were significantly reduced (41.4%). We showed that the increase of %α2,3-SA PSA in aggressive PCa is mostly produced by the reduction of disialylated core fucosylated structures with α2,6-SA in combination with the increase of α2,3-disialylated core fucosylated and α2,3-monosialylated non-core fucosylated structures.

In a second study, half-strip LFA was designed to recognise PSA α2,6-sialoforms. Standard PSA was used to set up the system. The capture bioreceptors were SNA lectin, dispensed in the test line (TL) to detect α2,6-SA and M-36 anti-tPSA antibody as an inner control (IC). 16.7 nm gold nanoparticles (AuNPs) were conjugated with MCA5842 monoclonal anti-PSA antibody as the detection bioreceptor. Samples were pre-incubated with the detection bioreceptor before inserting the half-strip LFA. Once optimised, a calibration curve with standard PSA (containing 80% of PSA α2,6 sialoforms) was obtained for SNA lectin (TL) and M-36 antibody (IC). The dynamic range of SNA was narrower than the M-36, from 65 ng/mL to 228 ng/mL (SNA) vs

from 3 to 282 ng/mL (M-36). Next, PSA fractions containing 100% and 0% α2,6-SA were prepared and tested by LFA. Results showed that the biosensor system was specific for α 2,6-SA PSA. In a first assessment with serum samples, it was necessary to dilute at least 50 times the samples to detect α2,6-SA PSA. Thus, before testing, it is necessary to remove other α2,6-SA glycoproteins from the serum sample that can interact with the SNA and hamper the binding of α2,6-SA PSA. The developed methodology did not allow us to analyse patient samples and for this reason the multicenter study of the %α2,3-SA biomarker was carried out with the affinity chromatography methodology previously developed by the biochemistry of cancer group of the University of Girona. (Llop, E and Peracaula R. Glycosylation: Methods and Protocols, Methods in Molecular Biology, 2022).

In the third study, %α2,3-SA PSA and PHI biomarkers were analysed and compared with tPSA in a multicentre study of four hospitals with 379 serum samples, which included 262 PCa (77 low-risk, 87 intermediate-risk and 98 high-risk PCa) and 117 BPH patients, to differentiate high-risk PCa from low/intermediate-risk PCa and BPH patients. Both biomarkers, %α2,3-SA PSA and PHI outperformed tPSA to distinguish high-risk PCa from the rest of samples (area under the curve (AUC) 0.804 and 0.870 vs 0.782, respectively), PHI being the one with highest performance. In the subcohort of samples with tPSA ≤10 ng/mL, both markers decreased their performance but interestingly %α2,3-SA PSA showed a lower decrease on its performance (AUC 0.744 vs 0.804) than PHI (AUC 0.763 vs 0.870). The combination of %α2,3-SA PSA+PHI increased the AUC up to 0.893 resulting in 90% sensitivity and 77% specificity, also in the cohort of tPSA ≤10 ng/mL (AUC of 0.812, 76% sensitivity and 79% specificity). Most of high-risk PCa (87%) presented α2,3-SA PSA >30% or PHI >75, while most csPCa (85%) showed α2,3-SA PSA >25% or PHI≥65. In both scenarios, biopsies would be recommended for patients above these cut-offs to confirm their diagnosis. Moreover, the potential of mpMRI to differentiate high-risk PCa from low/intermediate-risk PCa and BPH patients was also analysed in a cohort of 172 patients and its performance was comparable to %α2,3-SA PSA and PHI with an AUC of 0.810, high sensitivity (90%) but low specificity (41%). Furthermore, their combination enhanced the potential for high-risk PCa diagnosis with an AUC of 0.882 yielding a sensitivity and specificity of 69% and 93%, respectively. This combination might be useful for biopsy decision in men with α2,3-SA PSA 20-25% or PHI 50-65 due to in these ranges, 89% of csPCa patients present Pi-Rads ≥4. In addition, %α2,3-SA PSA and PHI are useful

biomarkers to differentiate PCa from BPH (AUC 0.775 and 0.811, respectively) and csPCa from non-csPCa (AUCs 0.774 and 0.807, respectively) and they outperformed the %fPSA biomarker in both studies (N=306 subjects).

In the analysed cohort, 92% of patients with α2,3-SA PSA <20% and PHI <50 presented BPH or non-csPCa, so in these cases the use of these novel biomarkers would be able to reduce unnecessary biopsies up to 22% compared with tPSA and %fPSA. Altogether, these results demonstrate that %α2,3-SA PSA combined with PHI are useful non-invasive markers to assist in PCa risk classification, reduce unnecessary biopsies and help in treatment decisions.

3. Relevance of the project and possible future implications

The research developed in this project has allowed us to characterise the main PSA N-glycans of aggressive PCa serum samples compared with those of the standard PSA (purified from healthy individuals' seminal plasma). Moreover, we have developed a proof of concept of a paper-based biosensor (LFA) to quantify α2,6-SA PSA that, although at present it only allows the analysis of pure PSA samples, paves the way for future research for the detection of glycoproteins as clinical biomarkers.

Serum-based biomarkers %α2,3-SA PSA and PHI, alone or in combination, were analysed in a large multicentre cohort of samples (4 hospitals) and outperformed tPSA and %fPSA potential for PCa detection and risk stratification, even in the subcohort of tPSA ≤10 ng/mL, in which it is most difficult to make the diagnosis. Overall, these results indicate that the implementation of the serum biomarkers PHI (currently commercially available) and %α2,3-SA PSA (current method not yet translated) in clinical practice would help in the detection of benign prostatic hyperplasia and clinically non-significant prostate cancers; reducing the number of unnecessary biopsies and improving the quality of life of these patients since biopsies are invasive techniques that entail associated morbidity.

The use of the biomarkers %α2,3-SA PSA and PHI would also have an important impact on risk stratification in patients with prostate pathology, helping to considerably

reduce the number of multiparametric magnetic resonance imaging (mpMRI) and diagnose aggressive cancers more accurately.

Further analyses of the multicentre cohort including 5-year survival and clinical relapse data would also be necessary to evaluate the prognostic potential of the studied biomarkers %α2,3-SA PSA, PHI and Pi-Rads. The data from this study would allow to personalise a patient's therapeutic plan more effectively since treatments applied early in patients with aggressive PCa would reduce the number of rescues due to tumour recurrence.

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