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ORIGINAL ARTICLE

Analysis of serum glycome by lectin microarrays for prostate cancer patients - a search for aberrant glycoforms

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Abstract

This is the first work focused on glycoprofiling of whole *N*- and *O*- glycome using lectins in an array format applied for analysis of serum samples from healthy individuals, benign prostate hyperplasia (BPH) patients, and prostate cancer (PCa) patients. Lectin microarray was prepared using traditional lectins with the incorporation of 2 recombinant bacterial lectins and 3 human lectins (17 lectins in total). Clinical validation of glycans as biomarkers was done in two studies: discrimination of healthy individuals with BPH patients vs. PCa patients (**C vs. PCa**) and discrimination of healthy individuals vs. BPH and PCa patients (**H vs. PCond**). Single lectins (17 lectins) and a combination of two lectins (136 binary lectin combinations) were applied in the clinical validation of glycan biomarkers providing 153 AUC values from ROC curves for both studies (C vs. PCa and H vs. PCond). Potential *N*- and *O*-glycans as biomarkers were identified and possible carriers of these glycans are shortly discussed.

Keywords Serum · *N*- and *O*-glycome · Prostate cancer · Lectin microarrays · Diagnostics · Clinical validation

Introduction

Prostate cancer (PCa) is a leading cause of deaths among men with an incidence of 1.1 million cases a year resulting in 366,000 deaths annually [1]. With ageing of the population, there is a projection that by 2035 there will be 2.1 million PCa cases with up to 633,328 associated deaths [2]. The number of deaths can be significantly reduced only by accurate early stage PCa diagnostics. The main reason behind the anticipated increase of PCa incidence and associated deaths is the fact that the current PCa biomarker - the level of prostate specific antigen (PSA) in the blood is not reliable enough for accurate early stage PCa diagnostics [3, 4]. The PSA protein is exclusively produced only by the prostate gland and thus it is a tissue specific biomarker, but elevated PSA level can be a result of any disruption of the basement membrane of the prostate (inflammation, benign form of the disease, etc.) [3, 4].

Currently we recognise three groups, when considering serological PSA concentration: "normal" (PSA ≤ 4 ng/mL), "intermediate" (4 ng/mL < PSA ≤ 10 ng/mL) and "high" (PSA > 10 ng/mL) [5]. While men having PSA level in the "high" group had advanced disease with 67% probability [5], PCa was confirmed also in the "intermediate" (30–35%) and even in the "normal" (15%) group [5, 6]. Professor Richard Ablin, who discovered PSA, is against the use of PSA as a diagnostic or as a prognostic PCa biomarker (the protein level does not give information if the PCa is aggressive or indolent) and should be used only as a biomarker for monitoring of recurrence of the disease [7, 8].

The use of PSA for PCa diagnostics is still highly controversial due to the following facts: in 1994, the US Food and

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Drug Administration (FDA) agency approved PSA as a diagnostic PCa biomarker [2]; in 2012 the US Preventative Services Task Force (USPSTF) advised not to use PSA as a diagnostic PCa biomarker due to PCa over-diagnosis [9]; and in 2017 the USPSTF recommended selective use of PSA tests for men aged from 55 to 70 years [10, 11] to deal with a high incidence of aggressive PCa appearing since 2012 [12]. In order to decrease the incidence of PCa in the "normal" group, the cut-off value was changed to 2.5 or 3 ng/mL in the past, but this caused an increase of false positive cases and/or an increasing number of unnecessary biopsies [13].

Due to high false negative and false positive cases depending on the PSA cut off value it is essential to identify new PCa biomarkers offering much higher accuracy. This is why new biomarkers are under development and some of them have been approved by the regulators [2, 5, 14, 15]. It is obvious that for accurate cancer diagnostics multiple biomarkers of different origins need to be combined, such as proteins and cell-free DNA [16]. Glycans proved to have the potential to become robust diagnostic and prognostic PCa biomarkers [3, 4, 17]. Numerous studies focused on identification of changes in the glycosylation profile of PSA applicable as robust diagnostic and prognostic PCa biomarkers detected in blood/serum or urine [4, 18, 19], but there is still a need to find other glycan-based biomarkers. It can be anticipated that a panel of glycan-based biomarkers can provide more robust PCa diagnostics compared to PSA glycoprofiling itself.

There is only one study focused on the analysis of serum *N*-glycome of PCa patients by using a fully quantitative high-throughput instrumental-based approach with AUC well below 0.8 [20]. The other study combined several types of biomarkers detected both in serum and tissues of PCa patients using multi-omics approach with AUC up to 0.91 [17]. Lectin microarray was applied for glycoprofiling of two proteins isolated from PCa tissues, but not in serum and without any clinical validation [21]. Furthermore, lectin microarray was applied for the analysis of serum glycome of serum samples from patients with colorectal patients [22] and breast cancer patients [23], but in both cases, discrimination power of lectin microarrays was not examined in a form of AUC curves. In this paper, a simple method for glycoprofiling of whole serum without any treatment using lectin microarrays is introduced for the first time with the aim to identify promising glycan biomarkers, protein carriers for these glycans, for more accurate future PCa diagnostics.

Material and methods

Materials

Human serum samples

Serum samples of 22 individuals (all males; 5 healthy with no comorbidities diagnosed at that time, 4 with no malignancy confirmed by a biopsy - diagnosed as benign prostate hyperplasia (BPH) with high grade PIN in 2 cases; 13 PCa patients diagnosed with prostate adenocarcinoma) were collected by a private urological ambulance in Trencin, Slovak Republic. Ethics Committee approved the use of the samples and all participants signed an informed consent document *prior* to sample collection. The procedure was done in accordance with the ethical guidelines of the last revision of the Helsinki Declaration. Untreated serum samples were taken during the morning fasted state into a gel and clot activator tube (Vacutest Kima, Piove di Sacco, IT). After 30 min, the tubes were centrifuged at 25 °C for 10 min at 2,500 g. The sera were transferred into sterile plastic vials and were stored in the form of aliquots at -80 °C until use.

Chemicals

All common chemicals (*e.g.* buffer components, bovine serum albumin BSA, etc.) were purchased from Sigma Aldrich (USA). All solutions were freshly prepared *prior* to experiments in 0.055 μS deionized water (DW) and filtered using 0.2 μm sterile filters. Biotin conjugation kits for biotinylation of unconjugated lectins were purchased from Abcam (UK). Lectins RPL-Fuc1 and RPL-Sia2 were obtained from GlycoSelect (Ireland). P-selectin, L-selectin, and E-selectin (only P-selectin was able to bind sufficiently to the samples used in the study) were used in a form of chimera proteins fused to IgG1 tail and obtained from Prof. Borsig with details provided in Ref. [24]. All the other lectins used in this study were purchased in their biotinylated form from Vector Labs (USA). A conjugate streptavidin-CF647 was provided from Biotium (USA).

Methods

Lectin microarrays

Lectin microarray experiments were performed with PBS (0.01 M, pH 7.4) as a printing buffer. For blocking purposes, 3% BSA was used, as we observed lower background fluorescence intensity compared to Carbo-free blocking solution (Vector Labs, U.S.) for some of the lectins used in the study. Each sample with 50x dilution was spotted in triplicates in two different wells (*i.e.* 6 spots in total for every sample) using SpotBot3 Microarray Protein edition (Arrayit, USA) on epoxide-coated slides Nexterion E (Schott, Germany) using a previously optimized protocol (*i.e.* sample dilutions 2-128x were tested during optimisation experiment and dilution of 50x was selected based on signal to background ratio). Shortly, after spotting and blocking the slides (1 h, RT, and shaking), biotinylated lectins (c = 5 μg/mL in PBS) were added and incubated at RT for 1 h. After a washing step, the slides were gently washed three times with PBS, and then a streptavidin-CF647 conjugate (c = 0.1 μg/mL in PBS) was added for 15 min. After a washing step

and an additional wash with DW, fluorescence was read at 635 nm using an InnoScan microarray reader (Arrayit, USA). A signal evaluated and ascribed to individual samples using Mapix software was an average value of six independent spots after background fluorescence subtraction.

Data evaluation

Receiver operating characteristic curves (ROC) and area under the curve parameter (AUC), sensitivity, specificity, and accuracy for individual lectins as well as for their binary combinations were constructed in R software (version 3.4.4) [25] using additional software packages such as MASS [26], ROCR [27] and pROC [28] using a bootstrap method described elsewhere [29]. All confidence intervals (CIs) for AUC values presented are 95% two-sided bootstrap intervals [30] (Table S1 and Table S2).

Results

Lectin microarray was prepared using 17 lectins of bacterial, fungi, plant, and human origin (see Table 1). A typical output from lectin microarray is shown in Fig. 1a, with an intensity of the spot proportional to the binding of lectins to spotted serum samples. Lectins applied in this study were selected in a way to cover cancer-associated changes in the glycan structures: α2,3-sialylation; fucosylation; the presence of Lewis antigens; and *N*- and *O*-glycan branching; LacdiNAc (Table 1) [3, 4, 31–35].

Control (healthy + BPH) group vs. PCa patients (C vs. PCa)

Single lectins

There are only two lectins able to discriminate the control group from PCa patients with AUC well above the value of 0.74 (AUC value of PHI *i.e.* Prostate Health Index, a gold standard for second opinion testing just *prior* a prostate biopsy) [18]), which are *Aleuria aurantia* lectin AAL (0.92) and P-selectin (0.84) (Fig. 1b). AAL recognizes fucose containing glycans, while P-selectin binds to sLe^x and negatively charged glycans (*i.e.* containing sialic acid and/or sulfo-groups) (Table S1).

It is quite interesting to compare the performance of AAL, a plant lectin, to another fucose binding lectin of bacterial origin used in this work such as RPL-Fuc1 (isolated from *Aspergillus fumigatus*), which shows a much lower AUC of 0.59 (Fig. 1b). RPL-Fuc1 exhibits affinity mainly towards antennary fucose and with a minor affinity for core fucose and towards all Lewis epitopes with preference to bind shorter non-branched glycan structures [38]. RPL-Fuc1 recognizes both *O*- and *N*-glycans [38]. AAL binds preferentially to core

Table 1 Lectin specificity, taken from Vector Laboratories leaflet and from Refs. [24, 36–40]

Lectin abbreviation	Source	Glycan specificity
AAL	*Aleuria aurantia* mushrooms	Fucα6GlcNAc (core Fuc), Fucα3(Galβ4)GlcNAc (Le^x)
RPL-Fuc1	*Aspergillus fumigatus*	Fucα3GlcNAc, Fucα4GlcNAc, Le^a, Le^b, Le^x, Le^y
PHA-E (erythroagglutinin)	*Phaseolus vulgaris* seeds	*N*-glycans with outer Gal and bisecting GlcNAc
PHA-L (leucoagglutinin)	*Phaseolus vulgaris* seeds	tri/tetra-antennary *N*-glycans
ConA	*Canavalia ensiformis* bean seeds	αMan, αGlc; high-Man; Manα6(Manα3)Man; Manα6Man; Manα3Man
NPL	*Narcissus pseudonarcissus* bulbs	αMan; high-Man; Manα6Man
GNL	*Galanthus nivalis* bulbs	αMan; high-Man; Manα3Man
HHL	*Hippeastrum* hybrid bulbs	αMan; high-Man; Manα3Man or Manα6Man
DBL	*Dolichos biflorus* seeds	αGalNAc; terminal GalNAc; GalNAcα3GalNAc
DSL	*Datura stramonium* seeds	$(GlcNAcβ4)_n$; tri/tetra-antennary *N*-glycans
GSL-II	*Griffonia simplicifolia* seeds	α or βGlcNAc; agalactosylated tri/tetra antennary glycans; core 3 *O*-glycans
WGA	*Triticum vulgaris*	$(GlcNAcβ4)_n$, Neu5Ac; poly(*N*-acetyllactosamine)
RCA-I	*Ricinus communis* seeds	Gal; Galβ4GlcNAc
MAA-II	*Maackia amurensis* seeds	Neu5Acα3Galβ4GalNAc; 3-*O*-Suα3Galβ4GalNAc; sT antigen
P-selectin	human	sLe^x (Neu5Acα3Galβ4(Fucα3)GlcNAc); sLe^a (Neu5Acα3Galβ4(Fucα4)GlcNAc); sulfo groups
RPL-Sia2	*Streptococcus gordonii* M99	Neu5Acα3 on *O*-glycans; Neu5Acα3Galβ3GalNAc (*O*-glycans) > Neu5Acα2-3Galβ4Glc (*N*-glycans)
SNA-I	*Sambucus nigra* bark	Neu5Acα6Galβ4GalNAc; 6-*O*-Suα3Galβ4GalNAc; sTn antigen

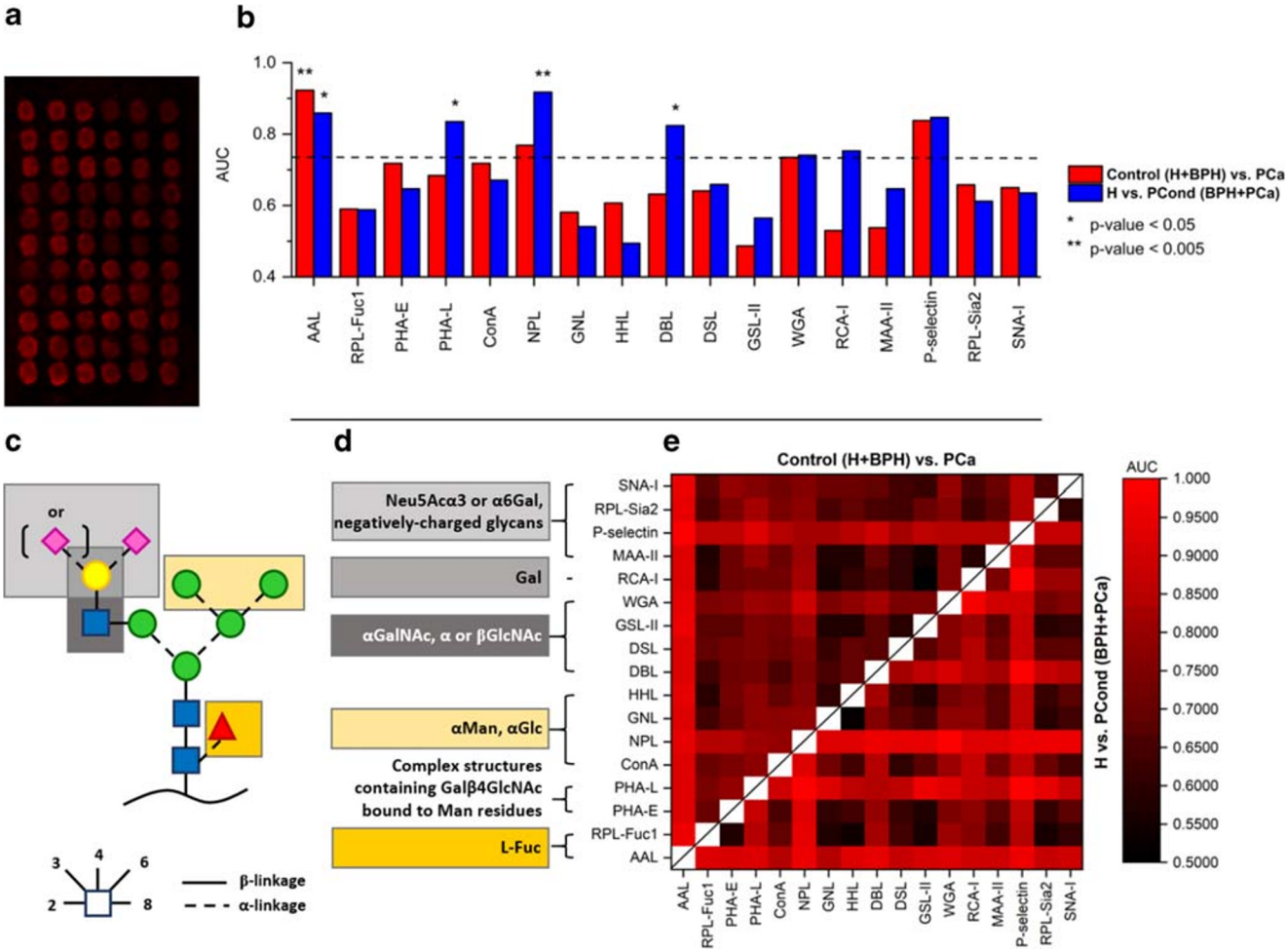

Fig. 1 **a** A typical output (scan read at 635 nm) from lectin microarray experiment of triplicates for 22 samples used in this study; **b** Clinical performance of single lectins to discriminate C vs. PCa and H. vs. PCond; **c** and **d** Typical glycan structures recognised by lectins applied in lectin microarray experiment; and **e** A "heat map" of binary lectin combinations to discriminate C vs. PCa and H. vs. PCond. All results shown were obtained by measurement of serum samples of 22 individuals (5 from healthy individuals, 4 from benign prostate hyperplasia (BPH) patients, and 13 from PCa patients). In Fig. 1b there are no SD values shown due to clarity of presentation, but the reader is advised to see Table S1 and Table S2 for SD of AUC values

fucose linked *via* (α-1,6) bond or to fucose linked *via* (α-1,3) bond to *N*-acetyllactosamine related structures and recognizes both *O*- and *N*-glycans [41] (Table S1).

While P-selectin offered a good clinical performance (AUC = 0.84), other sialic acid binding lectins including SNA-I, MAA-II and RPL-Sia2 showed poor clinical performance with AUC in the range from 0.54 to 0.66 (Fig. 1b).

Other lectins recognizing other types of glycans offered only a moderate performance with AUC well below or around value of 0.74 (*i.e.* 0.49–0.77) (Fig. 1b).

Combination of two lectins

Five binary lectin combinations increased value of AUC above 0.95: AAL + Selectin-P (1.00), AAL + SNA-I (0.97), AAL + GSL-II (0.97), AAL + RPL-Fuc1 (0.96) and AAL + NPL (0.95) (Fig. 1e, Table S1). Thus for *N*-glycans, especially the presence of core fucose combined with Manα6Man glycans, antennary fucose, Neu5Acα6-bound or shorter branched glycans is a good predictor. For *O*-glycans especially fucose/Lewis antigens combined with sialic acid, sulfo-groups or core 3 structures are good predictors.

Healthy vs. PCond (BPH + PCa) group (H vs. PCond)

In order to discriminate H vs. PCond, a wider variability of lectins can render reliable solutions either as single lectins or as a binary combination of lectins.

Single lectins

There are several lectins NPL (0.92), AAL (0.86), P-selectin (0.85), PHA-L (0.84), and DBL (0.82) able to discriminate healthy people from those having prostate disease either

BPH or PCa (PCond) with AUC significantly higher than the value of 0.74 (Fig. 1b).

NPL lectin recognizes high mannose containing glycans (Manα6Man), while other mannose binding lectins showed the much lower performance to discriminate H vs. PCond with AUC values of 0.67 (ConA, Manα6(Manα3)Man), 0.54 (GNL, Manα3Man) and 0.49 (HHL, Manα3Man or Manα6Man) (Fig. 1b, Table S2). This means that especially high mannose glycans containing Manα6Man structures provide better discrimination power for H vs. PCond.

AAL lectin can discriminate H. vs. PCond with much higher AUC (0.86) compared to RPL-Fuc1 (0.59) (Fig. 1b, Table S2), suggesting that core Fuc (*N*-glycans) and some Lewis antigens (*O*-glycans) are very good discriminants.

P-selectin provided much better discrimination power (0.85), compared to other sialic acid binding lectins MAA-II (0.65), SNA-I (0.64) and RPL-Sia2 (0.61) (Fig. 1b, Table S2). Thus, it seems that good discriminants are branched *O*-glycans such sLe^x, sLe^a or sulfo-containing glycans.

PHA-L recognizing tri/tetra-antennary *N*-glycans provided better AUC (0.84) compared to PHA-E (0.65) (Fig. 1b, Table S2), recognizing *N*-glycans with outer Gal and bisecting GlcNAc.

DBL recognising terminal GalNAc showed much better discrimination performance (AUC = 0.82), when compared to GlcNAc recognizing lectins – WGA ($(GlcNAc\beta4)_n$) with AUC = 0.74, DSL ($(GlcNAc\beta4)_n$) with AUC = 0.66; and GSL-II (α or βGlcNAc) with AUC = 0.57 (Fig. 1b, Table S2). This really suggests that especially *O*-glycans can be good discriminants over *N*-glycans.

Combination of two lectins

Mainly binary combination of lectins from the group of strong performers (NPL, AAL, P-selectin, PHA-L and DBL) provided AUC above 0.95 (*i.e.* 7 different combinations) (Table S2). There are 5 binary lectin combinations when the lectin from the group of strong biomarkers can provide good discrimination with lectins outside the group of strong performers (PHA-L + GSL-II; P-selectin + RCA; NPL + SNA-I; NPL + WGA and NPL + RPL-Sia2). Surprisingly there is one binary lectin combination based on lectins outside strong performers (RCA-I + WGA), also offering a good discrimination performance. The best lectin performer is NPL (7 different binary combinations), followed by P-selectin (5 combinations), PHA-L (3 combinations); AAL, and DBL (2 combinations). The ideal discrimination power with AUC = 1 was achieved by these combinations: P-selectin + PHA-L, P-selectin + RCA-I, P-selectin + DBL, NPL + PHA-L and NPL + WGA. So we can conclude that especially *O*-glycans having Lewis antigens, poly(*N*-acetyllactosamine), sialic acids and/or sulfo groups or short *O*-glycans (like Tn antigen) have a good discrimination potential. When *N*-glycans are considered, the following glycan types have a good discrimination performance: negatively charged, Lewis antigen containing, tri-/tetra antennary *N*-glycans with Manα6Man residues.

Discussion

Human serum contains 35–50 g/L of albumin (which is not glycosylated, but might be glycated), followed by immunoglobulins with the level of 20–25 g/L. The globulin fraction consists of 16–20 g/L of IgG, 3–4 g/L of IgA, and 1 g/L of IgM, the level of IgD and IgE are significantly lower. Immunoglobulins together with transferrin and α-2-macroglobulin represent 75% of all serum glycoproteins [42]. The other glycoproteins with significant abundance are: α-1-antitrypsin, α-1-acid glycoprotein, haptoglobin, ceruloplasmin, etc. [42], and such proteins could be potential carriers of *N*-glycans and/or of *O*-glycans in serum. Besides immunoglobulins, other glycoproteins such as α-1-acid glycoprotein were suggested as a carrier of altered *N*-glycans associated with PCa within serum *N*-glycome [43]. AUC values might be misleadingly high in case two cohorts are imbalanced. Still, the information yielded by these analyses can be very useful, like in the case of P-selectin (AUC above 0.8 in both cases). Here, a discussion about sensitivity and specificity is of great importance. In the case of C vs. PCa analysis, sensitivity (true positive rate) of 92% was achieved, while specificity (true negative rate) was only moderate (78%). In case BPH patients were moved to a PCa patient cohort (PCond, *i.e.* analysis H vs. PCond), sensitivity fell down to 82%, but specificity increased to 100%. This kind of results suggest a strong predictive value of glycan isoforms recognized by P-selectin as cancer markers, since a higher positive predictive value could be achieved while BPH (*i.e.* non-cancerous samples) were categorized as a control group.

Changes in *N*-glycans

The following *N*-glycans showed a good discrimination power: core fucose combined with mannose-containing glycans, antennary fucose, α6Neu5Ac or shorter branched glycans for discrimination **C** vs. **PCa** (see Section above); or negatively charged, Lewis antigen containing, tri-/tetra antennary *N*-glycans with mannose-containing glycan residues for discrimination of **H** vs. **PCond** (see Section above).

Since IgGs are glycoproteins containing *N*-glycans in the Fc domain and also in the Fab domain (25% of IgGs under physiological conditions contain *N*-glycans in the Fab domain) [44], most likely some changes associated with serum *N*-glycome can be attributed to changes in the IgG glycome. *N*-glycans in the Fc domain of IgG are of a complex biantennary type with quite a low level of sialic acid and bisecting GlcNAc, while having a high level of core fucose

[44]. IgG´s Fab *N*-glycans are rich in bisecting GlcNAc, galactose, sialic acid, mannose with a low level of core-fucose [44]. When these two sites are compared, *N*-glycans of Fab fragment are more accessible for binding with lectins compared to *N*-glycans of Fc fragment [44].

The fact that changes in the *N*-glycome found out in our study might be associated with changes in the *N*-glycome of IgG´s can be underlined by the following facts: there is an increasing proportion of *N*-glycans present in Fab fragment and increased level of sialic acid, bisecting GlcNAc and fucose associated with several cancer types [44, 45].

IgA1s contain 2 *N*-glycans in the Fc region and in IgA2s there are 5 *N*-glycans with 2 *N*-glycans in the Fab fragment and 3 *N*-glycans in the Fc region [46]. *N*-glycans present in the IgA1 Fc region are much richer from a compositional point of view when compared to IgG, which consist of biantennary and triantennary structures (Asn263) with triantennary or even tetraantennary glycans present at the tailpiece site of IgA1. Additionally, IgA1 *N*-glycans quite frequently contain sialic acid [46].

IgMs contain 5 *N*-glycosylation sites with 3 of them composed of biantennary complex *N*-glycans and 2 *N*-glycans with oligomannose structures [46].

The only report showing the potential of *N*-glycans of immunoglobulins (*i.e.* IgG) for PCa diagnostics was based on MALDI-TOF and lectin-based methods for the glycoprofiling of IgG isolated from human serum [47]. The part of the study dealing with MS-based assays identified that a biantennary *N*-glycan of IgG isolated from serum of PCa patients lacked terminal Gal. Furthermore, BPH patients and healthy individuals had higher amounts of Gal or sialic acid attached to glycan on IgG. Three lectins were applied in the study *i.e.* AAL, SNA and WGA and two of them offered high AUC value of 0.84 (WGA) or even 0.95 (SNA) [47]. There are other reports showing changed IgG glycosylation associated with ovarian cancer, lung, and gastric cancer [48, 49], but without providing AUC values. Changes in the glycosylation of IgG (increased core-fucosylation and decreased galactosylation) could be applied for diagnostics of breast cancer with AUC = 0.94 [50]. Moreover, such glycan changes in the Fc fragment of IgG supress cell mediated cytotoxicity allowing tumour cells to spread out in the body [50]. Diagnostics of colorectal cancer could be achieved from IgG glycome (AUC = 0.76) [51] or from serum *N*-glycome (AUC = 0.77–0.81) [42, 52]. Association of changes in the IgG glycosylation with the progression of other non-cancerous diseases was recently briefly summarised [53].

Changes in *O*-glycans

For *O*-glycans especially fucose/Lewis antigens combined with poly(*N*-acetyllactosamine), sialic acid, sulfo- groups, short *O*-glycans (like Tn antigen), and/or core 3 structures are good predictors.

Information regarding *O*-glycan composition of immunoglobulins is quite scarce. IgG3 hinge region contains 3 *O*-glycans with the detailed glycan composition not known due to several challenges associated with such analysis [54]. IgA1 is heavily *O*-glycosylated in the hinge region with up to 6 *O*-glycosylation sites [55]. *O*-glycans of IgA1 contain GalNAc, terminal Gal and either or both GalNAc or Gal saccharides can be modified by sialic acid [46].

Conclusions

Several glycans recognised by five binary combinations of lectins provide very good discrimination power to distinguish C vs. PCa with AUC above 0.95 and five binary combinations of lectins were able to discriminate H. vs. PCond with AUC = 1.00. It is worth comparing the clinical performance of the approach applied in this study with a typical clinical performance of PSA test with AUC of 0.68 [56] and of PHI test with AUC of 0.74 [18]. A clinical performance of serum glycome analysis by lectins is better compared to PSA glycoprofiling using lectins offering AUC values from 0.63 to 0.85 [4], depending on lectin applied. From the lectin binding preference, it was possible to identify the following *N*-glycans with a good discrimination power: core fucose combined with Manα6Man glycans, antennary fucose, α6Neu5Ac, shorter branched glycans (C vs. PCa) or negatively charged, Lewis antigen containing, tri-/tetra antennary *N*-glycans with Manα6Man residues (H vs. PCond). From *O*-glycans especially fucose/Lewis antigens combined with poly(*N*-acetyllactosamine), sialic acid, sulfo- groups, short *O*-glycans (like Tn antigen), and/or core 3 structures are prospective PCa biomarkers. From the literature survey, we concluded that especially immunoglobulins are the most likely carriers of these glycan biomarkers. Our work shows that while there are several studies focusing on serum *N*-glycome or IgG *N*-glycome in connection with PCa or other cancer diseases, a systematic effort needs to be devoted to examine *N*- and/or *O*-glycome of IgA1/IgA2 as potential cancer biomarkers. The main reason behind that is the fact that IgAs are more complex glycoproteins compared to IgGs with a possibility to display additional new cancer-related glycoforms with a significant discrimination power. At the same time, it is worth mentioning that lectins able to discriminate healthy individuals (BPH and/or H) from PCa patients found in the current study can be then applied for selective enrichment of glycoproteins from serum samples with subsequent identification of such glycoproteins by peptide mass fingerprinting. This can lead to the identification of potential new glycan-based biomarkers, which could be selectively glycoprofiled in a sandwich configuration using antibodies and lectins [3, 4, 57].

Glycoprofiling of specific glycoproteins using antibodies (to selectively fish out the glycoprotein of interest from serum sample) and lectins in a sandwich configuration will allow tracing the origin of changed glycans from many different glycoproteins to tissues the glycoproteins are released from or to identify if changed glycans are produced by other than cancerous conditions (autoimmune and inflammatory diseases) [58].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Collaboration, G.B.o.D.C.: Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2017: A systematic analysis for the global burden of disease study. JAMA Oncol. **5**(12), 1749–1768 (2019). https://doi.org/10.1001/jamaoncol.2019.2996
2. O'Reilly, J.-A., O'Kennedy, R.J.: Prostate cancer detection: complexities and strategies. J. Cancer Treat. Diagn. **2**(1), 18–25 (2017)
3. Tkac, J., Bertok, T., Hires, M., Jane, E., Lorencova, L., Tkac, J., Bertok, T., Lorencova, L., Kasak, P.: Glycomics of prostate cancer: updates. Exp. Rev. Proteomics. **16**(1), 65–76 (2019). https://doi.org/10.1080/14789450.2019.1549993. Accessed 20 Mar 2020
4. Tkac, J., Gajdosova, V., Hroncekova, S., Bertok, T., Hires, M., Jane, E., Lorencova, L., Kasak, P.: Prostate-specific antigen glycoprofiling as diagnostic and prognostic biomarker of prostate cancer. Interface Focus **9**(2), ID: 20180077: (2019). https://doi.org/10.1098/rsfs.2018.0077
5. Rodríguez, J.Z., O'Kennedy, R.: New approaches for the develoment of diagnostic systems for prostate cancer. Asian Hosp. Healthc. Manag., 18–23 (2017)
6. Garnick, M.B., Abrahamsson, P.-A., Dewolf, W.C., Kacker, R., Kaplan, I., Louglin, K.R., Srougi, M., Sternberg, C.N., Zietman, A.L.: Harvard Medical School 2018 Annual report on prostate diseases. Harvard, US: Harvard Medical School. Harvard Health Publishing, Boston (2018)
7. Ablin, R.J., Piana, R.: The great prostate hoax: How big medicine hijacked the PSA test and caused a public health disaster. Palgrave McMillian, New York (2014)
8. Ablin, R.J.: Prostate cancer test has been misused for money. https://www.newscientist.com/article/mg22129564-400-prostate-cancer-test-has-been-misused-for-money/ (2014)
9. Moyer, V.A.: Screening for prostate cancer: US Preventive Services Task Force recommendation statement. Ann. Intern. Med. **157**(2), 120–134 (2012). https://doi.org/10.7326/0003-4819-157-2-201207170-00459
10. Van Der Kwast, T.H., Roobol, M.J.: Prostate cancer: Draft USPSTF 2017 recommendation on PSA testing-a sea-change? Nat. Rev. Urol. **14**(8), 457–458 (2017). https://doi.org/10.1038/nrurol.2017.89
11. Grossman, D.C., Curry, S.J., Owens, D.K., Bibbins-Domingo, K., Caughey, A.B., Davidson, K.W., Doubeni, C.A., Ebell, M., Epling, J.W. Jr., Kemper, A.R., Krist, A.H., Kubik, M., Landefeld, C.S., Mangione, C.M., Silverstein, M., Simon, M.A., Siu, A.L., Tseng, C.-W.: Screening for prostate cancer: Us preventive services task force recommendation statement. JAMA. **319**(18), 1901–1913 (2018). https://doi.org/10.1001/jama.2018.3710
12. Fleshner, K., Carlsson, S.V., Roobol, M.J.: The effect of the USPSTF PSA screening recommendation on prostate cancer incidence patterns in the USA. Nat. Rev. Urol. **14**(1), 26–37 (2017). https://doi.org/10.1038/nrurol.2016.251
13. Early Detection of Prostate Cancer, American Urological Association (AUA) Guideline. https://www.auanet.org/Documents/Guidelines/PDF/EarlyProstateCancerGuideline_71318%20PDF.pdf (2018). Accessed 16 Mar 2020
14. Sharma, S., Zapatero-Rodríguez, J., O'Kennedy, R.: Prostate cancer diagnostics: Clinical challenges and the ongoing need for disruptive and effective diagnostic tools. Biotechnol. Adv. **35**(2), 135–149 (2017). https://doi.org/10.1016/j.biotechadv.2016.11.009
15. Kohaar, I., Petrovics, G., Srivastava, S.: A rich array of prostate cancer molecular biomarkers: opportunities and challenges. Int. J. Mol. Sci. **20**(8), 1813 (2019). https://doi.org/10.3390/ijms20081813
16. Cohen, J.D., Li, L., Wang, Y., Thoburn, C., Afsari, B., Danilova, L., Douville, C., Javed, A.A., Wong, F., Mattox, A.: Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science, eaar3247 (2018). https://doi.org/10.1126/science.aar3247
17. Murphy, K., Murphy, B.T., Boyce, S., Flynn, L., Gilgunn, S., O'Rourke, C.J., Rooney, C., Stöckmann, H., Walsh, A.L., Finn, S., O'Kennedy, R.J., O'Leary, J., Pennington, S.R., Perry, A.S., Rudd, P.M., Saldova, R., Sheils, O., Shields, D.C., Watson, R.W.: Integrating biomarkers across omic platforms: an approach to improve stratification of patients with indolent and aggressive prostate cancer. Mol. Oncol. **12**(9), 1513–1525 (2018). https://doi.org/10.1002/1878-0261.12348
18. Ferrer-Batallé, M., Llop, E., Ramírez, M., Aleixandre, R.N., Saez, M., Comet, J., De Llorens, R., Peracaula, R.: Comparative study of blood-based biomarkers, α2, 3-sialic acid PSA and PHI, for high-risk prostate cancer detection. Int. J. Mol. Sci. **18**(4), 845 (2017)
19. Llop, E., Ferrer-Batalle, M., Barrabes, S., Enrique Guerrero, P., Ramirez, M., Saldova, R., Rudd, P.M., Aleixandre, R.N., Comet, J., de Llorens, R., Peracaula, R.: Improvement of prostate cancer diagnosis by detecting PSA glycosylation-specific changes. Theranostics **6**(8), 1190–1204 (2016). https://doi.org/10.7150/thno.15226
20. Saldova, R., Fan, Y., Fitzpatrick, J.M., Watson, R.W.G., Rudd, P.M.: Core fucosylation and α2–3 sialylation in serum N-glycome is significantly increased in prostate cancer comparing to benign prostate hyperplasia. Glycobiology. **21**(2), 195–205 (2011). https://doi.org/10.1093/glycob/cwq147
21. Li, Y., Tao, S.-C., Bova, G.S., Liu, A.Y., Chan, D.W., Zhu, H., Zhang, H.: Detection and verification of glycosylation patterns of glycoproteins from clinical specimens using lectin microarrays and

lectin-based immunosorbent assays. Anal. Chem. **83**(22), 8509–8516 (2011). https://doi.org/10.1021/ac201452f
22. Zamorova, M., Holazova, A., Miljus, G., Robajac, D., Sunderic, M., Malenkovic, V., Dukanovic, B., Gemeiner, P., Katrlik, J., Nedic, O.: Analysis of changes in the glycan composition of serum, cytosol and membrane glycoprotein biomarkers of colorectal cancer using a lectin-based protein microarray. Anal. Methods. **9**(18), 2660–2666 (2017). https://doi.org/10.1039/c7ay00159b
23. Fry, S.A., Afrough, B., Lomax-Browne, H.J., Timms, J.F., Velentzis, L.S., Leathem, A.J.C.: Lectin microarray profiling of metastatic breast cancers. Glycobiology. **21**(8), 1060–1070 (2011). https://doi.org/10.1093/glycob/cwr045
24. Beauharnois, M.E., Lindquist, K.C., Marathe, D., Vanderslice, P., Xia, J., Matta, K.L., Neelamegham, S.: Affinity and kinetics of sialyl Lewis-X and core-2 based oligosaccharides binding to L- and P-selectin. Biochemistry. **44**(27), 9507–9519 (2005). https://doi.org/10.1021/bi0507130
25. Team, R.C.: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna (2017). https://www.R-project.org. Accessed 3 June 2020
26. Venables, W.N., Ripley, B.D.: Modern applied statistics with S, 4th edn. Springer, New York (2002)
27. Sing, T., Sander, O., Beerenwinkel, N., Lengauer, T.: ROCR: visualizing classifier performance in R. Bioinformatics **21**(20), 7881 (2005)
28. Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J., Müller, M.: pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics **12**, 77 (2011)
29. Carpenter, J., Bithell, J.: Bootstrap confidence intervals: when, which, what? A practical guide for medical statisticians. Stat. Med. **19**(9), 1141–1164 (2000)
30. Ström, P., Nordström, T., Aly, M., Egevad, L., Grönberg, H., Eklund, M.: The Stockholm-3 model for prostate cancer detection: algorithm update, biomarker contribution, and reflex test potential. Eur. Urol. **74**(2), 204–210 (2018)
31. Pinho, S.S., Reis, C.A.: Glycosylation in cancer: mechanisms and clinical implications. Nat. Rev. Cancer. **15**, 540–555 (2015). https://doi.org/10.1038/nrc3982
32. Tkac, J., Bertok, T., Hires, M., Jane, E., Lorencova, L., Kasak, P.: Glycomics of prostate cancer: updates. Exp. Rev. Proteomics. (2018). https://doi.org/10.1080/14789450.2019.1549993
33. Stowell, S.R., Ju, T., Cummings, R.D.: Protein glycosylation in cancer. In: Abbas, A.K., Galli, S.J., Howley, P.M. (eds.) Annual review of pathology: Mechanisms of disease, vol 10. Annual review of pathology-mechanisms of disease, pp. 473–510 (2015)
34. Teoh, S.T., Ogrodzinski, M.P., Ross, C., Hunter, K.W., Lunt, S.Y.: Sialic acid metabolism: A key player in breast cancer metastasis revealed by metabolomics. Front. Oncol. **8**(174) (2018). https://doi.org/10.3389/fonc.2018.00174
35. Blanas, A., Sahasrabudhe, N.M., Rodríguez, E., van Kooyk, Y., van Vliet, S.J.: Fucosylated antigens in cancer: An alliance toward tumor progression, metastasis, and resistance to chemotherapy. Front. Oncol. **8**(39) (2018). https://doi.org/10.3389/fonc.2018.00039
36. Hirabayashi, J., Yamada, M., Kuno, A., Tateno, H.: Lectin microarrays: concept, principle and applications. Chem. Soc. Rev. **42**(10), 4443–4458 (2013). https://doi.org/10.1039/C3CS35419A
37. Cummings, R.D., Darvill, A.G., Etzler, M.E., G., H.M.: Glycan-recognizing probes as tools. In: Varki, A., Cummings, R., Esko, J. (eds.) Essentials of glycobiology [Internet]. 3rd edition, pp. 2015–2017. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (2017)
38. Houser, J., Komarek, J., Kostlanova, N., Cioci, G., Varrot, A., Kerr, S.C., Lahmann, M., Balloy, V., Fahy, J.V., Chignard, M., Imberty, A., Wimmerova, M.: A soluble fucose-specific lectin from Aspergillus fumigatus conidia - structure, specificity and possible role in fungal pathogenicity. PLoS One. **8**(12), e83077/83071-e83077/83015, 83015 pp (2013). https://doi.org/10.1371/journal.pone.0083077
39. Ruhl, S., Sandberg, A.L., Cole, M.F., Cisar, J.O.: Recognition of immunoglobulin A1 by oral actinomyces and streptococcal lectins. Infect. Immun. **64**(12), 5421–5424 (1996). https://doi.org/10.1128/iai.64.12.5421-5424.1996
40. Läubli, H., Borsig, L.: Selectins promote tumor metastasis. Semin. Cancer Biol. **20**(3), 169–177 (2010). https://doi.org/10.1016/j.semcancer.2010.04.005
41. Shimomura, M., Nakayama, K., Azuma, K., Terao, N., Nishino, K., Takamatsu, S., Nakano, M., Takahashi, S., Kobayashi, Y., Murata, K., Kamada, Y., Miyoshi, E.: Establishment of a novel lectin-antibody ELISA system to determine core-fucosylated haptoglobin. Clin. Chim. Acta. **446**, 30–36 (2015). https://doi.org/10.1016/j.cca.2015.03.037
42. de Vroome, S.W., Holst, S., Girondo, M.R., van der Burgt, Y.E.M., Mesker, W.E., Tollenaar, R.A.E.M., Wuhrer, M.: Serum N -glycome alterations in colorectal cancer associate with survival. Oncotarget **9**(55) (2018). https://doi.org/10.18632/oncotarget.25753
43. Matsumoto, T., Hatakeyama, S., Yoneyama, T., Tobisawa, Y., Ishibashi, Y., Yamamoto, H., Yoneyama, T., Hashimoto, Y., Ito, H., Nishimura, S.-I., Ohyama, C.: Serum N-glycan profiling is a potential biomarker for castration-resistant prostate cancer. Sci. Rep. **9**(1), 16761 (2019). https://doi.org/10.1038/s41598-019-53384-y
44. van de Bovenkamp, F.S., Hafkenscheid, L., Rispens, T., Rombouts, Y.: The emerging importance of IgG Fab glycosylation in immunity. J. Immunol. **196**(4), 1435–1441 (2016). https://doi.org/10.4049/jimmunol.1502136
45. Kinoshita, N., Ohno, M., Nishiura, T., Fujii, S., Nishikawa, A., Kawakami, Y., Uozumi, N., Taniguchi, N.: Glycosylation at the Fab portion of myeloma immunoglobulin G and increased fucosylated biantennary sugar chains: structural analysis by high-performance liquid chromatography and antibody-lectin enzyme immunoassay using Lens culinaris agglutinin. Cancer Res. **51**(21), 5888–5892 (1991)
46. Epp, A., Sullivan, K.C., Herr, A.B., Strait, R.T.: Immunoglobulin glycosylation effects in allergy and immunity. Curr. Alergy Asthma Rep. **16**(11), 1–13 (2016). https://doi.org/10.1007/s11882-016-0658-x
47. Kazuno, S., Murayama, K., Ueno, T., Fujimura, T., Furukawa, J.-I., Shinohara, Y., Fujime, M.: Glycosylation status of serum immunoglobulin G in patients with prostate diseases. Cancer Med. **5**(6), 1137–1146 (2016). https://doi.org/10.1002/cam4.662
48. Saldova, R., Royle, L., Radcliffe, C.M., Abd Hamid, U.M., Evans, R., Arnold, J.N., Banks, R.E., Hutson, R., Harvey, D.J., Antrobus, R., Petrescu, S.M., Dwek, R.A., Rudd, P.M.: Ovarian cancer is associated with changes in glycosylation in both acute-phase proteins and IgG. Glycobiology **17**(12), 1344–1356 (2007). https://doi.org/10.1093/glycob/cwm100
49. Kodar, K., Stadlmann, J., Klaamas, K., Sergeyev, B., Kurtenkov, O.: Immunoglobulin G Fc N-glycan profiling in patients with gastric cancer by LC-ESI-MS: relation to tumor progression and survival. Glycoconjugate J. **29**(1), 57–66 (2012). https://doi.org/10.1007/s10719-011-9364-z
50. Gebrehiwot, A.G., Melka, D.S., Kassaye, Y.M., Gemechu, T., Lako, W., Hinou, H., Nishimura, S.-I.: Exploring serum and immunoglobulin G N-glycome as diagnostic biomarkers for early detection of breast cancer in Ethiopian women. BMC Cancer. **19**(1), 588 (2019). https://doi.org/10.1186/s12885-019-5817-8
51. Vučković, F., Theodoratou, E., Thaçi, K., Timofeeva, M., Vojta, A., Štambuk, J., Pučić-Baković, M., Rudd, P.M., Đerek, L., Servis, D., Wennerström, A., Farrington, S.M., Perola, M., Aulchenko, Y.,

Dunlop, M.G., Campbell, H., Lauc, G.: IgG glycome in colorectal cancer. Clin. Cancer Res. **22**(12), 3078–3086 (2016). https://doi.org/10.1158/1078-0432.Ccr-15-1867

52. Doherty, M., Theodoratou, E., Walsh, I., Adamczyk, B., Stockmann, H., Agakov, F., Timofeeva, M., Trbojevic-Akmacic, I., Vuckovic, F., Duffy, F., McManus, C.A., Farrington, S.M., Dunlop, M.G., Perola, M., Lauc, G., Campbell, H., Rudd, P.M.: Plasma N-glycans in colorectal cancer risk. Sci. Rep. **8**(1), 1–12 (2018). https://doi.org/10.1038/s41598-018-26805-7
53. Yu, H., Shu, J., Li, Z.: Lectin microarrays for glycoproteomics: an overview of their use and potential. Exp. Rev. Proteomics. **17**(1), 27–39 (2020). https://doi.org/10.1080/14789450.2020.1720512
54. de Haan, N., Falck, D., Wuhrer, M.: Monitoring of immunoglobulin N- and O-glycosylation in health and disease. Glycobiology.(2020). https://doi.org/10.1093/glycob/cwz1048
55. Plomp, R., de Haan, N., Bondt, A., Murli, J., Dotz, V., Wuhrer, M.: Comparative glycomics of immunoglobulin A and G from saliva and plasma reveals biomarker potential. Front. Immunol. **9**, 2436/2431–2436/2412 (2018). https://doi.org/10.3389/fimmu.2018.02436
56. Hatakeyama, S., Yoneyama, T., Tobisawa, Y., Ohyama, C.: Recent progress and perspectives on prostate cancer biomarkers. Int. J. Clin. Oncol. **22**(2), 214–221 (2017)
57. Chen, S., LaRoche, T., Hamelinck, D., Bergsma, D., Brenner, D., Simeone, D., Brand, R.E., Haab, B.B.: Multiplexed analysis of glycan variation on native proteins captured by antibody microarrays. Nat. Methods. **4**(5), 437–444 (2007). https://doi.org/10.1038/nmeth1035
58. Lauc, G., Pezer, M., Rudan, I., Campbell, H.: Mechanisms of disease: The human N-glycome. Biochim. Biophys. Acta-Gen. Subj. **1860**(8), 1574–1582 (2016). https://doi.org/10.1016/j.bbagen.2015.10.016