



**Serum tri- and tetra-antennary N-glycan is a potential predictive biomarker for castration-resistant prostate cancer**

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7 Title: Serum sialyl tri-antennary N-glycan is a potential predictive biomarker for  
8 castration-resistant prostate cancer.

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10 Corresponding Author: Dr. Tohru Yoneyama

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13 July 28, 2014

14 John T. Isaacs

15 Editor-in-Chief

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18 *The Prostate*

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21 Dear Dr. Isaacs,

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24 Thank you very much for your letter suggesting the revision of our manuscript.  
25 The critiques are generally positive and useful and we have revised the  
26 manuscript accordingly as follows:  
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31 Comments:   Reviewer 1

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33 Comments to the Author

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35 This paper present important data for diagnosis of prostate cancer. The authors  
36 uses patient serum and analyzed N-glycan structures using sophisticated  
37 methods. However the study has some weakness, which needs to be addressed  
38 by revision.  
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43 1. It is well-written that malignant cancer cells produce triantennary and  
44 tetraantennary glycans. This work is therefore consistent with the previous  
45 reports. This should be stated in the manuscript.

46  
47 [Response]

48 We added new reference #27 and added following sentence in page17;

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50 In prostate cancer, Kyselova Z et al [27] investigated that N-glycomic profiles (50  
51 types of N-glycan) derived from human blood sera of 10 healthy males were  
52 compared to those from 24 metastatic PC patients. Although the sample size  
53 was very small, they report tri- and tetra-antennary N-glycans of metastatic PC  
54 patients were significantly higher than those of healthy males. This was  
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consistent with our present result. In the present study, the recently established technology of *N*-glycan analysis with the glycoblotting method and MALDI-TOF was used for high-throughput, comprehensive, and quantitative serum *N*-glycan profiling in PC patients. To the best of our knowledge, this is the first report to identify serum *N*-glycans as biomarkers in CRPC patients by using high-throughput quantitative *N*-glycomics.

2. Structure of *N*-glycans were identified by mass spectrometry, but no mass spectrometry data are presented. The authors should present representative mass data each for HLT, BPH, esPC, PC with ADT, and CRPC, as supplemental figures.

[Response]

We added supplemental figure 2 and 3 including representative mass spectra data each for HLT, BPH, esPC, PC with ADT, and CRPC.

We added 5 words in page 4 as follows:

Nine *N*-glycans (*m/z* 1362, 1566, 1753, 1794, 3049, 3414, 3560, 3719, and 3865) were significantly different between PC with ADT and CRPC groups (Table 2, Supplementary Figs 2 and 3).

We also added supplemental figs 2 and 3 legends in supplementary figure legend section as follows;

Supplementary Figure 2. Representative MALDI-TOF MS spectra (range of *m/z* 1000 to 4000) of BOA-labeled *N*-glycans derived from HLT, BPH, esPC, PC with ADT and CRPC patient serum. Significantly different *N*-glycans (*m/z* 1362, 1566, 1753, 1794, 3049, 3414, 3560, 3719, and 3865) between CRPC and other groups were shown in mass spectra. Symbols: yellow circles, galactose (Gal); green circles, mannose (Man); blue squares, *N*-acetylgulucosamine (GlcNAc); purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).

Supplementary Figure 3. Representative MALDI-TOF MS spectra (range of *m/z* 2500 to 4000) of BOA-labeled *N*-glycans derived from HLT, BPH, esPC, PC with ADT and CRPC patient serum. Significantly different *N*-glycans (*m/z* 3049, 3414,

3560, 3719, and 3865) between CRPC and other groups were shown in mass spectra. Symbols: yellow circles, galactose (Gal); green circles, mannose (Man); blue squares, *N*-acetylglucosamine (GlcNAc); purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).

3. Manuscript need to be edited extensively. It is difficult to understand legend to figure 1.

[Response]

We deeply apologize for our mistake in Figure 1. Putative *N*-glycan structure of *m/z* 3414 (sialyl or Lactosaminy l tri-antennary *N*-glycan) upper left corner of graph B was wrong. Correct putative *N*-glycan structure of *m/z* 3414 was three terminal sialic acid attached tetra-antennary *N*-glycan ((Hex)<sub>4</sub>(HexNAc)<sub>4</sub>(NeuAc)<sub>3</sub> + (Man)<sub>3</sub>(GlcNAc)<sub>2</sub>) shown in Supplementary Table 1. Therefore, we revised correct *N*-glycan structure of *m/z* 3414 was shown in Figure 1, panel D. Putative structure of *m/z* 3049 was also transfer in panel D.

In according to above revision, we revised in title and manuscript as follows;

Previous title

Serum sialyl tri-antennary *N*-glycan is a potential predictive biomarker for castration-resistant prostate cancer.

Revised title

Serum tri-and tetra- antennary *N*-glycan is a potential predictive biomarker for castration-resistant prostate cancer.

In manuscript and legend, “sialyl tri-antennary” was instead of “tri- and tetra-antennary”.

To explain detail of fig.1, we added a few sentence in manuscript (page14) as follows;

To investigate predictive potential for CRPC, nine *N*-glycans were analyzed using logistic regression analysis. The tri- and tetra-antennary *N*-glycans *m/z* 3049 (odds ratio, 3.326) and *m/z* 3414 (odds ratio, 13.189) showed higher odds

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5 ratio than other glycans, therefore  $m/z$  3049 and  $m/z$  3414 were selected as  
6 specific *N*-glycans for the prediction of CRPC (Table 3). Fig.1A and B showed  
7 serum level of  $m/z$  3049 and  $m/z$  3414 glycans in each group.  
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13 We also revise Figure 1 legend as follows;

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15 **Figure 1.** Serum levels of significant tri- and tetra-antennary *N*-glycans  
16 associated with the prediction of CRPC that were selected using logistic  
17 regression analysis. A, serum  $m/z$  3049 level in HLT, BPH, esPC, PC with ADT,  
18 and CRPC patients. B, serum  $m/z$  3414 level in HLT, BPH, esPC, PC with ADT,  
19 and CRPC patients. C, receiver operating characteristics (ROC) curve for the  
20 prediction of CRPC. The AUCs of  $m/z$  3049 and  $m/z$  3414 were 0.697 and  
21 0.748, respectively. D, Putative structures of  $m/z$  3049 and  $m/z$  3414 are  
22 represented as monosaccharide symbols. Yellow circles, galactose (Gal);  
23 green circles, mannose (Man); blue squares, *N*-acetylgalucosamine (GlcNAc);  
24 purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).  
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33 By taking into consideration the critiques of the reviewers, we believe that the  
34 manuscript has improved, and we hope that it is now acceptable for publication in  
35 The Prostate. We thank you very much for your editorial efforts.  
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39 Sincerely yours,

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43 Assistant professor, Department of Advanced Transplant and Regenerative  
44 Medicine, Hirosaki University Graduate School of Medicine  
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6 **Serum tri- and tetra-antennary *N*-glycan is a potential predictive biomarker**  
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9 **for castration-resistant prostate cancer**  
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22 Shin-Ichiro Nishimura<sup>3</sup>, Chikara Ohyama<sup>1,2</sup>, and Tohru Yoneyama<sup>1,2</sup>.  
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22 **Running title:** Serum *N*-glycan profiling in CRPC  
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31 **Conflicts of interests:** All authors declare no conflicts of interests.  
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38 **Total number of figures and tables:** 3 figures and 4 tables, 1 supplementary  
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41 figure, and 1 supplementary table.  
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**ABSTRACT**

**BACKGROUND.** The U.S.FDA has approved several novel systemic agents including abiraterone acetate and taxoid cabazitaxel for metastatic castration-resistant prostate cancer (CRPC) result in a complicated decision-making while selecting an appropriate treatment. Therefore, a predictive biomarker for CRPC would provide useful information to physicians. The aim of this study is to evaluate the diagnostic potential of serum *N*-glycan profiling in CRPC.

**METHODS.** Serum *N*-glycomics was performed in 80 healthy volunteers and 286 benign prostatic hyperplasia, 258 early-stage PC, 46 PC with androgen deprivation therapy (ADT), and 68 CRPC patients using the glycoblotting method. A total of 36 types of *N*-glycan levels in each patient were analyzed using logistic regression analysis and receiver operating characteristic curves. We also examined the expression of *N*-glycan branching enzyme genes in PC cell lines using quantitative RT-PCR.

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6 **RESULTS.** We observed that tri- and tetra-antennary *N*-glycans were  
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9 significantly higher in CRPC patients than in any other groups. The longitudinal  
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11 follow-up of tri- and tetra- antennary *N*-glycan levels revealed that one PC with  
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13 ADT patient showed an increase that was more than the cut-off level and two  
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15 consecutive increases in tri- and tetra-antennary *N*-glycan levels 3 months apart;  
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19 resulted in biochemical recurrence despite the castrate level of testosterone, and  
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22 the patient was defined as CRPC. Expression of *N*-glycan branching enzyme  
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26 genes were significantly upregulated in CRPC cell lines.  
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32 **CONCLUSIONS.** These results suggest that the overexpression of tri- and  
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35 tetra-antennary *N*-glycan may be associated with the castration-resistant status  
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38 in PC and may be a potential predictive biomarker for CRPC.  
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41 **Keywords:** serum *N*-glycan; androgen deprivation therapy; biomarker;  
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44 castration-resistant prostate cancer; glycoblotting.  
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## 51 INTRODUCTION

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6 Prostate cancer (PC) is one of the most common cancers in men worldwide  
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9 [1]. The American Cancer Society estimated 241,740 new cases and 28,170  
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12 deaths in the United States in 2012 [2]. PC is a multifocal disease with a  
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15 moderate clinical progression. Localized early-stage PC (esPC) can be well  
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18 treated with radical prostatectomy. In contrast, advanced PC is mostly treated  
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21 with androgen deprivation therapy (ADT); however, ADT fails in approximately  
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24 10%–20% of patients, who then develop castration-resistant PC (CRPC) within 5  
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27 years of follow-up [3, 4]. CRPC is a heterogeneous and progressive stage of  
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30 PC and includes both symptomatic and asymptomatic male patients with or  
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33 without clinical metastases [5]. Although the mechanism underlying androgen  
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36 independence remains unclear, recent advances have led to a better  
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39 understanding of this mechanism. Over the past few years, several novel  
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42 systemic agents for metastatic CRPC, such as the androgen synthesis inhibitor  
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45 abiraterone acetate [6], the immunotherapeutic sipuleucel-T [7], the taxoid  
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48 cabazitaxel [8] and the enzalutamide [9], have been approved by the US Food  
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51 and Drug Administration (FDA). Therapeutic option for CRPC become  
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6 complicated treatment decision making. Therefore, a predictive biomarker for  
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10 CRPC would provide useful information to physicians for selecting the  
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12 appropriate therapy sequence at a given time as soon as possible. However,  
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16 no validated predictive biomarkers for CRPC have been reported.

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19 Glycosylation plays an important role in various biological functions.  
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22 Cancer-associated aberrant glycosylation has been frequently observed in  
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25 bladder cancer [10], germ cell tumors [11], PC [12], colorectal cancer [13],  
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28 hepatocellular cancer [14], pancreatic cancer [15], and renal cell carcinoma [16].  
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32 Recently, high-throughput, comprehensive, and quantitative *N*-glycomics based  
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35 on the glycoblotting method using Sweetblot revealed that serum *N*-glycomics is  
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38 promising to screen for a diagnostic and prognostic marker for renal cell  
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41 carcinoma [17]. It is also a promising prognostic tool in patients undergoing  
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44 hemodialysis [18] and patients with advanced hepatocellular carcinoma  
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47 undergoing treatment with sorafenib [19]. However, the use of serum  
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51 *N*-glycans as a predictive biomarker for PC has not yet been investigated. In  
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6 the present study, we performed serum *N*-glycomics in PC patients and  
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10 evaluated its potential as a predictive biomarker for CRPC.

## 11 12 13 14 15 16 **MATERIALS AND METHODS**

### 17 18 19 **Serum Samples**

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22 A total of 650 patients with benign prostatic hyperplasia (BPH), early-stage  
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25 PC (esPC), PC with ADT, or CRPC were treated at our hospital between June  
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28 2007 and December 2013. Serum samples from BPH ( $n = 286$ ) and esPC ( $n =$   
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31 258) patients were obtained at the time of biopsy. The final diagnosis of BPH  
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34 and esPC patients was confirmed using the histopathological findings of  
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37 prostate biopsies. Serum samples from PC with ADT ( $n = 46$ ) and CRPC ( $n =$   
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40 68) patients were obtained at the time of treatment. Biochemical recurrence  
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43 was defined as prostate-specific antigen (PSA) levels  $>0.2$  ng/mL after  
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46 prostatectomy or increase 2 ng/mL above the nadir PSA after radiotherapy  
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49 (RT). CRPC was defined by PSA or radiographic progression despite the  
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52 castrate levels of testosterone of  $<50$  ng/dL. All samples were stored at  $-80^{\circ}\text{C}$   
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6 until use. Serum samples from 80 healthy volunteers (HLT) were obtained from  
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10 our serum bank and were stored at  $-80^{\circ}\text{C}$  until use. The study was performed  
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13 in accordance with the ethical standards of the Declaration of Helsinki and was  
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16 approved by the Ethics Committee of Hirosaki University Graduate School of  
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19 Medicine. Informed consent was obtained from all patients. Patient  
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22 demographics are shown in Table 1.  
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### 24 25 26 27 28 29 **Glycoblotting Method and Mass Spectrometry** 30

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32 Serum *N*-glycan analysis was performed as described previously using  
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34 SweetBlot™ (System Instruments, Hachijo, Japan) [17] (Supplementary Figure  
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36 1). Briefly, 10  $\mu\text{L}$  of serum samples containing 40 pmol of the internal standard  
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38 disialo-galactosylated biantennary *N*-glycan, which has amidated sialic acids (A2  
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40 amide glycans) (Supplementary Table 1), were reduced and alkylated using DTT  
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42 and iodoacetamide (Wako Pure Chemical Industries, Osaka, Japan),  
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45 respectively. The resulting mixture was then trypsinized and heat inactivated.  
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55 After cooling down to room temperature, peptide *N*-glycanase F (New England  
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6 BioLabs, Ipswich, MA, USA) was added to the mixture to release total serum  
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9 *N*-glycans. After incubating for 360 min at 37°C, 20 μL of the resulting mixture,  
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12 equivalent to 2.5 μL of serum. An aliquot of each pretreated sample was mixed  
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15 with 500 μL of BlotGlyco H beads (Sumitomo Bakelite, Co., Tokyo, Japan) to  
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18 capture glycans via stable hydrazone bonds on MultiScreen Solvinert® filter  
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21 plate (MerkMillipore, Billerica, MA, USA). Then, acetyl capping of unreacted  
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24 hydrazide functional groups on the beads and methyl esterification of sialic acid  
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27 carboxyl groups, which exist in the terminal of the captured glycans, were  
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30 performed sequentially; serial washes were then performed before each step, as  
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33 described previously [17, 19, 20-24]. The captured *N*-glycans were labeled  
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36 with benzyloxiamine (BOA, Sigma-Aldrich, St. Louis, MO, USA) by  
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39 transiminization and were eluted in 150 μL of water. The BOA-labeled glycans  
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42 were detected using MALDI-TOF MS (Ultraflex 3 TOF/TOF mass spectrometer,  
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45 Bruker Daltonics, Bremen, Germany). Compositions and structures of glycans  
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48 were predicted using GlycoMod Tool (<http://br.expasy.org/tools/glcomod>).  
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### Quantitative Reproducibility Test of Sweetblot

Each quantitative reproducibility test of Sweetblot was performed as described previously [25]. Briefly, serum samples and serially diluted standard human serum (Sigma-Aldrich) were added to the plate, and the whole process of *N*-glycomics was performed with Sweetblot. The peak area of each glycan detected at 0.5x, 0.75x, 1x, 1.25x, 1.5x, 1.75x, 2x, and 2.25x concentrations was plotted. This assay was repeated twice, and quantitative reliability was then judged based on following parameters: outliers were allowed <3 points, slope  $\sigma$  of <3.0, and the significance level of the correlation coefficient  $r$  was <0.05. Glycan peaks were judged to be useful when the abovementioned criteria of the assay were met, and the resulting glycans were used for statistical analysis.

### Statistical Analysis

Statistical calculations for clinical data were performed using SPSS ver. 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.03 (GraphPad Software, San Diego, CA, USA). Intergroup differences were statistically

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6 compared using the Student's *t*-test for normally distributed models or the  
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9 Mann–Whitney U-test for nonnormally distributed models. *N*-glycan levels  
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12 were analyzed using logistic regression analysis and receiver operating  
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15 characteristic (ROC) curves to select *N*-glycans that were associated with CRPC  
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18 status in PC. The optimal cut-off points were calculated using the following  
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21 formula:  $(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2$  [26].  $P < 0.05$  was considered  
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24 significant.  
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### 32 Real-time Quantitative RT-PCR

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35 The normal prostate epithelial cell line RWPE-1 and the PC cell lines  
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38 LNCaP, DU145, and PC-3 were obtained from the American Type Culture  
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41 Collection. RWPE-1 was grown at 37°C with 5% CO<sub>2</sub> in Keratinocyte-SFM  
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44 medium supplemented with penicillin, streptomycin, bovine pituitary extract, 5  
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47 ng/ml epidermal growth factor. LNCaP, DU145, and PC-3 were grown at 37°C  
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51 with 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with penicillin, streptomycin,  
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54 and 10% FBS. LNCaP-androgen independent (AI) cell were grown at 37°C  
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6 with 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with penicillin, streptomycin,  
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9 and 10% charcoal-stripped FBS. Total RNA was isolated from RWPE-1, LNCaP,  
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11 LNCaP-AI, DU145, and PC-3 cells using ISOGEN II (Wako Pure Chemical  
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13 Industries) according to the manufacturer's instructions. First-strand cDNA was  
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16 synthesized from 0.5 µg of total RNA using ReverTra Ace® qPCR RT Master  
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18 Mix with gDNA Remover (Toyobo, Kita-ku, Osaka, Japan) according to the  
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20 manufacturer's instructions. Real-time qRT-PCR assays were performed in  
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22 triplicate using GeneAce SYBR® qPCR Mix α No ROX (Nippon Gene,  
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24 Chiyoda-ku, Tokyo, Japan) and 500 nM gene-specific primers. Reactions were  
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26 processed on a CFX connect™ Real-Time System (Bio-Rad Laboratories, Inc.,  
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28 Hercules, CA, USA) under the following conditions: 95°C for 10 min, followed by  
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30 40 cycles of 95°C for 15 s and 60°C for 45 s. PrimeTime® qPCR primer pairs  
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32 for human *N*-acetylglucosaminyltransferase I (*MGAT1*) (Hs.PT.58.4702749),  
33  
34 human *N*-acetylglucosaminyltransferase II (*MGAT2*) (Hs.PT.58.24612062.g),  
35  
36 human *N*-acetylglucosaminyltransferase III (*MGAT3*) (Hs.PT.58.26307986.g),  
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38 human *N*-acetylglucosaminyltransferase IVa (*MGAT4A*) (Hs.PT.58.3289156),  
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6 human *N*-acetylglucosaminyltransferase IVb (*MGAT4B*) (Hs.PT.58.19371732),  
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9 human *N*-acetylglucosaminyltransferase IVc (*MGAT4C*) (Hs.PT.58.2945729),  
10  
11  
12 human *N*-acetylglucosaminyltransferase V (*MGAT5A*) (Hs.PT.58.4758371),  
13  
14  
15 human *N*-acetylglucosaminyltransferase Vb (*MGAT5B*) (Hs.PT.58.27758528),  
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17  
18 and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)  
19  
20 (Hs.PT.39a.22214847) were purchased from Integrated DNA Technologies, Inc.  
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22  
23  
24  
25 (Coralville, IA, USA). Relative expression levels of *MGAT* genes were  
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28  
29 normalized to expression of the *GAPDH* gene.  
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## 35 RESULTS

### 36 37 38 Tri- and Tetra-Antennary *N*-glycans Significantly Increased in CRPC

#### 39 40 41 Patients.

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44 Serum *N*-glycan analysis performed using the glycoblotting method and  
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46  
47 mass spectrometry identified 45 types of BOA-labeled *N*-glycans in all serum  
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50 samples. We then performed quantitative reproducibility tests. Finally, 36  
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53 types of *N*-glycans (Supplementary Table 1) had good quantitative  
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6 reproducibility among all samples and could be used for statistical analysis.  
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10 Table 1 summarizes the demographics of the study cohort. No significant  
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12 differences were observed in age between BPH and esPC groups. The iPSA  
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14 level in the esPC group was significantly higher than that in the BPH group ( $P =$   
15  
16 0.0002). The age of patients in the PC with ADT group was significantly higher  
17  
18 than that in the CRPC group ( $P = 0.033$ ). No significant differences were  
19  
20 observed in the *N*-glycan profiles of HLT, BPH, esPC, and PC with ADT patients.  
21  
22 We observed significant differences in the *N*-glycan profiles between CRPC and  
23  
24 the other groups. Nine *N*-glycans ( $m/z$  1362, 1566, 1753, 1794, 3049, 3414,  
25  
26 3560, 3719, and 3865) were significantly different between PC with ADT and  
27  
28 CRPC groups (Table 2, Supplementary Figs 2 and 3). To investigate predictive  
29  
30 potential for CRPC, nine *N*-glycans were analyzed using logistic regression  
31  
32 analysis. The tri- and tetra-antennary *N*-glycans  $m/z$  3049 (odds ratio, 3.326)  
33  
34 and  $m/z$  3414 (odds ratio, 13.189) showed higher odds ratio than other glycans,  
35  
36 therefore  $m/z$  3049 and  $m/z$  3414 were selected as specific *N*-glycans for the  
37  
38 prediction of CRPC (Table 3). Fig.1A and B showed serum level of  $m/z$  3049 and  
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6 *m/z* 3414 glycans in each group. ROC curves were then used to compare the  
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9 predictive potential of *m/z* 3049 and *m/z* 3414 for CRPC (Fig. 1C). The area  
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11  
12 under the curve (AUC) of *m/z* 3049 and *m/z* 3414 could be used to discriminate  
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14  
15 between PC with ADT and CRPC patients (AUC, 0.697 and 0.748, respectively).  
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17

### 22 **Longitudinal Follow-Up of Tri- and Tetra-antennary *N*-glycan Levels in 16**

#### 25 **PC with ADT Patients**

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27  
28 The optimal cut-off levels of *m/z* 3049 and *m/z* 3414 were determined to be  
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31 >1.60  $\mu$ M and >1.36  $\mu$ M, respectively, for the prediction of CRPC based on ROC  
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34 curves (Table 4). To evaluate the predictive potential of *m/z* 3049 and *m/z*  
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36  
37 3414, we followed-up *m/z* 3049 and *m/z* 3414 levels in 16 PC with ADT patients  
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40 every 3 or 6 months (Fig. 2A, B). Total PSA and testosterone levels were also  
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43 followed-up at the same time points (Fig. 2C, D). We found that one PC with  
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46 ADT patient showed two consecutive increases in *m/z* 3049 and *m/z* 3414 levels  
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49 3 months apart. This patient also showed two consecutive increases in PSA  
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52 levels and was finally defined as CRPC because the testosterone level was <50  
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6 ng/dL. This finding suggests that the overexpression of serum tri- and  
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10 tetra-antennary *N*-glycans may be associated with the castration-resistant status  
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13 in PC.

### 14 15 16 17 18 19 **Transcription Levels of *N*-glycan Branching Enzyme Genes Were** 20 21 22 **Significantly Upregulated in CRPC Cell Lines** 23

24  
25 We also examined transcription levels of *MGAT1*, *MGAT2*, *MGAT3*,  
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28 *MGAT4A*, *MGAT4B*, *MGAT5A*, and *MGAT5B*, which are medial Golgi enzymes  
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31 that initiate the  $\beta$ 1,6GlcNAc branching in bi-, tri-, and tetra-branched *N*-glycans,  
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34 in PC cell lines using qRT-PCR (Fig. 3). The CRPC-like cell lines DU145 and  
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37 PC-3 showed significantly increased transcription of *MGAT1*, *MGAT2*, *MGAT4B*,  
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40 *MGAT5A*, and *MGAT5B* genes. Particularly, the expression of the *MGAT5B*  
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43 gene was 20-fold higher in CRPC like LNCaP-AI, DU145 and PC-3 cells than in  
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46 androgen-dependent LNCaP cells and normal prostate epithelial RWPE-1 cells.  
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## 54 55 **DISCUSSION**

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6 High-throughput, comprehensive, and quantitative *N*-glycomics is an important  
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9 and promising method. Several studies have reported that differences in  
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12 glycan profiling between diseased and benign states may be useful in the  
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15 diagnosis or prognosis of diseases [17-19, 23-25]. In prostate cancer,  
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18 Kyselova Z et al [27] investigated that *N*-glycomic profiles (50 types of *N*-glycan)  
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21 derived from human blood sera of 10 healthy males were compared to those  
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24 from 24 metastatic PC patients. Although the sample size was very small, they  
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27 report tri- and tetra-antennary *N*-glycans of metastatic PC patients were  
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30 significantly higher than those of healthy males. This was consistent with our  
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33 present result. In the present study, the recently established technology of  
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36 *N*-glycan analysis with the glycoblotting method and MALDI-TOF was used for  
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39 high-throughput, comprehensive, and quantitative serum *N*-glycan profiling in  
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42 PC patients. To the best of our knowledge, this is the first report to identify  
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45 serum *N*-glycans as biomarkers in CRPC patients by using high-throughput  
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48 quantitative *N*-glycomics. Our results demonstrate that serum levels of tri- and  
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51 tetra-antennary *N*-glycans (*m/z* 3049 and *m/z* 3414) were statistically and  
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6 significantly different between PC with ADT and CRPC patients using the  
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9 optimal cut-off points (Figs 1 and 2). A previous study reported that  
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12 cancer-associated aberrant glycosylation increases the transcription of the  
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15 *MGAT5* gene, which initiates  $\beta$ 1,6GlcNAc branching in tri- and tetra-branched  
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18 *N*-glycans in PC and plays an important role in metastasis of PC [28]. Zavareh  
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20  
21 et al [29] reported that the knockdown of *N*-acetylglucosaminyltransferase I,  
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24 which is encoded by the *MGAT1* gene and is the first branching enzyme  
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27 required for additional branching on *N*-glycan, decreased levels of branched  
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32 *N*-glycan on the surface of PC-3 cells. In addition, their orthotopic xenograft  
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35 model exhibited significantly decreased primary tumor growth and incidence of  
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38 lung metastasis. In the current study, we demonstrated that transcription levels  
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41 of *MGAT1*, *MGAT2*, *MGAT4B*, *MGAT5A*, and *MGAT5B* genes were significantly  
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44 upregulated in CRPC cell lines (Fig. 3).  
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48 Results of several reports and the current study indicated that the  
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51 overexpression of tri- and tetra-branched *N*-glycans on the surface of CRPC  
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54 cells due to upregulation of *N*-glycan branching enzymes (*MGATs*) was strongly  
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6 correlated with metastatic PC, and this overexpression may be associated with  
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9 the castration-resistant status in PC.  
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12 These results suggest that the use of the glycoblotting method may provide  
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14 insight into new factors predicting CRPC. Although serum tri- and  
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16 tetra-antennary *N*-glycan expression was revealed as a useful predictive  
17  
18 biomarker in CRPC patients in the current study, this study has several  
19  
20 limitations. First, this study is small and preliminary. Second, it is very  
21  
22 important to determine the carrier protein for tri- and tetra-antennary *N*-glycans  
23  
24 that enables it to be released into the circulation from tumor tissues or circulating  
25  
26 tumor cells. Otherwise, the altered serum *N*-glycan profile could be a  
27  
28 systematic immunogenic reaction of the released tumor-associated antigen.  
29  
30 Future studies should address whether these alterations are a direct result of the  
31  
32 castration-resistant status in PC. Third, longitudinal patterns of changes in tri-  
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34 and tetra-antennary *N*-glycan from PC with ADT to CRPC patients were  
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36 investigated in only 16 patients. To validate these predictive biomarkers for  
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38 CRPC, an increased number of patients is required. Despite these limitations,  
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6 the overexpression of tri- and tetra-antennary *N*-glycans was clearly  
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9 demonstrated to be a potential biomarker for the prediction of CRPC in this study.  
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12 Future large-scale prospective validation studies may determine the clinical  
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15 significance of these carbohydrate biomarkers.  
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## 23 **CONCLUSIONS**

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25 Although the present study is small and preliminary, quantitative whole  
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27 serum *N*-glycan profiling may have the potential to predict castration-resistant  
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29 status in PC. Glycoblotting with MALDI-TOF mass spectrometry may be a  
30  
31 promising method for screening of new predictive biomarkers. At present, no  
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34 validated predictive biomarkers for CRPC have been reported. Therefore, a  
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37 predictive biomarker for CRPC would provide useful information to physicians to  
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## 16 REFERENCES

- 17  
18  
19 1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer  
20  
21  
22 statistics. *CA Cancer J Clin* 2011;61:69-90.  
23  
24  
25 2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*  
26  
27  
28 2012;62:10-29.  
29  
30  
31  
32 3. Hirst CJ, Cabrera C, Kirby M. Epidemiology of castration resistant prostate  
33  
34  
35 cancer: a longitudinal analysis using a UK primary care database. *Cancer*  
36  
37  
38 *Epidemiol* 2012;36:e349-e353.  
39  
40  
41 4. Kirby M, Hirst C, Crawford ED. Characterising the castration-resistant prostate  
42  
43  
44 cancer population: a systematic review. *Int J Clin Pract* 2011;65:1180-1192.  
45  
46  
47  
48 5. Toren PJ, Gleave ME. Evolving landscape and novel treatments in metastatic  
49  
50  
51 castrate-resistant prostate cancer. *Asian J Androl* 2013;15:342-349.  
52  
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58  
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- 1  
2  
3  
4  
5  
6 6. de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones  
7  
8  
9 RJ, Goodman OB Jr, Saad F, Staffurth JN, Mainwaring P, Harland S, Flaig TW,  
10  
11  
12 Hutson TE, Cheng T, Patterson H, Hainsworth JD, Ryan CJ, Sternberg CN,  
13  
14  
15 Ellard SL, Fléchon A, Saleh M, Scholz M, Efstathiou E, Zivi A, Bianchini D, Loriot  
16  
17  
18 Y, Chieffo N, Kheoh T, Haqq CM, Scher HI; COU-AA-301 Investigators.  
19  
20  
21  
22 Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med*  
23  
24  
25 2011;364:1995-2005.  
26  
27  
28  
29 7. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF,  
30  
31  
32 Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer  
33  
34  
35 PF; IMPACT Study Investigators. Sipuleucel-T immunotherapy for  
36  
37  
38 castration-resistant prostate cancer. *N Engl J Med* 2010;363:411-422.  
39  
40  
41  
42 8. de Bono JS, Oudard S, Ozguroglu M, Hansen S, Machiels JP, Kocak I, Gravis  
43  
44  
45 G, Bodrogi I, Mackenzie MJ, Shen L, Roessner M, Gupta S, Sartor AO; TROPIC  
46  
47  
48 Investigators. Prednisone plus cabazitaxel or mitoxantrone for metastatic  
49  
50  
51 castration-resistant prostate cancer progressing after docetaxel treatment: a  
52  
53  
54 randomised open-label trial. *Lancet* 2010;376:1147-1154.  
55  
56  
57  
58  
59  
60

- 1  
2  
3  
4  
5  
6 9. Tombal B, Borre M, Rathenborg P, Werbrouck P, Van Poppel H, Heidenreich  
7  
8  
9 A, Iversen P, Braeckman J, Heracek J, Baskin-Bey E, Ouatas T, Perabo F,  
10  
11  
12 Phung D, Hirmand M, Smith MR. Enzalutamide monotherapy in hormone-naive  
13  
14  
15 prostate cancer: primary analysis of an open-label, single-arm, phase 2 study.  
16  
17  
18  
19 Lancet Oncol 2014;15:592-600.  
20  
21  
22 10. Ishimura H, Takahashi T, Nakagawa H, Nishimura S, Arai Y, Horikawa Y,  
23  
24  
25 Habuchi T, Miyoshi E, Kyan A, Hagsawa S, Ohyama C.  
26  
27  
28 *N*-acetylglucosaminyltransferase V and  $\beta$ 1-6 branching N-linked  
29  
30  
31 oligosaccharides are associated with good prognosis of patients with bladder  
32  
33  
34 cancer. Clin Cancer Res 2006;12:2506-2511.  
35  
36  
37  
38 11. Hatakeyama S, Kyan A, Yamamoto H, Okamoto A, Sugiyama N, Suzuki Y,  
39  
40  
41 Yoneyama T, Hashimoto Y, Koie T, Yamada S, Saito H, Arai Y, Fukuda M,  
42  
43  
44 Ohyama C. Core 2 *N*-acetylglucosaminyltransferase-1 expression induces  
45  
46  
47 aggressive potential of testicular germ cell tumor. Int J Cancer  
48  
49  
50  
51 2010;127:1052-1059.  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3  
4  
5  
6 12. Haggisawa S, Ohyama C, Takahashi T, Endoh M, Moriya T, Nakayama J,  
7  
8  
9 Arai Y, Fukuda M. Expression of core 2  $\beta$ 1,6-N-acetylglucosaminyltransferase  
10  
11 facilitates prostate cancer progression. *Glycobiology* 2005;1:1016-1024.  
12  
13  
14  
15  
16 13. D'Arrigo A, Belluco C, Ambrosi A, Digito M, Esposito G, Bertola A, Fabris M,  
17  
18 Nofrate V, Mammano E, Leon A, Nitti D, Lise M. Metastatic transcriptional  
19  
20 pattern revealed by gene expression profiling in primary colorectal carcinoma.  
21  
22  
23  
24  
25  
26 Int J Cancer 2005;115:256-262.  
27  
28  
29 14. Moriwaki K, Noda K, Nakagawa T, Asahi M, Yoshihara H, Taniguchi N,  
30  
31  
32 Hayashi N, Miyoshi E. A high expression of GDP-fucose transporter in  
33  
34  
35 hepatocellular carcinoma is a key factor for increases in fucosylation.  
36  
37  
38  
39  
40  
41  
42  
43  
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46  
47  
48  
49  
50  
51  
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55  
56  
57  
58  
59  
60
15. Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K,  
Murata K, Ohigashi H, Yokoyama S, Eguchi H, Ishikawa O, Ito T, Kato M,  
Kasahara A, Kawano S, Gu J, Taniguchi N, Miyoshi E. Fucosylated haptoglobin  
is a novel marker for pancreatic cancer: a detailed analysis of the

1  
2  
3  
4  
5  
6 oligosaccharide structure and a possible mechanism for fucosylation. Int J  
7  
8

9  
10 Cancer 2006;118:2803-2808.

11  
12 16. Saito S, Orikasa S, Ohyama C, Satoh M, Fukushi Y. Changes in glycolipids  
13  
14

15  
16 in human renal-cell carcinoma and their clinical significance. Int J Cancer  
17

18  
19 1991;49:329-334.  
20

21  
22 17. Hatakeyama S, Amano M, Tobisawa Y, Yoneyama T, Tsuchiya N, Habuchi T,  
23

24  
25 Nishimura S-I, Ohyama C. Serum N-glycan alteration associated with renal cell  
26

27  
28 carcinoma detected by high throughput glycan analysis. J Urol  
29

30  
31 2013;191:805-813.  
32

33  
34 18. Hatakeyama S, Amano M, Tobisawa Y, Yoneyama T, Tsushima M, Hirose K,  
35

36  
37 Yoneyama T, Hashimoto Y, Koie T, Saitoh H, Yamaya K, Funyu T, Nishimura  
38

39  
40 S-I, Ohyama C. Serum N-glycan profiling predicts prognosis in patients  
41

42  
43 undergoing hemodialysis. Sci World J 2013;5:1-10.  
44

45  
46 19. Miyahara K, Nouse K, Miyake Y, Nakamura S, Obi S, Amano M, Hirose K,  
47

48  
49 Nishimura S-I, Yamamoto K. Serum glycan as a prognostic marker in patients  
50

1  
2  
3  
4  
5  
6 with advanced hepatocellular carcinoma treated with sorafenib. *Hepatology*

7  
8  
9  
10 2014;59:355-356.

11  
12 20. Miura Y, Kato K, Takegawa Y, Kuroguchi M, Furukawa J, Shinohara Y,

13  
14  
15 Nagahori N, Amano M, Hinou H, Nishimura S-I. Glycoblotting-assisted

16  
17  
18  
19 O-glycomics: ammonium carbamate allows for highly efficient o-glycan release

20  
21  
22 from glycoproteins. *Anal Chem* 2010;82:10021-10029.

23  
24  
25 21. Amano M, Yamaguchi M, Takegawa Y, Yamashita T, Terashima M,

26  
27  
28  
29 Furukawa J, Miura Y, Shinohara Y, Iwasaki N, Minami A, Nishimura S-I.

30  
31  
32 Threshold in stage-specific embryonic glycotypes uncovered by a full portrait of

33  
34  
35 dynamic N-glycan expression during cell differentiation. *Mol Cell Proteomics*

36  
37  
38 2010;9:523-537.

39  
40  
41 22. Furukawa J, Shinohara Y, Kuramoto H, Miura Y, Shimaoka H, Kuroguchi M,

42  
43  
44 Nakano M, Nishimura S-I. Comprehensive approach to structural and functional

45  
46  
47 glycomics based on chemoselective glycoblotting and sequential tag conversion.

48  
49  
50  
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Anal Chem 2008;80:1094-1101.

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5  
6 23. Kamiyama T, Yokoo H, Furukawa J, Kuroguchi M, Togashi T, Miura N,  
7  
8  
9 Nakanishi K, Kamachi H, Kakisaka T, Tsuruga Y, Fujiyoshi M, Taketomi A,  
10  
11  
12 Nishimura S-I, Todo S. Identification of novel serum biomarkers of hepatocellular  
13  
14  
15 carcinoma using glycomic analysis. *Hepatology* 2013;57:2314-2325.  
16  
17  
18  
19 24. Nouse K, Amano M, Ito YM, Miyahara K, Morimoto Y, Kato H, Tsutsumi K,  
20  
21  
22 Tomoda T, Yamamoto N, Nakamura S, Kobayashi S, Kuwaki K, Hagihara H,  
23  
24  
25 Onishi H, Miyake Y, Ikeda F, Shiraha H, Takaki A, Nakahara T, Nishimura S-I,  
26  
27  
28 Yamamoto K. Clinical utility of high-throughput glycome analysis in patients with  
29  
30  
31 pancreatic cancer. *J Gastroenterol* 2013;48:1171-1179.  
32  
33  
34  
35 25. Takeuchi M, Amano M, Tsukamoto T, Masumori N, Hirose K, Ohashi T,  
36  
37  
38 Nishimura S-I. N- and O-glycome analysis of serum and urine from bladder  
39  
40  
41 cancer patients using a high-throughput glycoblotting method. *J Glycomics*  
42  
43  
44  
45 *Lipidomics* 2013;3:108.  
46  
47  
48 26. Akobeng AK. Understanding diagnostic tests 3: Receiver operating  
49  
50  
51 characteristic curves. *Acta Paediatr* 2007;96:644-647.  
52  
53  
54  
55  
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58  
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60

1  
2  
3  
4  
5  
6 27. Kyselova Z, Mechref Y, Al Bataineh MM, Dobrolecki LE, Hickey RJ, Vinson J,  
7  
8  
9  
10 Sweeney CJ, Novotny MV. (2007). Alterations in the serum glycome due to  
11  
12  
13 metastatic prostate cancer. *J Proteome Res* 2007;6:1822-1832.

14  
15  
16 28. Tsui KH, Chang PL, Feng TH, Chung LC, Sung HC, Juang HH. Evaluating  
17  
18  
19 the function of matriptase and *N*-acetylglucosaminyltransferase V in prostate  
20  
21  
22 cancer metastasis. *Anticancer Res* 2008;28:1993-1999.

23  
24  
25 29. Beheshti Zavareh R, Sukhai MA, Hurren R, Gronda M, Wang X, Simpson  
26  
27  
28 CD, Maclean N, Zih F, Ketela T, Swallow CJ, Moffat J, Rose DR, Schachter H,  
29  
30  
31 Schimmer AD, Dennis JW. Suppression of cancer progression by MGAT1  
32  
33  
34 shRNA knockdown. *PLoS ONE* 2012;7:e43721.  
35  
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### FIGURE LEGENDS

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45 **Figure 1.** Serum levels of significant tri- and tetra-antennary *N*-glycans  
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51 regression analysis. A, serum *m/z* 3049 level in HLT, BPH, esPC, PC with ADT,  
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54 and CRPC patients. B, serum *m/z* 3414 level in HLT, BPH, esPC, PC with ADT,  
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6 and CRPC patients. C, receiver operating characteristics (ROC) curve for the  
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9 prediction of CRPC. The AUCs of  $m/z$  3049 and  $m/z$  3414 were 0.697 and  
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12 0.748, respectively. D, Putative structures of  $m/z$  3049 and  $m/z$  3414 are  
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15 represented as monosaccharide symbols. Yellow circles, galactose (Gal);  
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18 green circles, mannose (Man); blue squares, *N*-acetylgulucosamine (GlcNAc);  
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22 purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).  
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**Figure 2.** The longitudinal follow-up of serum  $m/z$  3049,  $m/z$  3414, PSA, and  
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32 testosterone levels in PC with ADT patients. A, serum  $m/z$  3049 levels. The  
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35 red dashed line represents the optimal cut-off level of  $m/z$  3049 ( $>1.60 \mu\text{M}$ ). B,  
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38 serum  $m/z$  3414 levels. The red dashed line represents the optimal cut-off level  
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41 of  $m/z$  3414 ( $>1.36 \mu\text{M}$ ). C, total serum PSA levels. D, serum testosterone  
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44 levels. The red dashed line represents the castrate level of testosterone (50  
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47 ng/dL). Blue and pink bold lines in panels A and B indicate the PC patient who  
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50 was treated with ADT and then experienced two consecutive increases in tri-  
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53 and tetra-antennary *N*-glycan levels. Only the blue bold line shows the PC with  
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6 ADT patient who experienced two consecutive increases in PSA levels (panel C)  
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10 despite maintaining a castrate level of testosterone (panel D); he was finally  
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12 defined as CRPC.  
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19 **Figure 3.** Quantitative qRT-PCR of *N*-glycan branching enzymes (*MGATs*) in  
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21 PC cell lines. Relative expression levels of *MGAT* genes were normalized to  
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23 the expression of the *GAPDH* gene in each cell line. The expression of each  
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28 *MGAT* gene in LNCaP cells was used as control and was defined as 1.0.  
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32 Asterisk symbol indicate *P* value of LNCaP vs LNCaP-AI. Double asterisk  
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35 symbol indicate *P* value of LNCaP vs DU145. Triple asterisk symbol indicate *P*  
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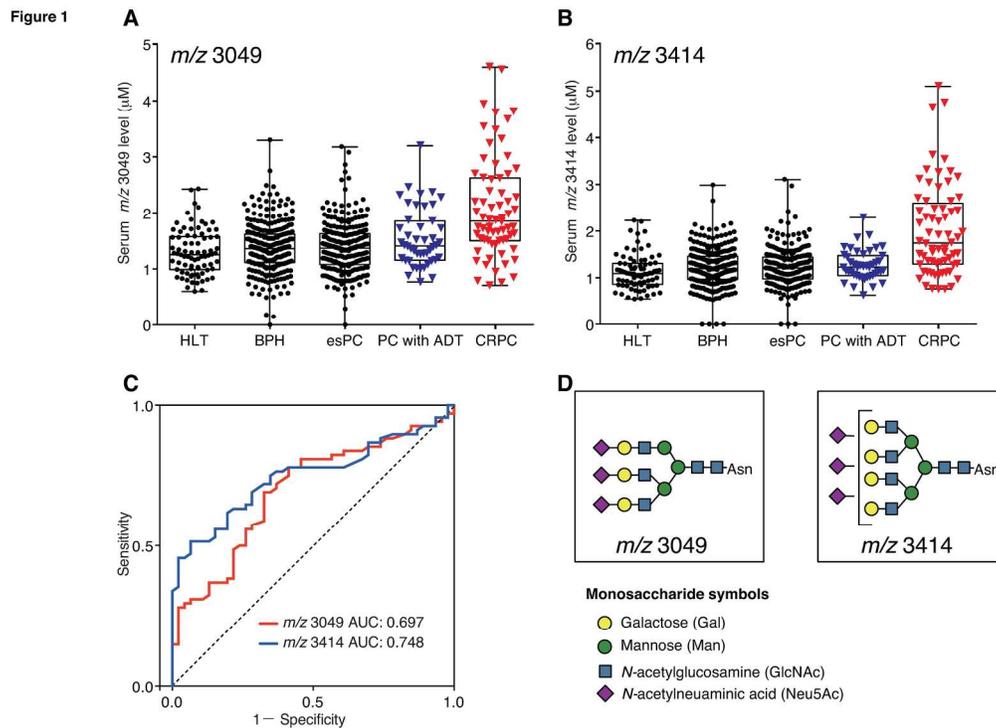


Figure 1. Serum levels of significant tri- and tetra-antennary N-glycans associated with the prediction of CRPC that were selected using logistic regression analysis. A, serum *m/z* 3049 level in HLT, BPH, esPC, PC with ADT, and CRPC patients. B, serum *m/z* 3414 level in HLT, BPH, esPC, PC with ADT, and CRPC patients. C, receiver operating characteristics (ROC) curve for the prediction of CRPC. The AUCs of *m/z* 3049 and *m/z* 3414 were 0.697 and 0.748, respectively. D, Putative structures of *m/z* 3049 and *m/z* 3414 are represented as monosaccharide symbols. Yellow circles, galactose (Gal); green circles, mannose (Man); blue squares, *N*-acetylglucosamine (GlcNAc); purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).  
205x149mm (300 x 300 DPI)

Figure 2

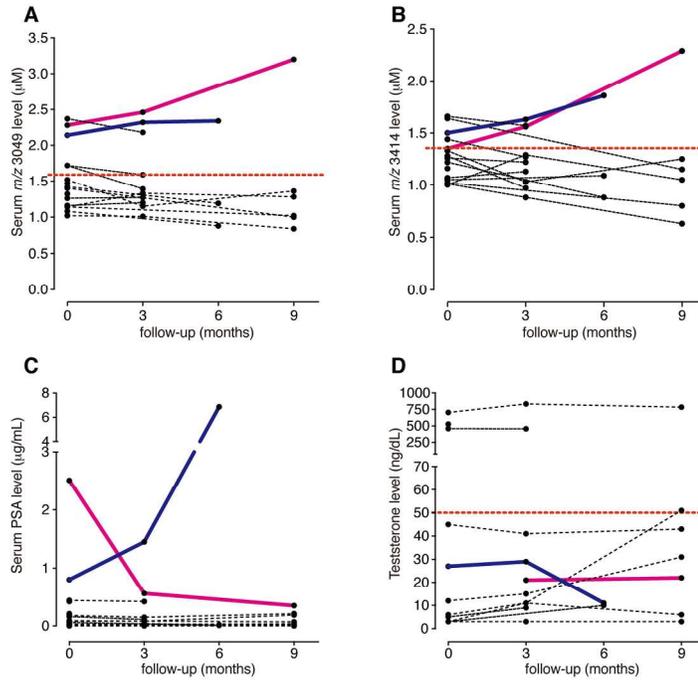


Figure 2. The longitudinal follow-up of serum m/z 3049, m/z 3414, PSA, and testosterone levels in PC with ADT patients. A, serum m/z 3049 levels. The red dashed line represents the optimal cut-off level of m/z 3049 (>1.60  $\mu\text{M}$ ). B, serum m/z 3414 levels. The red dashed line represents the optimal cut-off level of m/z 3414 (>1.36  $\mu\text{M}$ ). C, total serum PSA levels. D, serum testosterone levels. The red dashed line represents the castrate level of testosterone (50 ng/dL). Blue and pink bold lines in panels A and B indicate the PC patient who was treated with ADT and then experienced two consecutive increases in tri- and tetra-antennary N-glycan levels. Only the blue bold line shows the PC with ADT patient who experienced two consecutive increases in PSA levels (panel C) despite maintaining a castrate level of testosterone (panel D); he was finally defined as CRPC.

209x148mm (300 x 300 DPI)

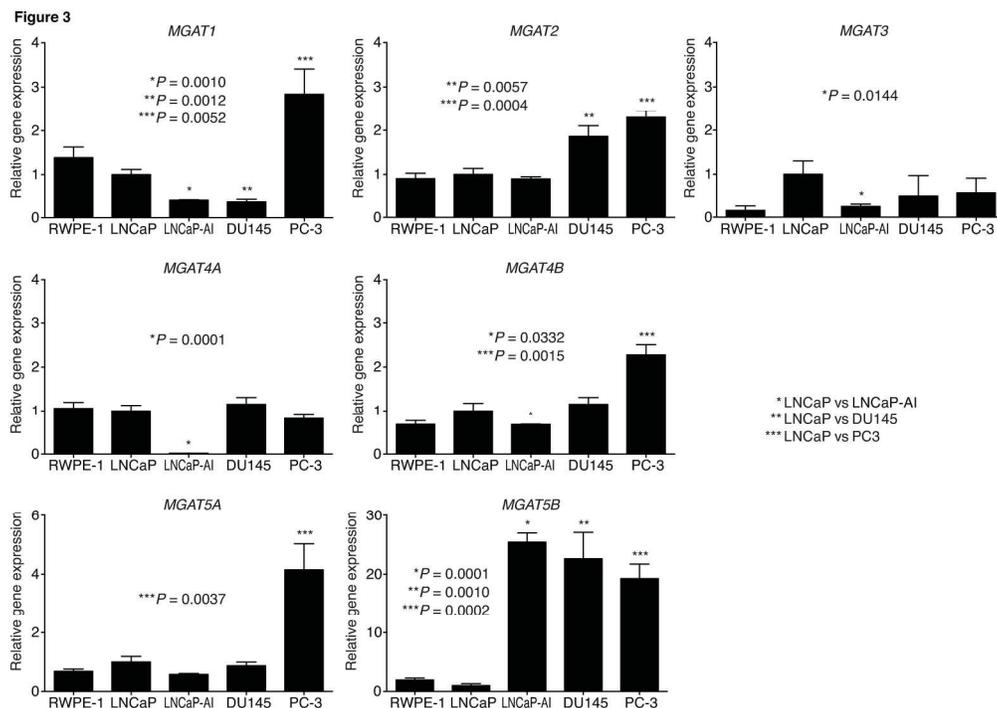


Figure 3. Quantitative qRT-PCR of N-glycan branching enzymes (MGATs) in PC cell lines. Relative expression levels of MGAT genes were normalized to the expression of the GAPDH gene in each cell line. The expression of each MGAT gene in LNCaP cells was used as control and was defined as 1.0. Asterisk symbol indicate P value of LNCaP vs LNCaP-AI. Double asterisk symbol indicate P value of LNCaP vs DU145. Triple asterisk symbol indicate P value of LNCaP vs PC-3.

205x145mm (300 x 300 DPI)

Table 1 Patient demographics of the study cohort.

	HLT	BPH	esPC	PC with ADT	CRPC
Patients ( <i>n</i> )	80	286	258	46	68
Age, mean $\pm$ SD	64 $\pm$ 13	67 $\pm$ 8	68 $\pm$ 7	77 $\pm$ 7	74 $\pm$ 7
No. of males/females	47/33	286/0	258/0	46/0	68/0
Median iPSA (range)		6.4 (0.6–19.7)	7.4 (2.2–17.9)	23.0 (5.5–4564)	127 (1.3–17340)
Median nPSA (range)				0.08 (0–2.6)	
Median ADT follow-up (months)				42.5	
Bone metastasis, <i>n</i> (%)				4 (8.7)	53 (77.9)
BCR, <i>n</i> (%)				19 (41.3)	68 (100)

iPSA: initial PSA value at diagnosis; nPSA: nadir PSA; BCR: biochemical recurrence.

Table 2 Results of serum *N*-glycomics were significantly different between PC with ADT and CRPC patients.

<i>m/z</i>	Mean $\pm$ SD level ( $\mu$ M)		<i>P</i> -value	ROC curve
	PC with ADT	CRPC		AUC (95%CI)
1362	1.79 $\pm$ 0.47	1.59 $\pm$ 0.41	0.042	0.612 (0.508–0.717)
1566	2.50 $\pm$ 1.22	2.21 $\pm$ 1.22	0.047	0.609 (0.503–0.716)
1753	2.23 $\pm$ 0.62	1.89 $\pm$ 0.73	0.022	0.626 (0.526–0.728)
1794	3.87 $\pm$ 2.46	2.95 $\pm$ 1.72	0.042	0.612 (0.508–0.716)
3049	1.54 $\pm$ 0.52	2.09 $\pm$ 0.91	0.0003	0.697 (0.599–0.794)
3414	1.27 $\pm$ 0.32	2.01 $\pm$ 0.99	<0.0001	0.748 (0.659–0.837)
3560	1.28 $\pm$ 0.99	1.98 $\pm$ 1.91	0.033	0.617 (0.515–0.720)
3719	1.14 $\pm$ 0.30	1.89 $\pm$ 0.95	<0.0001	0.753 (0.666–0.810)
3865	1.19 $\pm$ 0.78	1.89 $\pm$ 2.10	0.014	0.636 (0.534–0.738)

Table 3 Logistic regression analysis of serum *N*-glycans for prediction of CRPC.

<i>m/z</i>	Coefficient	Odds ratio	Odds ratio (95%CI)	<i>P</i> -value
1362	-0.476	0.621	0.313–1.234	0.174
1566	0.108	1.114	0.839–1.479	0.457
1753	-0.644	0.525	0.280–0.983	0.044
1794	0.265	1.304	1.002–1.697	0.048
3049	1.202	3.326	1.199–9.226	0.021
3414	2.579	13.189	3.477–50.030	<0.0001
3560	-0.189	0.828	0.465–1.475	0.521
3719	-1.535	0.215	0.073–0.633	0.005
3865	0.622	1.863	1.072–3.238	0.027

Table 4 Optimal cut-off levels of *m/z* 3049 and *m/z* 3414, sensitivity, specificity, accuracy, and predictive value.

<i>m/z</i>	Cut-off ( $\mu$ M)	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)
3049	>1.595	69.1	37.0	56.1	61.8	44.7
3414	>1.355	69.1	41.3	57.9	63.5	47.5

PPV: positive predictive value; NPV: negative predictive value.

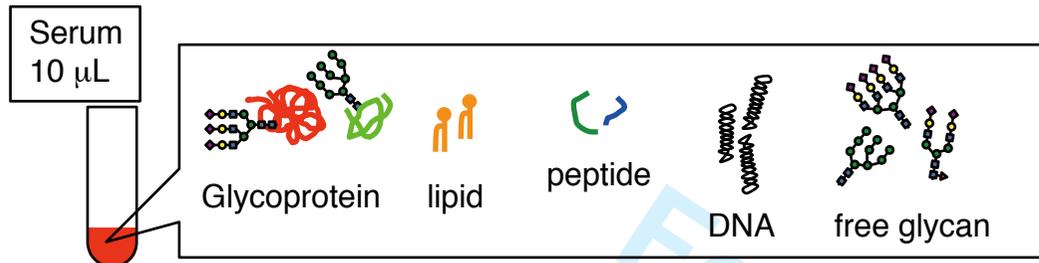
For Peer Review

**Supplementary Table 1.** Thirty-Six types of *N*-glycans demonstrated good quantitative reproducibility in all samples and could be analyzed statistically. *m/z* 2348.9 is the internal standard, disialo-galactosylated biantennary *N*-glycan, that contains amidated sialic acids (A2 amide glycans). Compositional annotations and putative structures are shown as abbreviations. Hex: hexose; HexNAc: *N*-acetylhexosamine; dHex: deoxyhexose.

Peak No.	<i>m/z</i>	Composition
1	1362.5	(Hex) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
2	1524.5	(Hex) <sub>3</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
3	1565.5	(Hex) <sub>5</sub> + (HexNAc) <sub>3</sub>
4	1590.6	(HexNAc) <sub>2</sub> (dHex) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
5	1606.6	(Hex) <sub>1</sub> (HexNAc) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
6	1647.6	(HexNAc) <sub>3</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
7	1686.6	(Hex) <sub>4</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
8	1708.6	(Hex) <sub>1</sub> (HexNAc) <sub>1</sub> (NeuAc) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
9	1752.6	(Hex) <sub>1</sub> (HexNAc) <sub>2</sub> (dHex) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
10	1768.6	(Hex) <sub>2</sub> (HexNAc) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
11	1793.7	(HexNAc) <sub>3</sub> (dHex) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
12	1809.7	(Hex) <sub>1</sub> (HexNAc) <sub>3</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
13	1848.6	(Hex) <sub>5</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
14	1870.7	(Hex) <sub>2</sub> (HexNAc) <sub>1</sub> (NeuAc) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
15	1914.7	(Hex) <sub>2</sub> (HexNAc) <sub>2</sub> (dHex) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
16	1955.7	(Hex) <sub>1</sub> (HexNAc) <sub>3</sub> (dHex) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
17	2010.7	(Hex) <sub>6</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
18	2032.7	(Hex) <sub>3</sub> (HexNAc) <sub>1</sub> (NeuAc) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
19	2057.8	(Hex) <sub>1</sub> (HexNAc) <sub>2</sub> (dHex) <sub>1</sub> (NeuAc) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
20	2073.8	(Hex) <sub>2</sub> (HexNAc) <sub>2</sub> (NeuAc) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
21	2219.8	(Hex) <sub>2</sub> (HexNAc) <sub>2</sub> (dHex) <sub>1</sub> (NeuAc) <sub>1</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
22	2336.9	(Hex) <sub>3</sub> (HexNAc) <sub>4</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
23	2348.9	Internal standard (BOA-labeled A2 amide)
24	2378.9	(Hex) <sub>2</sub> (HexNAc) <sub>2</sub> (NeuAc) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
25	2524.9	(Hex) <sub>2</sub> (HexNAc) <sub>2</sub> (dHex) <sub>1</sub> (NeuAc) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
26	2727.9	(Hex) <sub>2</sub> (HexNAc) <sub>3</sub> (dHex) <sub>1</sub> (NeuAc) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
27	2743.9	(Hex) <sub>3</sub> (HexNAc) <sub>3</sub> (NeuAc) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
28	2890.1	(Hex) <sub>3</sub> (HexNAc) <sub>3</sub> (dHex) <sub>1</sub> (NeuAc) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
29	3049.1	(Hex) <sub>3</sub> (HexNAc) <sub>3</sub> (NeuAc) <sub>3</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
30	3109.1	(Hex) <sub>4</sub> (HexNAc) <sub>4</sub> (NeuAc) <sub>2</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
31	3195.2	(Hex) <sub>3</sub> (HexNAc) <sub>3</sub> (dHex) <sub>1</sub> (NeuAc) <sub>3</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
32	3341.2	(Hex) <sub>3</sub> (HexNAc) <sub>3</sub> (Deoxyhexose) <sub>2</sub> (NeuAc) <sub>3</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
33	3414.2	(Hex) <sub>4</sub> (HexNAc) <sub>4</sub> (NeuAc) <sub>3</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
34	3560.3	(Hex) <sub>4</sub> (HexNAc) <sub>4</sub> (dHex) <sub>1</sub> (NeuAc) <sub>3</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
35	3719.3	(Hex) <sub>4</sub> (HexNAc) <sub>4</sub> (NeuAc) <sub>4</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>
36	3865.4	(Hex) <sub>4</sub> (HexNAc) <sub>4</sub> (dHex) <sub>1</sub> (NeuAc) <sub>4</sub> + (Man) <sub>3</sub> (GlcNAc) <sub>2</sub>

## Supplementary Figure 1

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Solubilized by PHM detergent in  $\text{NH}_4\text{HCO}_3$ ,  
Reduction-Alkylation  
Trypsin & PNGase digestion

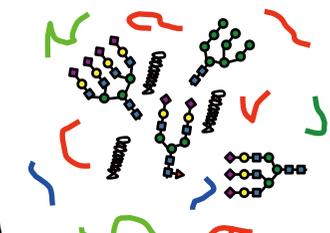
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SweetBlot runs integrated glycoblotting (A to F)



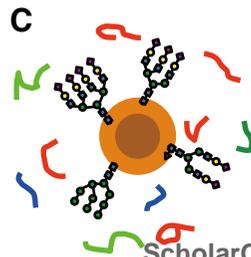
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N-glycan, peptide &  
DNA mixture



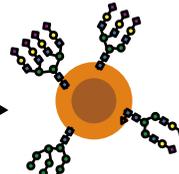
+

BlotGlyco beads



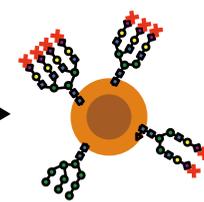
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Washout



E

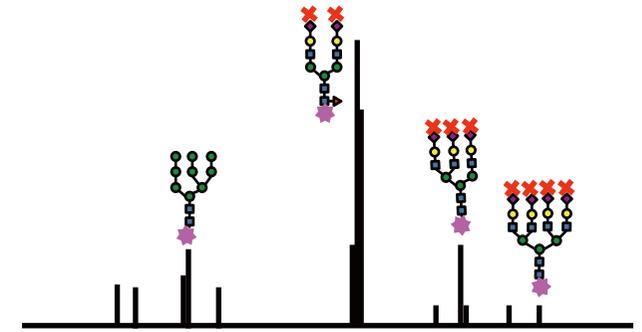
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(Methyl-esterified sialic acid)

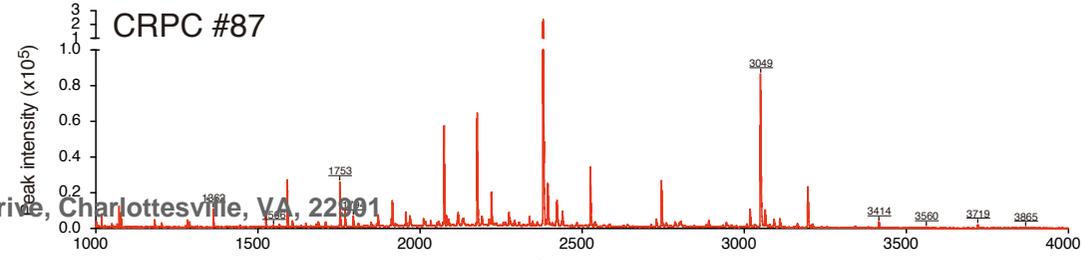
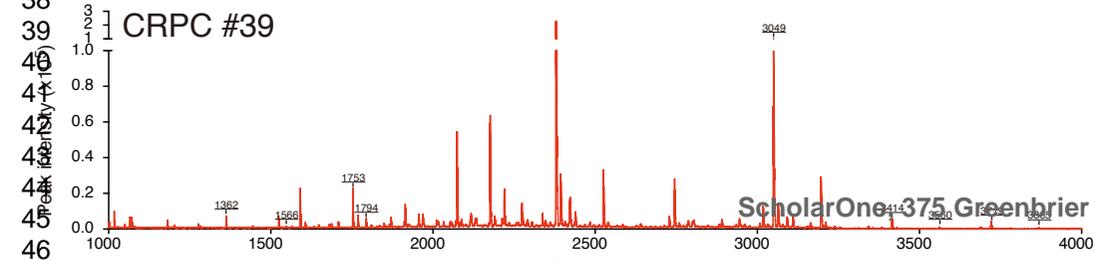
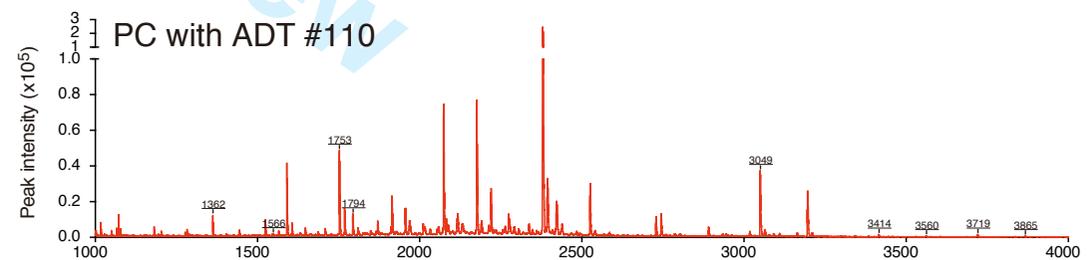
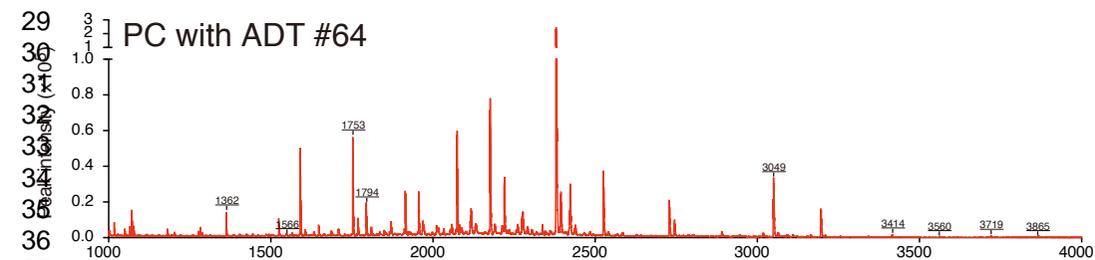
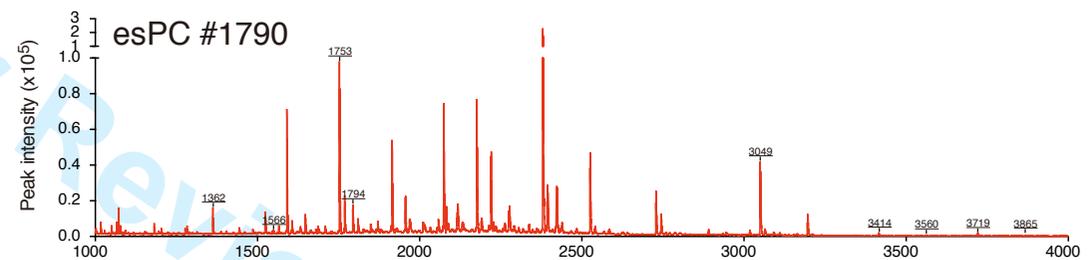
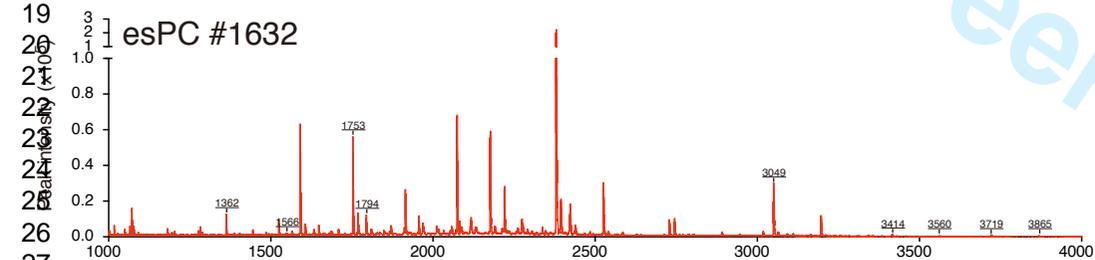
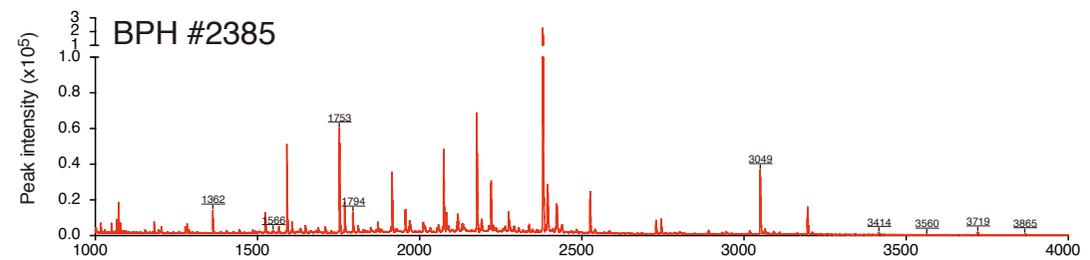
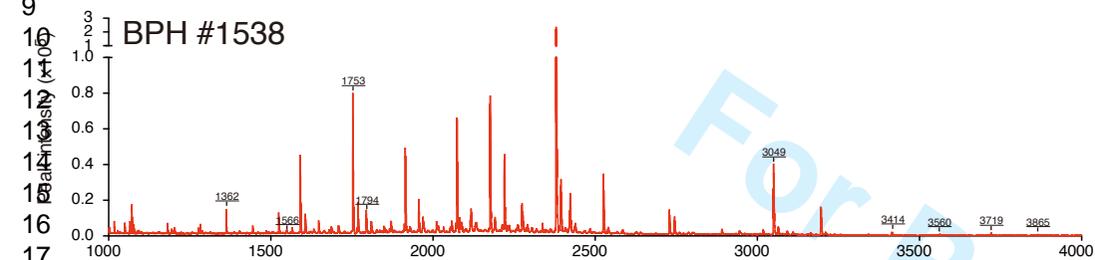
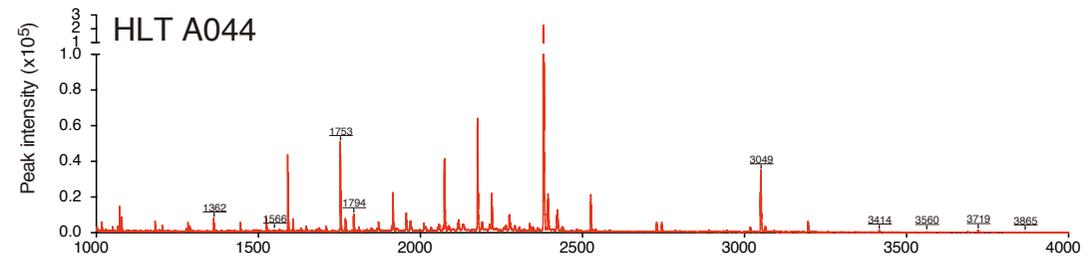
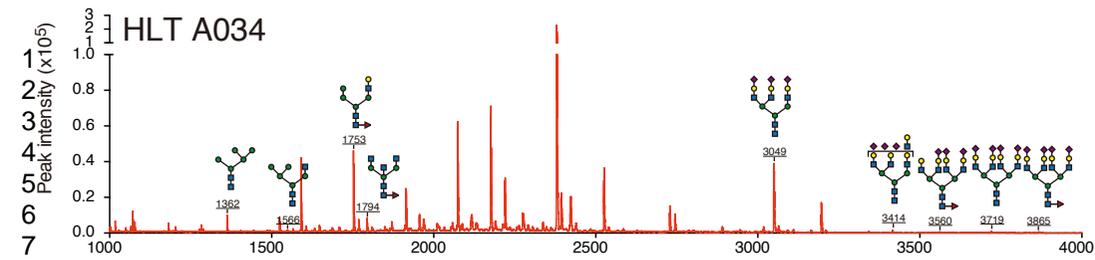


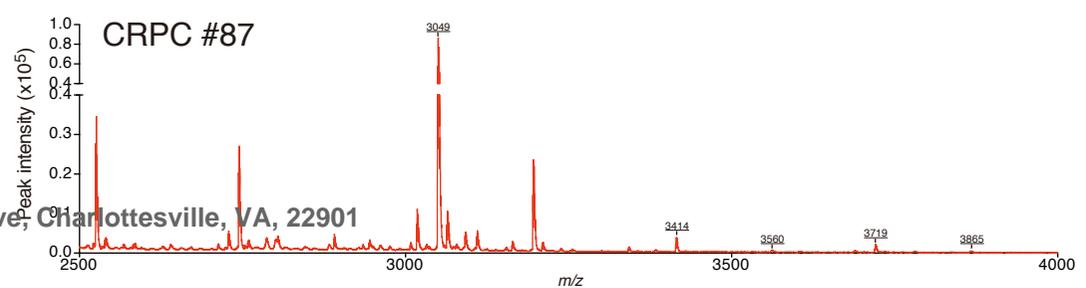
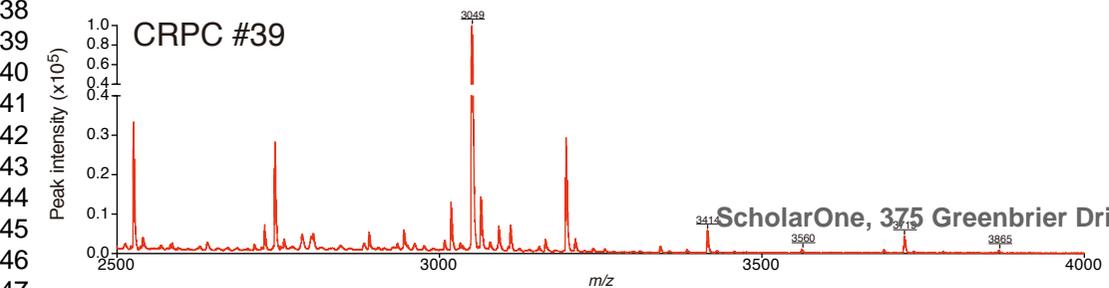
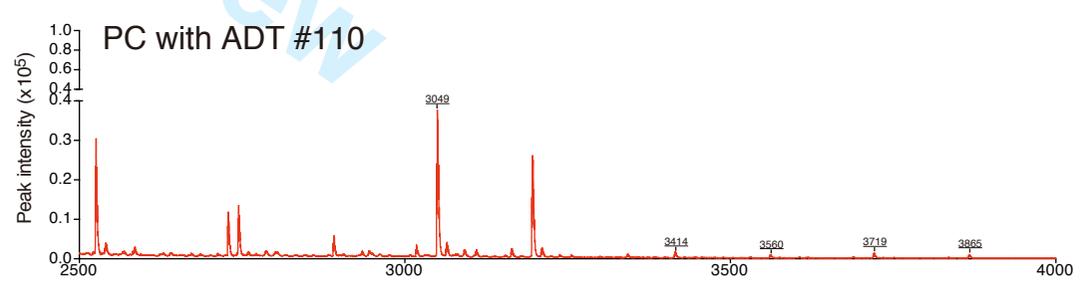
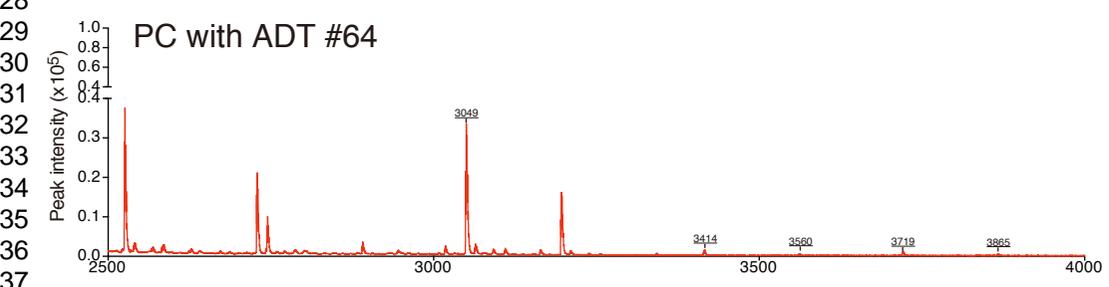
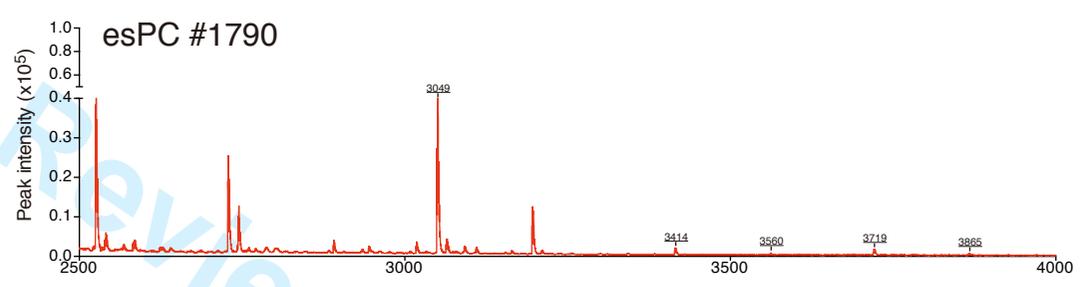
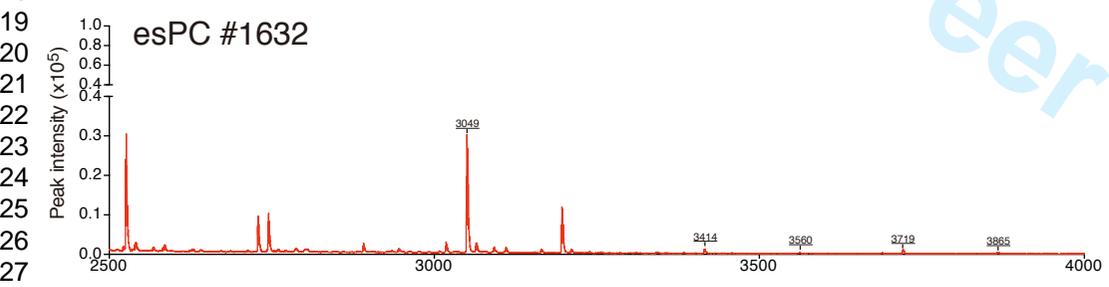
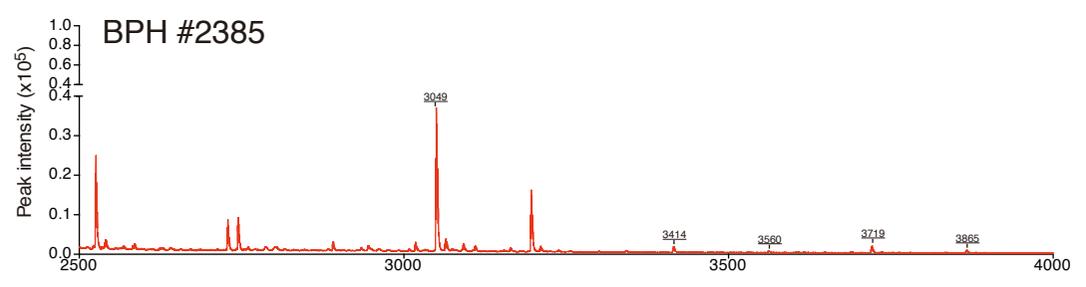
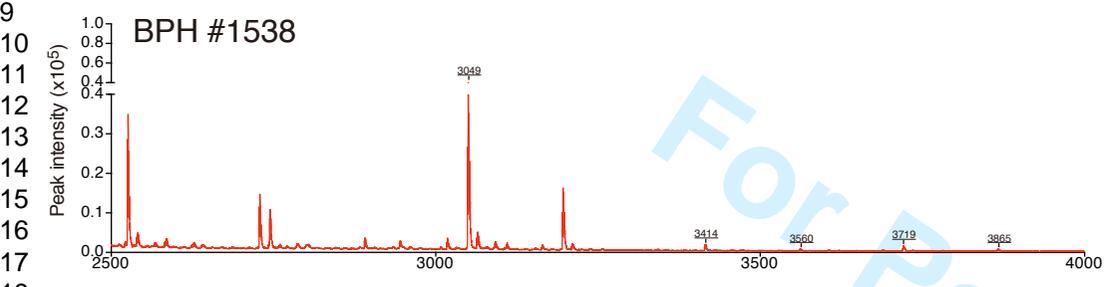
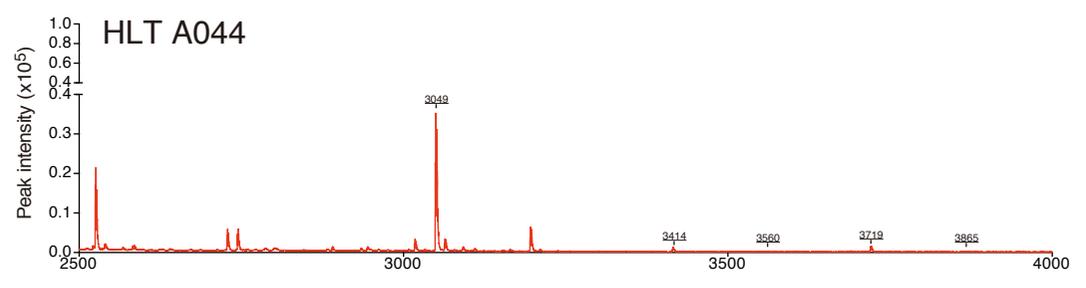
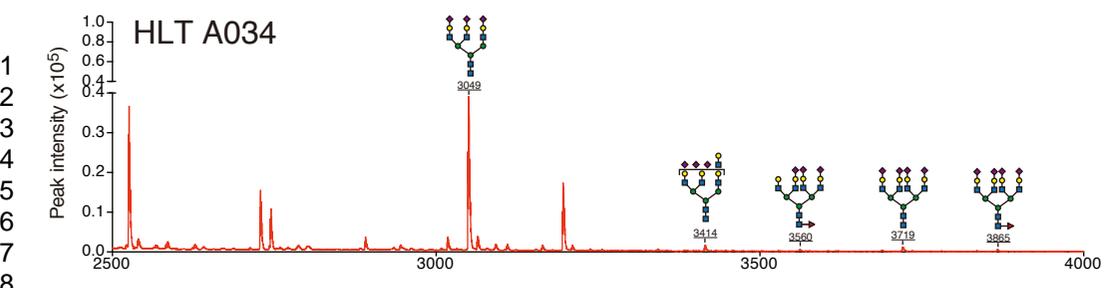
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Release of BOA-labeled N-glycan  
from BlotGlyco beads

G MALDI-TOF MS-based quantitative analysis







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Supplementary Figure 1. General protocol for the integrated glycoblotting technique and workflow for glycoblotting-based high-throughput clinical glycan analysis.

Ten-microliter serum samples (A) were applied to SweetBlot™ (System instruments, Hachijo, Japan) for glycoblotting. After enzymatic cleavage from serum protein, total serum *N*-glycans released into the digestion mixture (B) were directly mixed with BlotGlyco H beads (Sumitomo Bakelite, Co., Tokyo, Japan) to capture *N*-glycans (C). After the beads had been separated from other molecules by washing (D), sialic acid was methyl-esterified (E). These processed *N*-glycans were then labeled with benzyloxyamine (BOA) and released from BlotGlyco H beads (F). Mass spectra of BOA-labeled *N*-glycans were acquired using an Ultraflex III instrument (Bruker Daltonics, Germany) (G).

Supplementary Figure 2. Representative MALDI-TOF MS spectra (range of  $m/z$  1000 to 4000) of BOA-labeled *N*-glycans derived from HLT, BPH, esPC, PC with ADT and CRPC patient serum. Significantly different *N*-glycans ( $m/z$  1362, 1566, 1753, 1794, 3049, 3414, 3560, 3719, and 3865) between CRPC and other groups were shown in mass spectra. Symbols: yellow circles, galactose (Gal); green circles, mannose (Man); blue squares, *N*-acetylgulucosamine (GlcNAc); purple diamonds, *N*-acetylneuraminic acid (Neu5Ac); Red triangle, Fucose (Fuc).

Supplementary Figure 3. Representative MALDI-TOF MS spectra (range of  $m/z$  2500 to 4000) of BOA-labeled *N*-glycans derived from HLT, BPH, esPC, PC with

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6 ADT and CRPC patient serum. Significantly different *N*-glycans (*m/z* 3049, 3414,  
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8 3560, 3719, and 3865) between CRPC and other groups were shown in mass  
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10 spectra. Symbols: yellow circles, galactose (Gal); green circles, mannose (Man);  
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12 blue squares, *N*-acetylgulucosamine (GlcNAc); purple diamonds,  
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14 *N*-acetylneuraminic acid (Neu5Ac); Red triangle, Fucose (Fuc)..  
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6 **Serum tri- and tetra-antennary N-glycan is a potential predictive biomarker**  
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9 **for castration-resistant prostate cancer**  
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**ABSTRACT**

**BACKGROUND.** The U.S.FDA has approved several novel systemic agents including abiraterone acetate and taxoid cabazitaxel for metastatic castration-resistant prostate cancer (CRPC) result in a complicated decision-making while selecting an appropriate treatment. Therefore, a predictive biomarker for CRPC would provide useful information to physicians. The aim of this study is to evaluate the diagnostic potential of serum *N*-glycan profiling in CRPC.

**METHODS.** Serum *N*-glycomics was performed in 80 healthy volunteers and 286 benign prostatic hyperplasia, 258 early-stage PC, 46 PC with androgen deprivation therapy (ADT), and 68 CRPC patients using the glycoblotting method. A total of 36 types of *N*-glycan levels in each patient were analyzed using logistic regression analysis and receiver operating characteristic curves. We also examined the expression of *N*-glycan branching enzyme genes in PC cell lines using quantitative RT-PCR.

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6 **RESULTS.** We observed that tri- and tetra-antennary *N*-glycans were  
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9 significantly higher in CRPC patients than in any other groups. The longitudinal  
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12 follow-up of tri- and tetra- antennary *N*-glycan levels revealed that one PC with  
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15 ADT patient showed an increase that was more than the cut-off level and two  
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18 consecutive increases in tri- and tetra-antennary *N*-glycan levels 3 months apart;  
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22 resulted in biochemical recurrence despite the castrate level of testosterone, and  
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25 the patient was defined as CRPC. Expression of *N*-glycan branching enzyme  
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28 genes were significantly upregulated in CRPC cell lines.  
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32 **CONCLUSIONS.** These results suggest that the overexpression of tri- and  
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35 tetra-antennary *N*-glycan may be associated with the castration-resistant status  
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38 in PC and may be a potential predictive biomarker for CRPC.  
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41 **Keywords:** serum *N*-glycan; androgen deprivation therapy; biomarker;  
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44 castration-resistant prostate cancer; glycoblotting.  
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## 51 INTRODUCTION

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6 Prostate cancer (PC) is one of the most common cancers in men worldwide  
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9 [1]. The American Cancer Society estimated 241,740 new cases and 28,170  
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12 deaths in the United States in 2012 [2]. PC is a multifocal disease with a  
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15 moderate clinical progression. Localized early-stage PC (esPC) can be well  
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18 treated with radical prostatectomy. In contrast, advanced PC is mostly treated  
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21 with androgen deprivation therapy (ADT); however, ADT fails in approximately  
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24 10%–20% of patients, who then develop castration-resistant PC (CRPC) within 5  
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27 years of follow-up [3, 4]. CRPC is a heterogeneous and progressive stage of  
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30 PC and includes both symptomatic and asymptomatic male patients with or  
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33 without clinical metastases [5]. Although the mechanism underlying androgen  
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36 independence remains unclear, recent advances have led to a better  
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39 understanding of this mechanism. Over the past few years, several novel  
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42 systemic agents for metastatic CRPC, such as the androgen synthesis inhibitor  
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45 abiraterone acetate [6], the immunotherapeutic sipuleucel-T [7], the taxoid  
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48 cabazitaxel [8] and the enzalutamide [9], have been approved by the US Food  
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51 and Drug Administration (FDA). Therapeutic option for CRPC become  
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6 complicated treatment decision making. Therefore, a predictive biomarker for  
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9 CRPC would provide useful information to physicians for selecting the  
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12 appropriate therapy sequence at a given time as soon as possible. However,  
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15 no validated predictive biomarkers for CRPC have been reported.  
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19 Glycosylation plays an important role in various biological functions.  
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22 Cancer-associated aberrant glycosylation has been frequently observed in  
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25 bladder cancer [10], germ cell tumors [11], PC [12], colorectal cancer [13],  
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28 hepatocellular cancer [14], pancreatic cancer [15], and renal cell carcinoma [16].  
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31 Recently, high-throughput, comprehensive, and quantitative *N*-glycomics based  
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34 on the glycoblotting method using Sweetblot revealed that serum *N*-glycomics is  
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37 promising to screen for a diagnostic and prognostic marker for renal cell  
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40 carcinoma [17]. It is also a promising prognostic tool in patients undergoing  
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43 hemodialysis [18] and patients with advanced hepatocellular carcinoma  
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46 undergoing treatment with sorafenib [19]. However, the use of serum  
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49 *N*-glycans as a predictive biomarker for PC has not yet been investigated. In  
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6 the present study, we performed serum *N*-glycomics in PC patients and  
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10 evaluated its potential as a predictive biomarker for CRPC.

## 11 12 13 14 15 16 **MATERIALS AND METHODS**

### 17 18 19 **Serum Samples**

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22 A total of 650 patients with benign prostatic hyperplasia (BPH), early-stage  
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25 PC (esPC), PC with ADT, or CRPC were treated at our hospital between June  
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28 2007 and December 2013. Serum samples from BPH ( $n = 286$ ) and esPC ( $n =$   
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31 258) patients were obtained at the time of biopsy. The final diagnosis of BPH  
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34 and esPC patients was confirmed using the histopathological findings of  
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37 prostate biopsies. Serum samples from PC with ADT ( $n = 46$ ) and CRPC ( $n =$   
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40 68) patients were obtained at the time of treatment. Biochemical recurrence  
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43 was defined as prostate-specific antigen (PSA) levels  $>0.2$  ng/mL after  
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46 prostatectomy or increase 2 ng/mL above the nadir PSA after radiotherapy  
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49 (RT). CRPC was defined by PSA or radiographic progression despite the  
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52 castrate levels of testosterone of  $<50$  ng/dL. All samples were stored at  $-80^{\circ}\text{C}$   
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6 until use. Serum samples from 80 healthy volunteers (HLT) were obtained from  
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10 our serum bank and were stored at  $-80^{\circ}\text{C}$  until use. The study was performed  
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13 in accordance with the ethical standards of the Declaration of Helsinki and was  
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16 approved by the Ethics Committee of Hirosaki University Graduate School of  
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19 Medicine. Informed consent was obtained from all patients. Patient  
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22 demographics are shown in Table 1.  
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### 29 **Glycoblotting Method and Mass Spectrometry**

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32 Serum *N*-glycan analysis was performed as described previously using  
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35 SweetBlot<sup>TM</sup> (System Instruments, Hachijo, Japan) [17] (Supplementary Figure  
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38 1). Briefly, 10  $\mu\text{L}$  of serum samples containing 40 pmol of the internal standard  
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41 disialo-galactosylated biantennary *N*-glycan, which has amidated sialic acids (A2  
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44 amide glycans) (Supplementary Table 1), were reduced and alkylated using DTT  
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47 and iodoacetamide (Wako Pure Chemical Industries, Osaka, Japan),  
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50 respectively. The resulting mixture was then trypsinized and heat inactivated.  
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54 After cooling down to room temperature, peptide *N*-glycanase F (New England  
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6 BioLabs, Ipswich, MA, USA) was added to the mixture to release total serum  
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9 *N*-glycans. After incubating for 360 min at 37°C, 20 µL of the resulting mixture,  
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12 equivalent to 2.5 µL of serum. An aliquot of each pretreated sample was mixed  
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15 with 500 µL of BlotGlyco H beads (Sumitomo Bakelite, Co., Tokyo, Japan) to  
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18 capture glycans via stable hydrazone bonds on MultiScreen Solvinert® filter  
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21 plate (MerkMillipore, Billerica, MA, USA). Then, acetyl capping of unreacted  
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24 hydrazide functional groups on the beads and methyl esterification of sialic acid  
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27 carboxyl groups, which exist in the terminal of the captured glycans, were  
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30 performed sequentially; serial washes were then performed before each step, as  
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33 described previously [17, 19, 20-24]. The captured *N*-glycans were labeled  
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36 with benzyloxiamine (BOA, Sigma-Aldrich, St. Louis, MO, USA) by  
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39 transiminization and were eluted in 150 µL of water. The BOA-labeled glycans  
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43 were detected using MALDI-TOF MS (Ultraflex 3 TOF/TOF mass spectrometer,  
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46 Bruker Daltonics, Bremen, Germany). Compositions and structures of glycans  
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49 were predicted using GlycoMod Tool (<http://br.expasy.org/tools/glcomod>).  
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### Quantitative Reproducibility Test of Sweetblot

Each quantitative reproducibility test of Sweetblot was performed as described previously [25]. Briefly, serum samples and serially diluted standard human serum (Sigma-Aldrich) were added to the plate, and the whole process of *N*-glycomics was performed with Sweetblot. The peak area of each glycan detected at 0.5x, 0.75x, 1x, 1.25x, 1.5x, 1.75x, 2x, and 2.25x concentrations was plotted. This assay was repeated twice, and quantitative reliability was then judged based on following parameters: outliers were allowed <3 points, slope  $\sigma$  of <3.0, and the significance level of the correlation coefficient  $r$  was <0.05. Glycan peaks were judged to be useful when the abovementioned criteria of the assay were met, and the resulting glycans were used for statistical analysis.

### Statistical Analysis

Statistical calculations for clinical data were performed using SPSS ver. 20.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6.03 (GraphPad Software, San Diego, CA, USA). Intergroup differences were statistically

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6 compared using the Student's *t*-test for normally distributed models or the  
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9 Mann–Whitney U-test for nonnormally distributed models. *N*-glycan levels  
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12 were analyzed using logistic regression analysis and receiver operating  
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15 characteristic (ROC) curves to select *N*-glycans that were associated with CRPC  
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18 status in PC. The optimal cut-off points were calculated using the following  
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21 formula:  $(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2$  [26].  $P < 0.05$  was considered  
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24 significant.  
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### 32 Real-time Quantitative RT-PCR

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35 The normal prostate epithelial cell line RWPE-1 and the PC cell lines  
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38 LNCaP, DU145, and PC-3 were obtained from the American Type Culture  
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41 Collection. RWPE-1 was grown at 37°C with 5% CO<sub>2</sub> in Keratinocyte-SFM  
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44 medium supplemented with penicillin, streptomycin, bovine pituitary extract, 5  
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47 ng/ml epidermal growth factor. LNCaP, DU145, and PC-3 were grown at 37°C  
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51 with 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with penicillin, streptomycin,  
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54 and 10% FBS. LNCaP-androgen independent (AI) cell were grown at 37°C  
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6 with 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with penicillin, streptomycin,  
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9 and 10% charcoal-stripped FBS. Total RNA was isolated from RWPE-1, LNCaP,  
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12 LNCaP-AI, DU145, and PC-3 cells using ISOGEN II (Wako Pure Chemical  
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15 Industries) according to the manufacturer's instructions. First-strand cDNA was  
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18 synthesized from 0.5 µg of total RNA using ReverTra Ace® qPCR RT Master  
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21 Mix with gDNA Remover (Toyobo, Kita-ku, Osaka, Japan) according to the  
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24 manufacturer's instructions. Real-time qRT-PCR assays were performed in  
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28 triplicate using GeneAce SYBR® qPCR Mix α No ROX (Nippon Gene,  
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31 Chiyoda-ku, Tokyo, Japan) and 500 nM gene-specific primers. Reactions were  
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34 processed on a CFX connect™ Real-Time System (Bio-Rad Laboratories, Inc.,  
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37 Hercules, CA, USA) under the following conditions: 95°C for 10 min, followed by  
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41 40 cycles of 95°C for 15 s and 60°C for 45 s. PrimeTime® qPCR primer pairs  
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44 for human *N*-acetylglucosaminyltransferase I (*MGAT1*) (Hs.PT.58.4702749),  
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47 human *N*-acetylglucosaminyltransferase II (*MGAT2*) (Hs.PT.58.24612062.g),  
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50 human *N*-acetylglucosaminyltransferase III (*MGAT3*) (Hs.PT.58.26307986.g),  
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53 human *N*-acetylglucosaminyltransferase IVa (*MGAT4A*) (Hs.PT.58.3289156),  
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6 human *N*-acetylglucosaminyltransferase IVb (*MGAT4B*) (Hs.PT.58.19371732),  
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9 human *N*-acetylglucosaminyltransferase IVc (*MGAT4C*) (Hs.PT.58.2945729),  
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12 human *N*-acetylglucosaminyltransferase V (*MGAT5A*) (Hs.PT.58.4758371),  
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15 human *N*-acetylglucosaminyltransferase Vb (*MGAT5B*) (Hs.PT.58.27758528),  
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18 and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)  
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20 (Hs.PT.39a.22214847) were purchased from Integrated DNA Technologies, Inc.  
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25 (Coralville, IA, USA). Relative expression levels of *MGAT* genes were  
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29 normalized to expression of the *GAPDH* gene.  
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## 35 RESULTS

### 36 37 38 **Tri- and Tetra-Antennary *N*-glycans Significantly Increased in CRPC**

#### 39 40 41 **Patients.**

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45 Serum *N*-glycan analysis performed using the glycoblotting method and  
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48 mass spectrometry identified 45 types of BOA-labeled *N*-glycans in all serum  
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51 samples. We then performed quantitative reproducibility tests. Finally, 36  
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54 types of *N*-glycans (Supplementary Table 1) had good quantitative  
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6 reproducibility among all samples and could be used for statistical analysis.  
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10 Table 1 summarizes the demographics of the study cohort. No significant  
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12 differences were observed in age between BPH and esPC groups. The iPSA  
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14 level in the esPC group was significantly higher than that in the BPH group ( $P =$   
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16 0.0002). The age of patients in the PC with ADT group was significantly higher  
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18 than that in the CRPC group ( $P = 0.033$ ). No significant differences were  
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20 observed in the *N*-glycan profiles of HLT, BPH, esPC, and PC with ADT patients.  
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22 We observed significant differences in the *N*-glycan profiles between CRPC and  
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24 the other groups. Nine *N*-glycans ( $m/z$  1362, 1566, 1753, 1794, 3049, 3414,  
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26 3560, 3719, and 3865) were significantly different between PC with ADT and  
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28 CRPC groups (Table 2, Supplementary Figs 2 and 3). To investigate predictive  
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30 potential for CRPC, nine *N*-glycans were analyzed using logistic regression  
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32 analysis. The tri- and tetra-antennary *N*-glycans  $m/z$  3049 (odds ratio, 3.326)  
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34 and  $m/z$  3414 (odds ratio, 13.189) showed higher odds ratio than other glycans,  
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36 therefore  $m/z$  3049 and  $m/z$  3414 were selected as specific *N*-glycans for the  
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38 prediction of CRPC (Table 3). Fig.1A and B showed serum level of  $m/z$  3049 and  
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6 *m/z 3414 glycans in each group.* ROC curves were then used to compare the  
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10 predictive potential of *m/z 3049* and *m/z 3414* for CRPC (Fig. 1C). The area  
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12 under the curve (AUC) of *m/z 3049* and *m/z 3414* could be used to discriminate  
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16 between PC with ADT and CRPC patients (AUC, 0.697 and 0.748, respectively).  
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### 21 22 **Longitudinal Follow-Up of Tri- and Tetra-antennary N-glycan Levels in 16**

#### 23 24 25 26 **PC with ADT Patients**

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28 The optimal cut-off levels of *m/z 3049* and *m/z 3414* were determined to be  
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32  $>1.60 \mu\text{M}$  and  $>1.36 \mu\text{M}$ , respectively, for the prediction of CRPC based on ROC  
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The optimal cut-off levels of *m/z 3049* and *m/z 3414* were determined to be  $>1.60 \mu\text{M}$  and  $>1.36 \mu\text{M}$ , respectively, for the prediction of CRPC based on ROC curves (Table 4). To evaluate the predictive potential of *m/z 3049* and *m/z 3414*, we followed-up *m/z 3049* and *m/z 3414* levels in 16 PC with ADT patients every 3 or 6 months (Fig. 2A, B). Total PSA and testosterone levels were also followed-up at the same time points (Fig. 2C, D). We found that one PC with ADT patient showed two consecutive increases in *m/z 3049* and *m/z 3414* levels 3 months apart. This patient also showed two consecutive increases in PSA levels and was finally defined as CRPC because the testosterone level was  $<50$

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6 ng/dL. This finding suggests that the overexpression of serum tri- and  
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9 tetra-antennary *N*-glycans may be associated with the castration-resistant status  
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13 in PC.

### 14 15 16 17 18 19 **Transcription Levels of *N*-glycan Branching Enzyme Genes Were** 20 21 22 **Significantly Upregulated in CRPC Cell Lines** 23

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25 We also examined transcription levels of *MGAT1*, *MGAT2*, *MGAT3*,  
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28 *MGAT4A*, *MGAT4B*, *MGAT5A*, and *MGAT5B*, which are medial Golgi enzymes  
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32 that initiate the  $\beta$ 1,6GlcNAc branching in bi-, tri-, and tetra-branched *N*-glycans,  
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35 in PC cell lines using qRT-PCR (Fig. 3). The CRPC-like cell lines DU145 and  
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38 PC-3 showed significantly increased transcription of *MGAT1*, *MGAT2*, *MGAT4B*,  
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41 *MGAT5A*, and *MGAT5B* genes. Particularly, the expression of the *MGAT5B*  
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45 gene was 20-fold higher in CRPC like LNCaP-AI, DU145 and PC-3 cells than in  
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48 androgen-dependent LNCaP cells and normal prostate epithelial RWPE-1 cells.  
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## 55 **DISCUSSION**

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6 High-throughput, comprehensive, and quantitative *N*-glycomics is an important  
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9 and promising method. Several studies have reported that differences in  
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12 glycan profiling between diseased and benign states may be useful in the  
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15 diagnosis or prognosis of diseases [17-19, 23-25]. In prostate cancer,  
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19 Kyselova Z et al [27] investigated that *N*-glycomic profiles (50 types of *N*-glycan)  
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22 derived from human blood sera of 10 healthy males were compared to those  
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25 from 24 metastatic PC patients. Although the sample size was very small, they  
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28 report tri- and tetra-antennary *N*-glycans of metastatic PC patients were  
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31 significantly higher than those of healthy males. This was consistent with our  
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34 present result. In the present study, the recently established technology of  
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38 *N*-glycan analysis with the glycoblotting method and MALDI-TOF was used for  
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41 high-throughput, comprehensive, and quantitative serum *N*-glycan profiling in  
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44 PC patients. To the best of our knowledge, this is the first report to identify  
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47 serum *N*-glycans as biomarkers in CRPC patients by using high-throughput  
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50 quantitative *N*-glycomics. Our results demonstrate that serum levels of tri- and  
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53 tetra-antennary *N*-glycans (*m/z* 3049 and *m/z* 3414) were statistically and  
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6 significantly different between PC with ADT and CRPC patients using the  
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9 optimal cut-off points (Figs 1 and 2). A previous study reported that  
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12 cancer-associated aberrant glycosylation increases the transcription of the  
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15 *MGAT5* gene, which initiates  $\beta$ 1,6GlcNAc branching in tri- and tetra-branched  
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18 *N*-glycans in PC and plays an important role in metastasis of PC [28]. Zavareh  
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21 et al [29] reported that the knockdown of *N*-acetylglucosaminyltransferase I,  
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24 which is encoded by the *MGAT1* gene and is the first branching enzyme  
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27 required for additional branching on *N*-glycan, decreased levels of branched  
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32 *N*-glycan on the surface of PC-3 cells. In addition, their orthotopic xenograft  
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35 model exhibited significantly decreased primary tumor growth and incidence of  
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38 lung metastasis. In the current study, we demonstrated that transcription levels  
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41 of *MGAT1*, *MGAT2*, *MGAT4B*, *MGAT5A*, and *MGAT5B* genes were significantly  
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44 upregulated in CRPC cell lines (Fig. 3).  
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48 Results of several reports and the current study indicated that the  
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51 overexpression of tri- and tetra-branched *N*-glycans on the surface of CRPC  
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54 cells due to upregulation of *N*-glycan branching enzymes (*MGATs*) was strongly  
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6 correlated with metastatic PC, and this overexpression may be associated with  
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9 the castration-resistant status in PC.  
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12 These results suggest that the use of the glycoblotting method may provide  
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14 insight into new factors predicting CRPC. Although serum tri- and  
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16 tetra-antennary *N*-glycan expression was revealed as a useful predictive  
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18 biomarker in CRPC patients in the current study, this study has several  
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20 limitations. First, this study is small and preliminary. Second, it is very  
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22 important to determine the carrier protein for tri- and tetra-antennary *N*-glycans  
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24 that enables it to be released into the circulation from tumor tissues or circulating  
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26 tumor cells. Otherwise, the altered serum *N*-glycan profile could be a  
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28 systematic immunogenic reaction of the released tumor-associated antigen.  
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30 Future studies should address whether these alterations are a direct result of the  
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32 castration-resistant status in PC. Third, longitudinal patterns of changes in tri-  
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34 and tetra-antennary *N*-glycan from PC with ADT to CRPC patients were  
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36 investigated in only 16 patients. To validate these predictive biomarkers for  
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38 CRPC, an increased number of patients is required. Despite these limitations,  
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6 the overexpression of tri- and tetra-antennary *N*-glycans was clearly  
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9 demonstrated to be a potential biomarker for the prediction of CRPC in this study.  
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12 Future large-scale prospective validation studies may determine the clinical  
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15 significance of these carbohydrate biomarkers.  
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## 23 CONCLUSIONS

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25 Although the present study is small and preliminary, quantitative whole  
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27 serum *N*-glycan profiling may have the potential to predict castration-resistant  
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29 status in PC. Glycoblotting with MALDI-TOF mass spectrometry may be a  
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31 promising method for screening of new predictive biomarkers. At present, no  
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33 validated predictive biomarkers for CRPC have been reported. Therefore, a  
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35 predictive biomarker for CRPC would provide useful information to physicians to  
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37 decide the appropriate therapy sequence. Further clinical trials are warranted  
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39 to investigate the clinical significance of novel carbohydrate markers.  
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## 16 REFERENCES

- 17  
18  
19 1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer  
20  
21  
22 statistics. *CA Cancer J Clin* 2011;61:69-90.  
23  
24  
25 2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*  
26  
27  
28 2012;62:10-29.  
29  
30  
31  
32 3. Hirst CJ, Cabrera C, Kirby M. Epidemiology of castration resistant prostate  
33  
34  
35 cancer: a longitudinal analysis using a UK primary care database. *Cancer*  
36  
37  
38 *Epidemiol* 2012;36:e349-e353.  
39  
40  
41 4. Kirby M, Hirst C, Crawford ED. Characterising the castration-resistant prostate  
42  
43  
44 cancer population: a systematic review. *Int J Clin Pract* 2011;65:1180-1192.  
45  
46  
47  
48 5. Toren PJ, Gleave ME. Evolving landscape and novel treatments in metastatic  
49  
50  
51 castrate-resistant prostate cancer. *Asian J Androl* 2013;15:342-349.  
52  
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6 6. de Bono JS, Logothetis CJ, Molina A, Fizazi K, North S, Chu L, Chi KN, Jones  
7  
8  
9 RJ, Goodman OB Jr, Saad F, Staffurth JN, Mainwaring P, Harland S, Flaig TW,  
10  
11  
12 Hutson TE, Cheng T, Patterson H, Hainsworth JD, Ryan CJ, Sternberg CN,  
13  
14  
15 Ellard SL, Fléchon A, Saleh M, Scholz M, Efstathiou E, Zivi A, Bianchini D, Loriot  
16  
17  
18 Y, Chieffo N, Kheoh T, Haqq CM, Scher HI; COU-AA-301 Investigators.  
19  
20  
21  
22 Abiraterone and increased survival in metastatic prostate cancer. *N Engl J Med*  
23  
24  
25 2011;364:1995-2005.  
26  
27  
28  
29 7. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF,  
30  
31  
32 Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer  
33  
34  
35 PF; IMPACT Study Investigators. Sipuleucel-T immunotherapy for  
36  
37  
38 castration-resistant prostate cancer. *N Engl J Med* 2010;363:411-422.  
39  
40  
41  
42 8. de Bono JS, Oudard S, Ozguroglu M, Hansen S, Machiels JP, Kocak I, Gravis  
43  
44  
45 G, Bodrogi I, Mackenzie MJ, Shen L, Roessner M, Gupta S, Sartor AO; TROPIC  
46  
47  
48 Investigators. Prednisone plus cabazitaxel or mitoxantrone for metastatic  
49  
50  
51 castration-resistant prostate cancer progressing after docetaxel treatment: a  
52  
53  
54  
55 randomised open-label trial. *Lancet* 2010;376:1147-1154.  
56  
57  
58  
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2  
3  
4  
5  
6 9. Tombal B, Borre M, Rathenborg P, Werbrouck P, Van Poppel H, Heidenreich  
7  
8  
9 A, Iversen P, Braeckman J, Heracek J, Baskin-Bey E, Ouatas T, Perabo F,  
10  
11  
12 Phung D, Hirmand M, Smith MR. Enzalutamide monotherapy in hormone-naive  
13  
14  
15 prostate cancer: primary analysis of an open-label, single-arm, phase 2 study.  
16  
17  
18  
19 Lancet Oncol 2014;15:592-600.  
20  
21  
22 10. Ishimura H, Takahashi T, Nakagawa H, Nishimura S, Arai Y, Horikawa Y,  
23  
24  
25 Habuchi T, Miyoshi E, Kyan A, Hagsawa S, Ohyama C.  
26  
27  
28 *N*-acetylglucosaminyltransferase V and  $\beta$ 1-6 branching N-linked  
29  
30  
31 oligosaccharides are associated with good prognosis of patients with bladder  
32  
33  
34 cancer. Clin Cancer Res 2006;12:2506-2511.  
35  
36  
37  
38 11. Hatakeyama S, Kyan A, Yamamoto H, Okamoto A, Sugiyama N, Suzuki Y,  
39  
40  
41 Yoneyama T, Hashimoto Y, Koie T, Yamada S, Saito H, Arai Y, Fukuda M,  
42  
43  
44 Ohyama C. Core 2 *N*-acetylglucosaminyltransferase-1 expression induces  
45  
46  
47 aggressive potential of testicular germ cell tumor. Int J Cancer  
48  
49  
50  
51 2010;127:1052-1059.  
52  
53  
54  
55  
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58  
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- 1  
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4  
5  
6 12. Hagiwara S, Ohyama C, Takahashi T, Endoh M, Moriya T, Nakayama J,  
7  
8  
9 Arai Y, Fukuda M. Expression of core 2  $\beta$ 1,6-N-acetylglucosaminyltransferase  
10  
11 facilitates prostate cancer progression. *Glycobiology* 2005;1:1016-1024.  
12  
13  
14  
15  
16 13. D'Arrigo A, Belluco C, Ambrosi A, Digito M, Esposito G, Bertola A, Fabris M,  
17  
18 Nofrate V, Mammano E, Leon A, Nitti D, Lise M. Metastatic transcriptional  
19  
20 pattern revealed by gene expression profiling in primary colorectal carcinoma.  
21  
22  
23  
24  
25  
26 *Int J Cancer* 2005;115:256-262.  
27  
28  
29 14. Moriwaki K, Noda K, Nakagawa T, Asahi M, Yoshihara H, Taniguchi N,  
30  
31  
32 Hayashi N, Miyoshi E. A high expression of GDP-fucose transporter in  
33  
34  
35 hepatocellular carcinoma is a key factor for increases in fucosylation.  
36  
37  
38  
39 *Glycobiology* 2007;17:1311-1320.  
40  
41  
42 15. Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K,  
43  
44  
45 Murata K, Ohigashi H, Yokoyama S, Eguchi H, Ishikawa O, Ito T, Kato M,  
46  
47  
48 Kasahara A, Kawano S, Gu J, Taniguchi N, Miyoshi E. Fucosylated haptoglobin  
49  
50  
51 is a novel marker for pancreatic cancer: a detailed analysis of the  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6 oligosaccharide structure and a possible mechanism for fucosylation. Int J  
7  
8

9  
10 Cancer 2006;118:2803-2808.

11  
12 16. Saito S, Orikasa S, Ohyama C, Satoh M, Fukushi Y. Changes in glycolipids  
13  
14

15  
16 in human renal-cell carcinoma and their clinical significance. Int J Cancer  
17  
18

19  
20 1991;49:329-334.

21  
22 17. Hatakeyama S, Amano M, Tobisawa Y, Yoneyama T, Tsuchiya N, Habuchi T,  
23  
24

25  
26 Nishimura S-I, Ohyama C. Serum N-glycan alteration associated with renal cell  
27  
28

29  
30 carcinoma detected by high throughput glycan analysis. J Urol  
31  
32

33  
34 2013;191:805-813.

35  
36 18. Hatakeyama S, Amano M, Tobisawa Y, Yoneyama T, Tsushima M, Hirose K,  
37  
38

39  
40 Yoneyama T, Hashimoto Y, Koie T, Saitoh H, Yamaya K, Funyu T, Nishimura  
41  
42

43  
44 S-I, Ohyama C. Serum N-glycan profiling predicts prognosis in patients  
45  
46

47  
48 undergoing hemodialysis. Sci World J 2013;5:1-10.

49  
50 19. Miyahara K, Nouse K, Miyake Y, Nakamura S, Obi S, Amano M, Hirose K,  
51  
52

53  
54 Nishimura S-I, Yamamoto K. Serum glycan as a prognostic marker in patients  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6 with advanced hepatocellular carcinoma treated with sorafenib. *Hepatology*

7  
8  
9 2014;59:355-356.

10  
11  
12 20. Miura Y, Kato K, Takegawa Y, Kuroguchi M, Furukawa J, Shinohara Y,

13  
14  
15 Nagahori N, Amano M, Hinou H, Nishimura S-I. Glycoblotting-assisted

16  
17  
18 O-glycomics: ammonium carbamate allows for highly efficient o-glycan release

19  
20  
21 from glycoproteins. *Anal Chem* 2010;82:10021-10029.

22  
23  
24 21. Amano M, Yamaguchi M, Takegawa Y, Yamashita T, Terashima M,

25  
26  
27 Furukawa J, Miura Y, Shinohara Y, Iwasaki N, Minami A, Nishimura S-I.

28  
29  
30 Threshold in stage-specific embryonic glycotypes uncovered by a full portrait of

31  
32  
33 dynamic N-glycan expression during cell differentiation. *Mol Cell Proteomics*

34  
35  
36 2010;9:523-537.

37  
38  
39 22. Furukawa J, Shinohara Y, Kuramoto H, Miura Y, Shimaoka H, Kuroguchi M,

40  
41  
42 Nakano M, Nishimura S-I. Comprehensive approach to structural and functional

43  
44  
45 glycomics based on chemoselective glycoblotting and sequential tag conversion.

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6 23. Kamiyama T, Yokoo H, Furukawa J, Kuroguchi M, Togashi T, Miura N,  
7  
8  
9 Nakanishi K, Kamachi H, Kakisaka T, Tsuruga Y, Fujiyoshi M, Taketomi A,  
10  
11  
12 Nishimura S-I, Todo S. Identification of novel serum biomarkers of hepatocellular  
13  
14  
15 carcinoma using glycomic analysis. *Hepatology* 2013;57:2314-2325.  
16  
17  
18 24. Nouse K, Amano M, Ito YM, Miyahara K, Morimoto Y, Kato H, Tsutsumi K,  
19  
20  
21 Tomoda T, Yamamoto N, Nakamura S, Kobayashi S, Kuwaki K, Hagihara H,  
22  
23  
24 Onishi H, Miyake Y, Ikeda F, Shiraha H, Takaki A, Nakahara T, Nishimura S-I,  
25  
26  
27 Yamamoto K. Clinical utility of high-throughput glycome analysis in patients with  
28  
29  
30 pancreatic cancer. *J Gastroenterol* 2013;48:1171-1179.  
31  
32  
33 25. Takeuchi M, Amano M, Tsukamoto T, Masumori N, Hirose K, Ohashi T,  
34  
35  
36 Nishimura S-I. N- and O-glycome analysis of serum and urine from bladder  
37  
38  
39 cancer patients using a high-throughput glycoblotting method. *J Glycomics*  
40  
41  
42  
43  
44  
45  
46  
47 Lipidomics 2013;3:108.  
48  
49 26. Akobeng AK. Understanding diagnostic tests 3: Receiver operating  
50  
51  
52 characteristic curves. *Acta Paediatr* 2007;96:644-647.  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6 27. Kyselova Z, Mechref Y, Al Bataineh MM, Dobrolecki LE, Hickey RJ, Vinson J,  
7  
8  
9  
10 Sweeney CJ, Novotny MV. (2007). Alterations in the serum glycome due to  
11  
12  
13 metastatic prostate cancer. *J Proteome Res* 2007;6:1822-1832.

14  
15  
16 28. Tsui KH, Chang PL, Feng TH, Chung LC, Sung HC, Juang HH. Evaluating  
17  
18  
19 the function of matriptase and *N*-acetylglucosaminyltransferase V in prostate  
20  
21  
22 cancer metastasis. *Anticancer Res* 2008;28:1993-1999.

23  
24  
25 29. Beheshti Zavareh R, Sukhai MA, Hurren R, Gronda M, Wang X, Simpson  
26  
27  
28 CD, Maclean N, Zih F, Ketela T, Swallow CJ, Moffat J, Rose DR, Schachter H,  
29  
30  
31 Schimmer AD, Dennis JW. Suppression of cancer progression by MGAT1  
32  
33  
34 shRNA knockdown. *PLoS ONE* 2012;7:e43721.  
35  
36  
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## 42 **FIGURE LEGENDS**

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45 **Figure 1.** Serum levels of significant tri- and tetra-antennary *N*-glycans  
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48 associated with the prediction of CRPC that were selected using logistic  
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51 regression analysis. A, serum *m/z* 3049 level in HLT, BPH, esPC, PC with ADT,  
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54 and CRPC patients. B, serum *m/z* 3414 level in HLT, BPH, esPC, PC with ADT,  
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6 and CRPC patients. C, receiver operating characteristics (ROC) curve for the  
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9 prediction of CRPC. The AUCs of *m/z* 3049 and *m/z* 3414 were 0.697 and  
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12 0.748, respectively. D, Putative structures of *m/z* 3049 and *m/z* 3414 are  
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15 represented as monosaccharide symbols. Yellow circles, galactose (Gal);  
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18 green circles, mannose (Man); blue squares, *N*-acetylgulucosamine (GlcNAc);  
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22 purple diamonds, *N*-acetylneuraminic acid (Neu5Ac).  
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30 **Figure 2.** The longitudinal follow-up of serum *m/z* 3049, *m/z* 3414, PSA, and  
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32 testosterone levels in PC with ADT patients. A, serum *m/z* 3049 levels. The  
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34 red dashed line represents the optimal cut-off level of *m/z* 3049 (>1.60  $\mu$ M). B,  
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36 serum *m/z* 3414 levels. The red dashed line represents the optimal cut-off level  
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38 of *m/z* 3414 (>1.36  $\mu$ M). C, total serum PSA levels. D, serum testosterone  
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41 levels. The red dashed line represents the castrate level of testosterone (50  
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43 ng/dL). Blue and pink bold lines in panels A and B indicate the PC patient who  
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46 was treated with ADT and then experienced two consecutive increases in tri-  
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49 and tetra-antennary *N*-glycan levels. Only the blue bold line shows the PC with  
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6 ADT patient who experienced two consecutive increases in PSA levels (panel C)  
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9 despite maintaining a castrate level of testosterone (panel D); he was finally  
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12 defined as CRPC.  
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19 **Figure 3.** Quantitative qRT-PCR of *N*-glycan branching enzymes (*MGATs*) in  
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21 PC cell lines. Relative expression levels of *MGAT* genes were normalized to  
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23 the expression of the *GAPDH* gene in each cell line. The expression of each  
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28 *MGAT* gene in LNCaP cells was used as control and was defined as 1.0.  
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32 Asterisk symbol indicate *P* value of LNCaP vs LNCaP-AI. Double asterisk  
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35 symbol indicate *P* value of LNCaP vs DU145. Triple asterisk symbol indicate *P*  
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38 value of LNCaP vs PC-3.  
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