

Research Article
Development of urinary diagnostic biomarker for IgA nephropathy by lectin microarray

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Abstract

Introduction. The pathogenic roles of aberrantly glycosylated IgA1 have been reported. However, it is unexplored whether the profiling of urinary glycans contributes to the diagnosis of IgAN.

Methods. We conducted the retrospective study enrolling 493 patients who underwent renal biopsy at Okayama University Hospital between Dec. 2010 and Sep. 2017. We performed lectin microarray in urine samples and investigated whether c-statistics of the reference standard diagnosis model employing hematuria, proteinuria, and serum IgA was improved by adding the urinary glycan intensity.

Results. Among 45 lectins, 3 lectins showed a significant improvement of the models: *Amaranthus caudatus* lectin (ACA) with the difference of c-statistics 0.038 [95%CI, 0.019 - 0.058, $P < 0.001$], *Agaricus bisporus* lectin (ABA) 0.035 [95%CI, 0.015 - 0.055, $P < 0.001$], *Maackia amurensis* lectin (MAH) 0.035 [95%CI, 0.015 - 0.054, $P < 0.001$]. In 3 lectins, each signal plus reference standard showed good reclassification (category free NRI and relative IDI) and good model fitting associated with the improvement of AIC and BIC. Stratified by eGFR, the discriminatory ability of ACA plus reference standard was maintained, suggesting the robust renal function-independent diagnostic performance of ACA. By decision curve analysis, there was a 3.45% net benefit by adding urinary glycan intensity of ACA to reference standard at the pre-defined threshold probability of 40%.

Conclusions. The reduction of Gal(β 1-3)GalNAc (T-antigen), Sia(α 2-3)Gal(β 1-3)GalNAc (Sialyl T), and Sia(α 2-3)Gal(β 1-3)Sia(α 2-6)GalNAc (disialyl-T) was suggested by binding specificities of 3 lectins. C1GALT1 and COSMC were responsible for the biosynthesis of these glycans, and they were known to be downregulated in IgAN. The urinary glycan analysis by ACA is useful and robust non-invasive strategy for the diagnosis of IgAN.

Introduction

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis worldwide, characterized by persistent microscopic hematuria and/or proteinuria [1]. About 20-50% of the patients with IgAN develop end-stage renal disease (ESRD) within 20 years [2-4]. IgAN is defined by the dominant glomerular deposition of IgA [5], and renal biopsy is essential for the diagnosis. However, renal biopsy is an invasive diagnostic procedure, and it is not warranted in cases with isolated glomerular hematuria. In IgAN, the delay in diagnosis causes the development of proteinuria and disease progression over years.

Glycosylation of proteins is the most common and structurally diverse post-translational modification (PTM). In eukaryotes, over half of all proteins are glycosylated [6], and glycosylation plays an important role in immunity, infections, inflammation, development, and carcinogenesis through changes in biochemical interactions [7]. Glycosylation is also closely involved in the pathogenesis of IgAN, and the multi-hit hypothesis originating from glycosyl modification of IgA1 is widely accepted [8, 1]. In healthy subjects, the hinge region of IgA1 heavy chains are glycosylated with 3 to 6 O-glycans; Gal(β 1-3)GalNAc-Ser/Thr or its sialylated forms [9]. In the IgAN patients, O-glycosylation sites in the IgA1 hinge region were highly underglycosylated [10, 11]. Auto-antibodies against galactose-deficient IgA1 are produced, which form immune complexes and deposit in the mesangial region, leading to mesangial cell proliferation, cytokine secretion, and renal injury [12, 8].

The glycosylation of IgA1 is directly involved in the pathogenesis of IgAN and shown to be useful in diagnostic and prognostic biomarker [13, 14]. An evanescent-field fluorescence-assisted lectin microarray has been developed and it enables high-throughput analysis by direct measuring lectin-glycan interactions [15, 16]. We established urinary lectin array analyses and identified new urinary glycan biomarkers predicting cardiovascular and renal outcomes in diabetic kidney disease [17, 18]. Although unbiased global determination of glycan profile had not been performed in IgAN, we

performed urinary lection and identified the prognostic glycan markers Gal(β 1-4)GlcNAc and high-mannose including Man(α 1-6)Man, recognized by *Erythrina cristagalli* lectin (ECA) and *Narcissus pseudonarcissus* lectin (NPA), respectively [19]. We further promoted the urinary lectin array analyses in IgAN and established reliable diagnostic formulation containing glycan signals and established reference standard including the presence of hematuria, 24 hours urinary protein excretion (g/day), and concentration of serum IgA (mg/dl).

Materials and Methods

Study design and population

The current investigation was a retrospective study of the patients who underwent renal biopsy at Okayama University Hospital between Dec. 2010 and Sep. 2017. Among 514 patients, 19 patients with IgAN complicated with other renal diseases and 2 patients lacking data of serum IgA concentration were excluded. We conducted an exploratory analysis of 157 cases of primary IgAN and 336 cases of the control group: 332 other renal diseases and 4 healthy kidney donors (**Suppl. Fig. 1**). This study was conducted in accordance with the principles of the Declaration of Helsinki, and the protocol was approved by the ethics committee of Okayama University Hospital (authorization number: K1709-039) and was registered in University Hospital Medical Information Network on Sep 1, 2017 (UMIN clinical trial registry number: UMIN000029336). All study participants provided written informed consent.

In order to perform lectin fluorescence staining, 9 patients with renal biopsy were newly enrolled at Okayama University Hospital. Four patients with primary IgAN, 2 with primary membranous nephropathy, 1 with tubulointerstitial nephritis, and 2 kidney donors. The protocol was approved by the ethics committee of Okayama University Hospital (authorization number: K2106-027), and written informed consent was obtained from all study participants.

71

72 ***Variables and data sources***

73 The clinical characteristics at renal biopsy were collected from medical records. Hypertension was
74 defined as the systolic BP ≥ 140 mmHg and/or diastolic BP ≥ 90 mmHg or use of antihypertensive
75 agents. Diabetes mellites was defined according to ADA criteria and/or use of anti-diabetic
76 medications. History of cardiovascular events was obtained from medical records. For the patients at
77 18 years and older, the eGFR was calculated using the following estimation equation for Japanese
78 patients with CKD (eGFR [ml/min/ 1.73 m²] = $194 \times \text{age}^{-0.287} \times \text{sCr}^{-1.094} \times [0.739 \text{ for women}]$) [20]. For
79 the patients under the age of 18, the eGFR was calculated using the equation reported by the
80 Japanese Society for Pediatric Nephrology [21]. Urinary microscopic hematuria was defined as 5 and
81 over urinary red blood cells /high-powered field in multiple urinalyses before renal biopsy.

82

83 ***Diagnosis***

84 The diagnosis was established by a consensus of the team of nephrologists and a pathologist based
85 on clinical symptoms, laboratory data, light microscopy, immunofluorescence, and electron
86 microscopy [22, 19]. The pathological scoring system of IgAN was applied and determined by 3
87 nephrologists, including MEST scores by Oxford classification [5].

88

89 ***Lectin microarray analysis***

90 All of spot urine samples were collected during hospitalization for renal biopsy, stored at -80°C, and
91 thawed only once to perform this study. Urinary glycan moieties of glycoproteins were measured by
92 the evanescent-field fluorescence-assisted lectin microarray (urinary glycan intensity) [15, 17]. In
93 brief, 20 μL of urine samples were labeled with 100 μg of Cy3 monoreactive dye (GE Healthcare) for 1

hr. Then, free Cy3 was removed by Zeba Desalt Spin Column (Pearce). We applied urinary Cy3-labeled glycoproteins to the wells of LecChip Ver1.0 immobilized with 45 lectins (**Suppl. Table 1**). After binding the Cy3-labeled glycoprotein probes to immobilized lectins, we captured the fluorescence images of the array using GlycoStation Reader 1200 (GlycoTechnica Ltd, Yokohama). Glycan intensity was obtained as a 16-bit score and defined as raw glycan intensity [Raw-I]. We defined the net glycan intensity [Net-I] as Raw-I subtracted by background intensity. Since albumin lacks sugar chains, urinary albumin labeled by Cy3, which binds to primary amine, mainly contributes to the background reactivity. The background intensity correlated with urinary albumin ($r = 0.599$) and urinary protein ($r = 0.633$) with moderate association. Creatinine is another major urinary substance with primary amines. Previously, we showed that Net-I of spot urine samples is a more accurate predictor of 24 hours urinary glycan intensity compared to Net-I/urinary creatinine (UCr) ratio or Raw-I/UCr ratio[17] and we used Net-I in the analysis. To assess the assay-to-assay consistency, we measured the standard probes consisting of the following glycoproteins 17 times with different LecChips; asialofetuin, fetuin, Gal α 1-3Gal-BSA, N-acetylglucosamine BSA, 3'sialyl-N-acetyllactosamine-BSA, α 1-3, α 1-6mannose-BSA, 2'fucosyllactose-APD-BSA, GalNAc α -O-PAP-BSA.

Reference standards

To evaluate the predictive value of urinary glycan intensity in the diagnosis of IgAN, we defined the reference standards. Serum IgA is known to be associated with the diagnosis of IgA nephropathy [23]. Moreover, microscopic hematuria is the most typical manifestation of IgA nephropathy and is seen in 70-100% of cases, especially in children and young adults in the early stages of the clinical course [24]. In contrast, nephrotic range of proteinuria is not common at presentation [25]. Therefore, we used laboratory data including the presence of hematuria (categorical value), 24hr urinary protein excretion (g/day: continuous value), and concentration of serum IgA (mg/dl:

continuous value) as a reference standard. In addition, to evaluate the discriminatory ability of the single lectins, we compared them to a known diagnostic marker, the IgA/C3 ratio (continuous value) [26]. In addition, we compared them with 24hr urinary protein excretion (g/day: continuous value) and eGFR (ml/min/1.73m²: continuous value).

Lectin fluorescence staining

The human kidney tissues were embedded in the OCT compound and 4 µm-thick serial cryostat sections were prepared. They were blocked for 30 min with 3% bovine serum albumin, and stained overnight at 4°C with the several lectins or antibodies; FITC-conjugated *Amaranthus caudatus* lectin (ACA: F-8021-2, EY laboratories), FITC-conjugated *Agaricus bisporus* lectin (ABA: F-5001-1, EY laboratories), FITC-conjugated *Maackia amurensis* lectin (MAH: F-7801-2, EY laboratories), anti-C1GALT1 rabbit polyclonal antibody (ab237734, Abcam), anti-COSMC rabbit polyclonal antibody (ab229831, Abcam), anti-Aquaporin 1 mouse monoclonal antibody (ab9566), and anti-Uromodulin sheep polyclonal antibody (PA5-46959, Thermo Fisher Scientific). The sections were then incubated at room temperature for 1 hr with Alexa Fluor 488-conjugated anti-rabbit antibody (goat polyclonal antibody, A11008, Thermo Fisher Scientific), Alexa Fluor 555-conjugated anti-mouse antibody (goat polyclonal antibody, ab150118, Abcam), Alexa Fluor 647-conjugated anti-sheep antibody (donkey polyclonal antibody, ab150179, Abcam), and with DAPI (Sigma-Aldrich) for nuclear staining. The sections were then mounted with Fluoromount (Diagnostic BioSystems). Representative images were acquired with All-in-one Fluorescence Microscope BZ-X700 (Keyence).

Statistical analysis

Data were presented as the percentages, means $\pm$ SD, or medians [interquartile range; IQR].

Categorical variables of patient characteristics at baseline were analyzed with chi-square test and continuous variables were compared using *t*-test or Mann-Whitney U test, as appropriate. Pearson correlation analysis was used to assess the association between two variables. To assess the likelihood of IgAN, we developed a predictive equation, using multivariate logistic regression analysis. To compare the discrimination of regression equations with and without 45 urinary glycan intensity, we calculated the difference of c-statistics using STATA command “comproc” from <http://www.stata-journal.com/software/sj9-1> [27]. The bootstrap estimation of 95% confidence intervals was calculated according to the normal distribution. In the difference of c-statistics, significance thresholds were adjusted for multiple comparisons by Bonferroni methods. To compare the model fitness, we calculated Akaike information criterion (AIC) and Bayesian information criterion (BIC) in the multivariate logistic regression models. Furthermore, we calculated the category-free Net Reclassification Improvement (cfNRI) and the relative integrated discrimination improvement (rIDI) using the STATA command “nri” and “idi” from <http://personalpages.manchester.ac.uk/staff/mark.lunt> [28]. The 95% CIs for the differences of the c-statistics, cfNRI, and rIDI were computed from 1000 bootstrap samples. For the evaluation of clinical usefulness, we examined the net benefit using decision curve analysis [29, 30]. The net benefit was obtained by subtracting the proportion of patients who were false positive from the proportion who were true positive, weighting by the relative harm of a false-positive and a false-negative result. We calculated the net benefit using the STATA command “dca” from <https://www.mskcc.org/departments/epidemiology-biostatistics/biostatistics/decision-curve-analysis>. Two-tailed *p* values <0.05 were considered statistically significant. All statistical analyses were performed using Stata software (version 16.1; Stata Corporation, College Station, TX, USA).

Results

Clinical characteristics of the participants

A total of 493 patients were enrolled and classified as primary IgAN (IgAN group, n=157) and control group (renal diseases except for IgAN, n=332; normal kidney from living kidney donors, n=4) (**Suppl. Fig. 1**). ANCA-associated vasculitis, lupus nephritis, IgA vasculitis, and membranous nephropathy were leading biopsy-proven renal diseases except for IgAN (**Suppl. Fig. 2**). In comparison with control group, IgAN group demonstrated younger age (41.8 ± 16.1 vs 55.1 ± 17.7 years old, $P < 0.001$), lower eGFR (67.9 [IQR: $51.1 - 90.5$] vs 56.5 [IQR: $35.5 - 80.7$] ml/min/1.73m², $P < 0.001$), less urinary protein excretion (0.73 [IQR: $0.27 - 1.53$] vs 1.33 [IQR: $0.47 - 4.09$] g/day, $P < 0.001$), more frequent microscopic hematuria (84.7% vs 53.9% , $P < 0.001$), and higher levels of serum IgA (309.7 ± 118.5 vs 264.3 ± 126.7 mg/dl, $P < 0.001$) (**Suppl. Table 2**). Although IgA/C3 ratio is known as a diagnostic marker for IgAN, there was no significant difference in IgAN and control groups (3.22 ± 1.47 vs 3.14 ± 2.67 , $P = 0.738$). The distribution of histological lesions according to Oxford classification is shown in **Suppl. Table 3**. Compared to the original Oxford Classification cohort [31], there were less mesangial or endocapillary hypercellularity and more segmental sclerosis/adhesion, crescent and IFTA, reflecting the exclusion of patients with eGFR < 30 ml/min/1.73 m² in the Oxford Classification cohort.

Urinary glycan intensity as diagnostic marker for IgAN

The urinary glycan intensity of 45 lectins demonstrated weak to moderate positive correlations ($r = 0.1578 - 0.6676$) with urinary protein concentration and 42 lectins showed weak to moderate negative correlations with eGFR ($r = -0.4618$ to -0.0505) (**Suppl. Fig. 3**). The urinary glycan intensity showed strong correlations among the lectins with same monosaccharide specificity and weak correlations among the lectins with different specificity (**Suppl. Fig. 4**). Among 45 lectins, urinary

glycan intensity of 33 lectins were lower in IgAN group compared to control group (**Suppl. Fig. 5**). The urinary glycan intensity of 3 lectins, by adding to the established reference standard, showed a significant improvement in diagnostic performances for IgAN, exceeding the threshold of significance by the Bonferroni method ($\alpha = 0.0011$); *Amaranthus caudatus* lectin (ACA): the difference of c-statistics 0.038 [95%CI, 0.019 - 0.058, $P < 0.001$], *Agaricus bisporus* lectin (ABA): 0.035 [95%CI, 0.015 - 0.055, $P < 0.001$], *Maackia amurensis* lectin (MAH): 0.035 [95%CI, 0.015 - 0.054, $P < 0.001$] (**Fig. 1**), and they were promising candidates for diagnostic markers. In ACA, ABA, and MAH, the diagnostic performance of single lectins was significantly superior to the reported effective predictor of IgAN, *i.e.*, IgA/C3 ratio (**Suppl. Fig. 6**). Furthermore, the diagnostic performance of ACA was superior to that of 24hr urinary protein excretion and eGFR (**Suppl. Fig. 7, 8**).

Receiver operating characteristic (ROC) curve is shown in **Figure 2**, and equation, cut-off point, sensitivity, and specificity defined by Youden index are also shown. In ACA, ABA, and MAH, each lectin plus the reference standard provided excellent reclassification; category-free net reclassification index (cfNRI) ranging from 0.415 to 0.526 and the relative integrated discrimination improvement (rIDI) was 0.044 to 0.050 (**Table**). In the fitness of the model, the reference standard showed AIC 530.47, BIC 547.28. In all 3 lectins, each lectin plus the reference standard showed good model fitting, with a decrease in AIC and BIC. The urinary glycan intensity of ACA, ABA, MAH were significantly lower in IgA nephropathy than in the control group and showed mutual and tight correlations (**Suppl. Fig. 9**). For ACA and ABA, inter-assay variations in 17 independent measurements of standard glycoprotein probes were %CV < 15% (**Suppl. Table. 4**). The Gal(β 1-3)GalNAc (T-antigen) is recognized by ACA and ABA, Sia(α 2-3)Gal(β 1-3)GalNAc (Sialyl T) by ACA, and Sia(α 2-3)Gal(β 1-3)Sia(α 2-6)GalNAc (disialyl-T) by MAH. The reduction of urinary glycan intensity may reflect the reduced glycosylation by C1GALT1 and COSMC, which were known to be downregulated in IgAN (**Suppl. Fig. 10**).

Comparison of diagnostic performance by ACA, ABA, MAH stratified by eGFR and etiology

Since ACA, ABA, MAH negatively correlated with eGFR, we divided the patients into 2 groups by eGFR 60 ml/min/1.73m² in sensitivity analyses (**Suppl. Table 5**). In the group with eGFR < 60 ml/min/1.73 m², the discriminatory ability of each lectin plus reference standard remained similar (difference of c-statistics: ACA 0.052 [95%CI 0.013-0.092, *P* = 0.010], ABA 0.05 [95%CI 0.015-0.085, *P* = 0.005], MAH 0.055 [95%CI 0.015-0.095, *P* = 0.008]). In the group with eGFR ≥ 60 ml/min/1.73m², the discriminatory ability of ACA plus reference standard was also maintained (difference of c-statistics: ACA 0.036 [95%CI 0.004-0.068, *P* = 0.026]). We optimized the regression equation with reference standard plus lectins and eGFR. We found no improvements in c-statistics, cfNRI and rIDI for ACA and MAH (**Suppl. Table 6**). Taken together, the diagnostic performance of ACA was robust, independent of renal function.

Although the etiological basis for IgAN and IgA vasculitis might be common, the urinary glycan intensity by ACA and ABA were significantly higher in IgA vasculitis (**Suppl. Fig. 11**) and the diagnostic performance was not altered when we excluded IgA vasculitis from the control group (**Suppl. Table 7**). In the analysis of patients with IgAN (n=157) plus IgA vasculitis (n=17), 9 cases of IgA vasculitis were predicted as negative for IgAN by ACA and ABA; thus, specificity was low as 52.9% (**Suppl. Table 8**), suggesting the performance for diagnosis of 2 disease entities may not be sufficient.

Since the diagnosis of nephrotic syndrome, metabolic diseases, and tubulointerstitial nephritis is clinically apparent, we evaluated the diagnostic performance when these disease entities were excluded from the control group. The discrimination ability, reclassification metrics, and calibration were not altered, suggesting the usefulness of urinary glycan intensity by the lectins in the clinical setting (**Suppl. Table 9**).

The net benefit for the diagnosis of IgA nephropathy comparing the reference standard and prediction model by ACA using threshold probability

To investigate the clinical usefulness of ACA, we performed a decision curve analysis (DCA), which shows the net benefit based on the probabilities of IgAN. Since renal biopsy is an invasive test, it should be carefully considered when it is presumed that nephritis, such as IgA nephropathy, is not highly probable. A pre-test probability of 40-60% for IgA nephropathy is considered a clinically indeterminate level for renal biopsy. At the pre-defined threshold probability of 40%, there was a 3.45% net benefit of adding urinary glycan intensity of ACA to reference standard for diagnosis of IgAN (**Fig. 3**), which showed the ACA could be clinically useful as the diagnostic biomarker.

Lectin fluorescence staining of 3 identified lectins; ACA, ABA, and MAH

Finally, we evaluated the glycosylation changes in kidney tissue by fluorescence staining with 3 lectins using renal biopsy tissues (**Suppl. Fig. 12**). Staining of the kidney donor samples revealed that the proximal tubular cells were positive, and the renal glomeruli were negative for all 3 lectins. C1GALT1 and COSMC immunoreactivities were observed in glomeruli, proximal tubules, and distal tubules, but only proximal tubules co-localized with the lectin binding signals. In IgAN, membranous nephropathy (MN) and tubulointerstitial nephritis (TIN) samples, the proximal tubules demonstrated similar lectin binding fluorescent activities, but the glomeruli did not. These results suggest the alterations in urinary glycan excretion may reflect the secreted or truncated glycoproteins derived from proximal tubular cells.

Discussion/Conclusion

We showed that 3 lectins recognizing urinary O-glycan related to T-antigen were critical for the diagnosis of IgA nephropathy identified by lectin microarray. The urinary glycan signal intensity measured by ACA, ABA, MAH statistically improved discrimination ability (difference of c-statistics: **Fig. 1**), reclassification metrics (NRI and IDI: **Table**) and calibration (AIC and BIC: **Table**). Especially in

ACA, the results were robust when stratified by renal function. In addition, we found clinical usefulness of adding urinary glycan signal intensity to the reference standard using decision curve analysis (DCA), which, unlike conventional approaches, enables the clinical relevance to be assessed according to the probabilities of the outcome [30]. A good biomarker can be measured from a non-invasive and easily available source such as urine, be highly sensitive and specific, enable early diagnosis of disease, and be biologically plausible [32]. These requirements seem to be met by the identified lectins assisted by evanescent-field fluorescence.

The O-GalNAc glycans have four major core structures, which can be extended by various sugar residues. The most common O-GalNAc glycan is core 1 (T-antigen), which consists of O-GalNAc with galactose attached at the β 1-3 linkage and are found in glycoproteins by many different cell types. Core 1 O-GalNAc is generated by C1GALT1, which is stabilized by COSMC [33]. The 3 lectins found in this study recognize the glycans related to Core 1 O-GalNAc (**Suppl. Fig. 10**); ACA recognizes Gal(β 1-3)GalNAc (T-antigen), Sia(α 2-3)Gal(β 1-3)GalNAc (Sialyl T). ABA recognizes Gal(β 1-3)GalNAc (T-antigen). MAH recognizes Sia(α 2-3)Gal(β 1-3)Sia(α 2-6)GalNAc (disialyl-T). Core 1 O-GalNAc is the precursor of many cell surface mucin O-glycans and secreted glycoproteins, and C1GALT1, which generates Core 1 O-GalNAc, plays a role in normal development (such as angiogenesis, platelets production, and kidney development), inflammatory diseases, and carcinogenesis [34]. In the pathogenesis of IgAN, the association between core 1 O-glycans and the hinge region of IgA1 is well known [35]. In the patients with IgAN, galactose-deficit IgA1 (Gd-IgA1) are elevated [10], and autoantibodies recognizing Gd-IgA1 are produced and form immune complexes [12]. The method of detecting serum galactose-deficit IgA is reported to be diagnostic and prognostic markers; the lectin recognizing O-GalNAc, such as Helix aspersa lectin (HAA) [36], and the Gd-IgA1-specific monoclonal antibody, KM55 [37]. IgG-IgA immunocomplex and Gd-IgA1 are excreted in urine by circulatory load and have been reported as urinary diagnostic and prognostic biomarkers for IgAN.

Using lectin-microarray, we found alterations of glycans derived from O-linked glycoproteins, but it is still unexplored whether the glycan changes are restricted in IgA molecule or generally observed in other glycoproteins. The origin of these glycoproteins may be filtered proteins in circulating plasma or secreted from podocytes or tubular epithelium. In the patients with IgAN, C1GALT1 gene mutation is known [38]. Among lymphocytes, only B cells have reduced C1GALT1 activity [39]. In addition, the pattern of O-galactosylation of IgD, the only O-glycosylated human immunoglobulin isotype except for IgA, was not changed in IgAN [40], suggesting that the reduction in IgA1 O-galactosylation in IgAN is a specific feature of IgA1-secreting cells.

For the identification of the glycoproteins related to the pathogenesis of IgAN, phenotypes of C1GALT1 mutant mice (*plt1/plt1* mice) have been reported [41]. Alexander et al. showed the remarkable renal disease in *plt1/plt1* mice and identified the glycoproteins with O-GalNAc from renal extracts of *plt1/plt1* mice using affinity-purification with HPA-agarose and mass spectrometry. The major HPA-reactive O-linked glycoproteins in *plt1/plt1* were podocalyxin and CD13 (aminopeptidase N). Podocalyxin is expressed in the podocytes and plays an essential role in the formation and maintenance of foot processes [42]. In the patients with IgAN, urinary excretion of podocalyxin is associated with histological abnormalities [43]. CD13 is expressed in the proximal tubule segments visualized by ACA, ABA, and MAH staining. CD13 regulates Na⁺/K⁺ ATPase level and salt handling and influences blood pressure [44]. The urinary excretion of CD13 has been reported to be lower in the IgAN patients compared to in the thin basement membrane disease (TBMN) patients [45]. The identification of glycoproteins recognized by ACA, ABA, and MAH is a future challenge.

The three lectins, ACA, ABA, and MAH, found as diagnostic markers in this study are distinct from the prognostic markers previously reported, *i.e.* ECA recognizing Gal(β1-4)GlcNAc and NPA high-mannose including Man(α1-6)Man. Both glycans belong to intermediate products in the biosynthetic process of N-type glycans [19]. The alterations of N-type glycans on immunoglobulins and interacting molecules initiate complement activation and following inflammatory reactions, *i.e.* the multi-hit

hypothesis [8, 46]. For instance, the binding affinity of Fc α receptor (CD89) to IgA molecules was dependent on N-type glycans on CD89 [47]. Furthermore, the complement activation was mediated by the association between mannose-binding lectin (MBL) and N-type glycans on IgG/IgM [48, 49]. The line of evidence suggested that the disease progression of IgAN is mainly mediated by N-type glycans. In contrast, the urinary O-type glycans binding to 3 lectins found in this study may represent the disease-specific changes in O-glycosylation in IgA1 or unknown glycoproteins.

Several limitations of our study also warrant mention. First, this study included only the patients with biopsy-proven renal diseases and our findings may not be representative of the patients without renal biopsy. As such, interpretation and generalization of the results should be conducted with care, and the external validation study is needed. This was a single-gate diagnostic study with consecutively recruited participants, which is considered the optimal design for the evaluation of diagnostic accuracy [50]. Second, this study was conducted in a single center in Japan, and other races including Caucasians were not included. The racial and regional differences have been reported for the prevalence of IgAN, serum level of Gd-IgA, and the mutation of C1GALT1 [51-53], and investigations and validation in various ethnicities and regions are required. Third, we were not able to adjust other possible confounders in the multivariate models. Some of the previously reported biomarkers have not been measured; serum and urinary Gd-IgA [37], serum anti-glycan antibody [12, 54], urinary factor H level related to complement system [55], and serum level of a proliferation-inducing ligand (APRIL) [56]. The addition of these new biomarkers such as Gd-IgA to the equation may further improve the diagnostic performance in the future studies.

In conclusion, we newly demonstrated the diagnostic accuracy and significance of urinary excretion O-glycan related to T-antigen measured by lectin microarray by ACA, ABA and MAH. Especially, ACA is useful and robust for diagnosis of IgAN in clinical settings and it may provide non-invasive diagnostic methods of liquid biopsy using urine samples. Further research is needed to clarify the role of abnormal O-glycans related to T-antigen in the pathogenesis of IgAN.

Statements

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Statement of Ethics

This study was conducted in accordance with the principles of the Declaration of Helsinki, and the protocol was reviewed and approved by the ethics committee of Okayama University Hospital (authorization number: K1709-039, K2106-027). This study was registered in University Hospital Medical Information Network on Sep 1, 2017 (UMIN clinical trial registry number: UMIN000029336). All study participants provided written informed consent.

Conflict of Interest Statement

Haruhito A. Uchida belongs to the Department of Chronic Kidney Disease and Cardiovascular Disease which is endowed by Chugai Pharmaceutical, MSD, Boehringer Ingelheim, and Kawanishi Holdings. Jun Wada receives speaker honoraria from Astra Zeneca, Daiichi Sankyo, Novartis, Novo Nordisk Pharma, Tanabe Mitsubishi and receives grant support from Astellas, Baxter, Bayer, Chugai, Dainippon Sumitomo, Kyowa Kirin, Novo Nordisk Pharma, Ono, Otsuka, Tanabe Mitsubishi, and Teijin. M.Ya. is an employee of Glycotechnica. Any other authors declare no conflicts of interest.

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Author Contributions

The contributions of the authors are detailed as follows. YO formulated the analysis plan, collected clinical data, performed statistical analyses, interpreted data, performed the immunostaining of renal biopsy samples, and wrote the manuscript. KM designed the research, collected clinical data, performed statistical analyses, interpreted data, and edited the manuscript. CK collected clinical data and interpreted data. HU and HS recruited the patients and interpreted data. RS and SY performed the immunostaining of renal biopsy samples. M. Yoshida and TM supported the statistical

analyses. M. Yamada measured urinary glycan intensity, and analyzed the urinary lectin microarray data, and wrote the manuscript. JH interpreted the results and data. JW took responsibility for all the study, conceived the study, supervised the data collection, analyzed the data, and edited the manuscript. All authors contributed to the critical revision of the manuscript and approved the final version of the manuscript. All authors ensured personally accountable for the individual's own contributions and to ensure that questions pertaining to the accuracy or integrity of any portion of the work, even one in which the author was not directly involved, are appropriately investigated, and resolved, including with documentation in the literature if appropriate.

Availability of Data and Material

We have not obtained consent from the participants of this study to release their data to the public. The data may be available upon requests to the corresponding author.

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Figure Legends

Fig. 1. The multivariate ROC curve model for the diagnosis of IgA nephropathy by urinary glycan intensity using 45 lectins.

Comparison of diagnostic performance of reference standard plus urinary glycan intensity using difference of c-statistics. Reference standard is defined as the regression equation calculated by the presence of hematuria, the 24 hours urinary protein excretion, and the concentration of serum IgA. The urinary glycan intensity is continuous variable. The threshold for significance was adjusted for multiple comparisons using Bonferroni method ($\alpha = 0.0011$). Abbreviations: 95%CI: 95% confidence interval.

Fig. 2. The multivariate ROC curves of ACA, ABA, and MAH.

The ROC curves from the 4 models for the diagnosis of IgAN: the reference standard plus urinary glycan intensity by (A) ACA, (B) ABA, and (C) MAH. The curves are overlaid with the ROC curve from the reference standard only. Abbreviations: UPE, urinary protein excretion (g/day); IgA, serum IgA levels (mg/dl); URBC, presence of hematuria.

Fig. 3. The decision curve for the diagnosis of IgA nephropathy comparing the reference standard and prediction model by ACA.

The Black solid line is the net benefit of renal biopsies undergone in all patients. The dotted blue line is the net benefit of renal biopsies performed based on reference standard. The dash-dotted red line is the net benefit of renal biopsies performed according to reference standard plus ACA. The dashed gray line is the net benefit of no application of renal biopsies. As the reference standard plus ACA curve runs higher than clinical findings curve, decision curve analysis (DCA) shows a net benefit in identifying the patients with IgA nephropathy at threshold probabilities of 0.3 - 0.6.

Table. Diagnostic accuracy of regression equation by reference standard plus lectins for IgA nephropathy

Lectin	Sensitivity (95% CI)	Specificity (95% CI)	Category-free NRI (95% CI)	<i>p</i> value	Relative IDI (95% CI)	<i>p</i> value	AIC	BIC
Reference standard	79.0 (71.8 - 85.1)	65.2 (59.8 - 70.3)	Ref.		Ref.		530.47	547.28
Reference standard plus lectins								
ACA	75.2 (67.6 - 81.7)	75.9 (71.0 - 80.4)	0.526 (0.319 - 0.733)	<0.001	0.050 (0.130 - 0.877)	0.008	512.08	533.08
ABA	76.4 (69.0 - 82.8)	74.1 (69.1 - 78.7)	0.415 (0.209 - 0.621)	<0.001	0.048 (0.015 - 0.081)	0.004	511.93	532.93
MAH	76.4 (69.0 - 82.8)	73.8 (68.8 - 78.4)	0.437 (0.244 - 0.630)	<0.001	0.044 (0.011 - 0.079)	0.009	513.37	534.38

Reference standard is defined as the regression equation calculated by the presence of hematuria, the 24hr urinary protein excretion, and the concentration of serum IgA. Abbreviations: NRI: net reclassification improvement, IDI: integrated discrimination improvement, AIC: Akaike's Information Criterion, BIC: Bayesian information criterion

Figure 1.

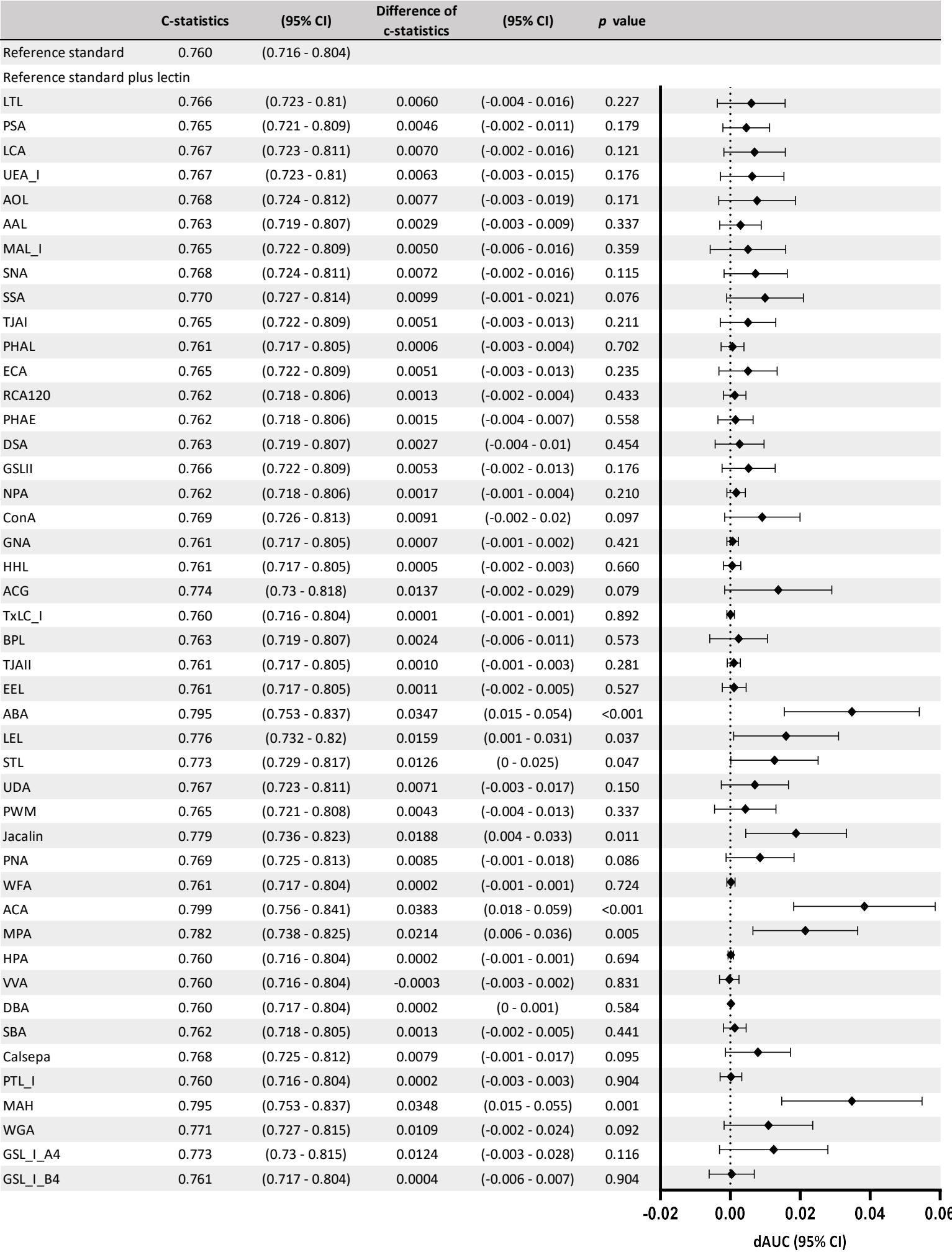


Figure 2.

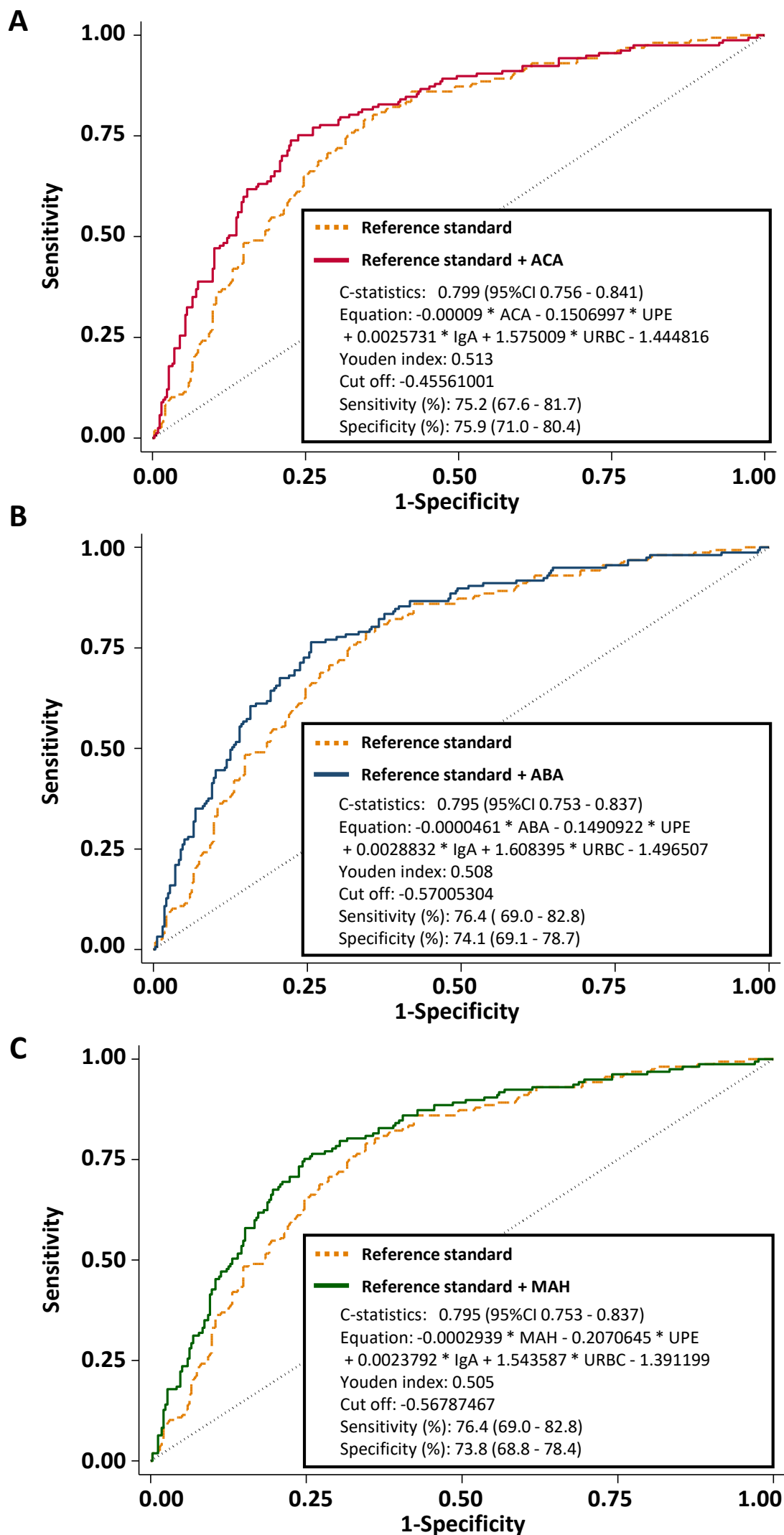


Figure 3.

