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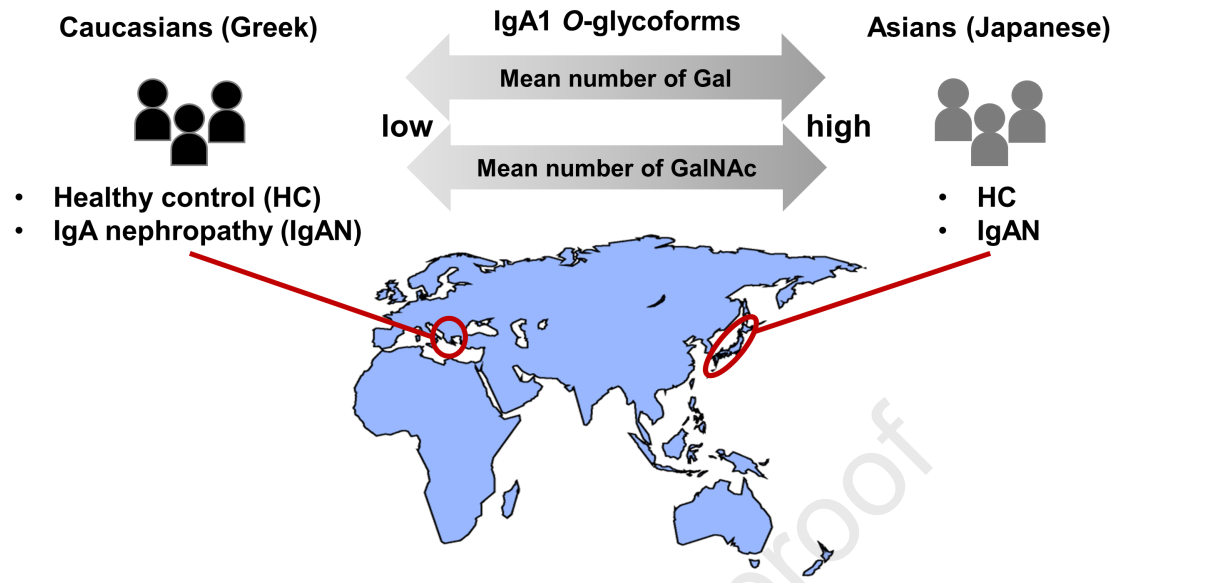

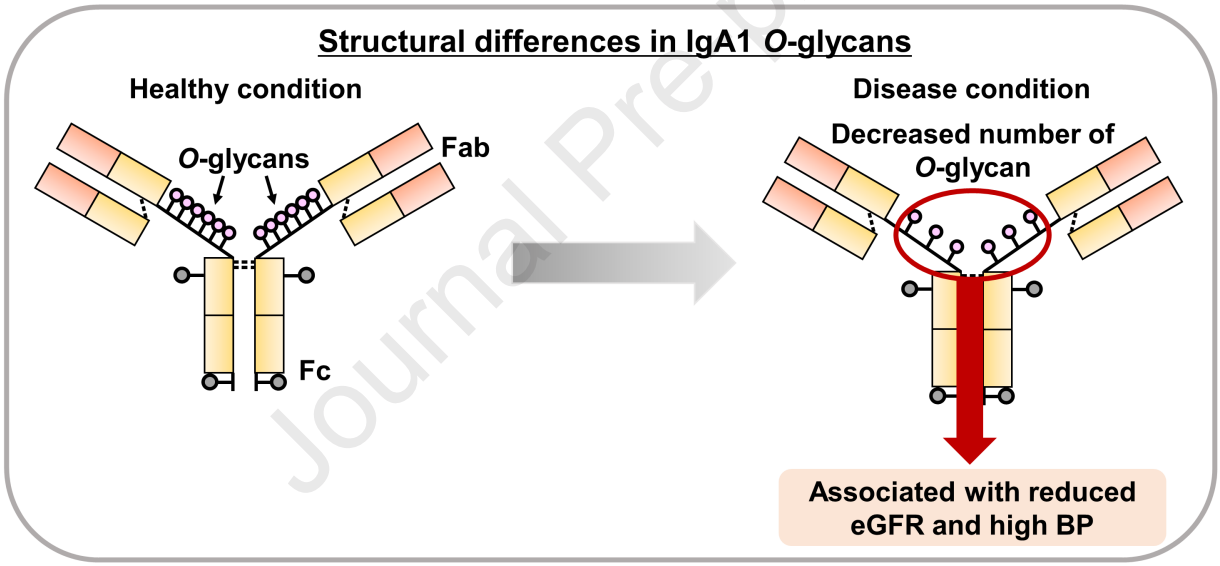

Racial heterogeneity of IgA1 hinge region *O*-glycoforms in patients with IgA nephropathy

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Summary

Galactose (Gal)-deficient IgA1 (Gd-IgA1) is involved in IgA nephropathy (IgAN) pathogenesis. To reflect racial differences in clinical characteristics, we assessed disease- and race-specific heterogeneity in the *O*-glycosylation of the IgA1 hinge region (HR). We determined serum Gd-IgA1 levels in Caucasians (healthy controls [HCs], n = 31; IgAN patients, n = 63) and Asians (HCs, n = 20; IgAN patients, n = 60) and analyzed profiles of serum IgA1 HR *O*-glycoforms. Elevated serum Gd-IgA1 levels and reduced number of Gal residues per HR were observed in Caucasians. Reduced number of *N*-acetylgalactosamine (GalNAc) residues per HR and elevated relative abundance of IgA1 with three HR *O*-glycans were common features in IgAN patients; these features were associated with elevated blood pressure and reduced renal function. We speculate that the mechanisms underlying the reduced GalNAc content in IgA1 HR may be relevant to IgAN pathogenesis.

INTRODUCTION

IgA nephropathy (IgAN), the most common form of primary glomerulonephritis worldwide, is characterized by glomerular mesangial deposition of IgA1, IgG, and complement C3 (Rizk et al., 2019). The disease onset occurs predominantly in young adults, and up to 20–40% patients progress to kidney failure within 20 years from diagnosis (Barbour and Reich, 2018; Wyatt and Julian, 2013). Although optimized supportive therapy, including administration of renin-angiotensin system blockers and/or corticosteroids showed potential renal benefits (Coppo et al., 2007; Tesar et al., 2015), corticosteroid therapy was associated with increased risk of serious adverse events, mostly due to infections (Lv et al., 2017; Rauen et al., 2015). Thus, assessment of the prognosis in individual patients is critical for providing adequate treatment. Although risk-prediction tools based on clinical, laboratory, and pathological parameters have been proposed recently (Barbour et al., 2019; Schena et al., 2021), biomarkers with prognostic significance should be developed.

Geographical diversity affects the incidence, severity (Kiryluk et al., 2012), and sex-specific prevalence of IgAN (Feehally and Cameron, 2011; Magistroni et al., 2015; Schena and Nistor, 2018; Wyatt and Julian, 2013) as well as the prevalence of incidental mesangial IgA1 deposits in necropsy studies and in renal allograft donors (Kiryluk et al., 2012; Suzuki et al., 2003; Waldherr et al., 1989). Furthermore, effective treatment for IgAN differs with race in European and Asian countries. While therapy targeting gut associated lymphoid tissue was effective in European countries (Fellstrom et al., 2017), therapy targeting nasal associated lymphoid tissue was effective in Asian patients (Hirano et al., 2019; Kawamura et al., 2014; Yang et al., 2016). These facts indicate that racial comparisons are important for elucidating the pathogenesis of the disease.

In humans, serum IgA1 usually has three to six core 1 *O*-glycans in the hinge region (HR) of each heavy chain (**Figure S1a**) (Reily et al., 2019). Serum and mesangial IgA1 of patients with IgAN are highly reactive with *Helix aspersa* (HAA) lectin, which binds to galactose-deficient (Gd)-*O*-glycans due to its specificity for terminal *N*-acetylgalactosamine (GalNAc) (Allen et al., 2001; Moldoveanu et al., 2007; Tomana et al., 1997). Polymeric IgA1, which constitutes the mesangial IgA1 deposits in patients with IgAN, is also highly reactive with HAA lectin (Oortwijn et al., 2006), suggesting

that pathogenic IgA1 contains Gd-*O*-glycans in its HR (Gd-IgA1). Gd-IgA1 is recognized by IgG autoantibodies specific for Gd-IgA1 (Tomana et al., 1999). Elevated amounts of circulating IgA1 immune complexes have been detected in patients with IgAN (Suzuki et al., 2009). Some of these complexes accumulate in the kidney and induce mesangial cell proliferation (Novak et al., 2011). Thus, aberrant *O*-glycosylation of IgA1 is believed to play an important role in the pathogenesis of IgAN (Suzuki *et al.*, 2009; Suzuki et al., 2011).

The inheritance pattern of Gd-IgA1 serum levels in familial and sporadic IgAN (Gharavi et al., 2008; Hastings et al., 2010; Lin et al., 2009) suggests that IgA1 glycosylation is genetically regulated. IgA1 HR *O*-glycosylation is controlled by specific glycosyltransferases in IgA1-secreting cells (Buck et al., 2008; Suzuki et al., 2008), particularly in the Golgi apparatus. As shown in **Figure S1b**, GalNAc residue(s) are attached to the serine (S) or threonine (T) residues of HR via polypeptide GalNAc-transferases (ppGalNAc-Ts). The attached GalNAc(s) are extended via the attachment of galactose (Gal) residues by core 1 β1,3-galactosyltransferase (C1GalT1). C1GalT1 activity depends on its molecular chaperone, Cosmc. Finally, each saccharide may be sialylated by sialyltransferases (Novak et al., 2008). Results from recent genome-wide association studies (GWAS) revealed an association between Gd-IgA1 levels and a common variation in *C1GALT1*, which encodes core 1 β1,3-galactosyltransferase 1 (Gale et al., 2017; Kiryluk et al., 2017). Kiryluk *et al*. showed that the allele rs13226913, which is associated with elevated serum levels of Gd-IgA1, is common in Europeans but rare in East Asians (Kiryluk *et al.*, 2017).

In addition to the genetic determinants of gene expression, enzyme activities of specific glycosyltransferases may be further altered by other factors (Suzuki et al., 2014; Yamada et al., 2020; Yamada et al., 2017), thereby further affecting the heterogeneity of IgA1 HR glycoforms in IgAN patients (Buck *et al.*, 2008; Ohyama et al., 2021). Thus, profiling of IgA1 HR *O*-glycoforms in IgAN is required to understand the pathological processes of this disease.

Studies have demonstrated alteration in IgA1 HR *O*-glycosylation using GalNAc-specific lectins (e.g., from *Helix aspersa* or *Helix pomatia*) or Gd-IgA1-specific monoclonal antibodies (KM55 and 35A12) (Hiki et al., 2015; Moldoveanu *et al.*, 2007;

Yasutake et al., 2015). Despite this, the heterogeneity of the IgA1 HR *O*-glycoforms in patients with IgAN is not well understood at the molecular level. We previously developed a high-throughput quantitative workflow (**Figure S2**) for profiling IgA1 *O*-glycosylation and identifying the IgA1 HR *O*-glycoform(s) specific for IgAN (Ohyama et al., 2020).

The purpose of this study was to analyze the heterogeneity in IgA1 HR *O*-glycoforms between Caucasian and Asian patients with IgAN and identify disease-specific IgA1 *O*-glycoforms via comparison of serum IgA1 from region-matching healthy controls. To determine specific *O*-glycoforms associated with the differences in severity between races, we selected Greeks, who show the lowest severity in Europe, and Japanese, who show the highest severity in Asia (Kiryluk et al., 2012).

We observed elevated serum levels of Gd-IgA1 and reduced Gal content per IgA1 HR in Caucasians. Furthermore, reduction in the number of GalNAc residues per HR and elevation in relative abundance of IgA1 HR with three *O*-glycans was observed in patients with IgAN. The decreased number of GalNAc residues per IgA1 HR was associated with elevated blood pressure, and elevated relative abundance of IgA1 HR with 3 *O*-glycans was associated with reduced estimated glomerular filtration rate (eGFR). Therefore, not only Gal-deficiency but also reduced number of *O*-glycans (i.e., reduced number of GalNAc residues per HR) characterize IgA1 in IgAN. Although we did not find a *O*-glycoform that contributes to the severity of IgAN in East Asians, we identified glycan structures involved in clinical features of IgAN common to both races. The profiling of IgA1 HR *O*-glycoforms may be useful for identifying new biomarkers and will likely provide new information regarding IgAN pathogenesis.

RESULTS

Clinical and laboratory information

The demographic and clinical characteristics of the total cohort, consisting of Japanese healthy controls (J-HC, n = 20), Japanese IgAN patients (J-IgAN, n = 60), Greek healthy controls (G-HC, n = 31), and Greek IgAN patients (G-IgAN, n = 63), are shown in **Table 1**. Comparison between J-IgAN and G-IgAN patients showed that age, sex distribution, proteinuria, eGFR, frequency of concomitant disease, such as dyslipidemia (DL), diabetes

mellitus (DM), and cardiovascular disease (CVD), frequency of past history of tonsillectomy, and past history of immunosuppressive therapy were similar. In contrast, systolic blood pressure (sBP), diastolic blood pressure (dBP), mean arterial pressure (MAP), serum creatinine (sCr), and antihypertensive medication use were higher in G-IgAN than in J-IgAN ($P = 0.001$, $= 0.003$, $= 0.001$, < 0.001, and $= 0.002$, respectively). Among histological findings, endocapillary hypercellularity (E1) and crescents (C1,2) score were more frequent in J-IgAN than in G-IgAN ($P < 0.001$ and $P < 0.001$, respectively), whereas mesangial hypercellularity (M1), segmental glomerulosclerosis (S1), and tubular atrophy/interstitial fibrosis (T1,2) were similar in both groups.

Presence of IgAN, race, and age are associated with serum Gd-IgA1 levels

To compare the serum levels of Gd-IgA1 among the four groups, we measured serum Gd-IgA1 levels in the total cohort using the Gd-IgA1-specific monoclonal antibody KM55. Gd-IgA1 levels differed significantly among the four groups ($P < 0.001$). Comparisons of each group with the reference group revealed that serum Gd-IgA1 levels in J-IgAN, G-HC, and G-IgAN were significantly higher than those in J-HC (Dunn's correction $P < 0.001$, $= 0.005$, and < 0.001, respectively) (**Figure 1**). Multiple linear regression analyses showed that elevated Gd-IgA1 level was associated with IgAN diagnosis, Greek race, and higher age when each of the independent variables were mutually adjusted (**Table 2**). Furthermore, among patients with IgAN (n = 123), only Greeks exhibited elevated serum Gd-IgA1 levels when age, sex, races, MAP, eGFR, urinary protein level, and antihypertensive medication use were mutually adjusted (**Table 3**).

Identification of disease-specific IgA1 HR *O*-glycoforms

To identify disease-specific IgA1 HR *O*-glycoforms and assess the heterogeneity in IgA1 HR *O*-glycoforms between Caucasians and Asians, individual profiles of serum IgA1 HR *O*-glycoforms were analyzed in a subset of Caucasians (G-HC, n = 16; G-IgAN, n = 23) and Asians (J-HC, n = 10; J-IgAN, n = 36), using randomly selected samples. The profiles were obtained via liquid chromatography (LC)-mass spectrometry (MS) analysis. The clinical and laboratory findings for the donor subset are shown in **Table S1**.

Twelve glycoforms of IgA1 HR *O*-glycopeptides (3GalNAc2Gal, 3GalNAc3Gal,

4GalNAc2Gal, 4GalNAc3Gal, 4GalNAc4Gal, 5GalNAc2Gal, 5GalNAc3Gal, 5GalNAc4Gal, 5GalNAc5Gal, 6GalNAc3Gal, 6GalNAc4Gal, and 6GalNAc5Gal) were detected in the mass spectra of HCs and patients with IgAN. In the Japanese group, 3GalNAc3Gal was the only glycoform that was elevated in the patients ($P < 0.001$). In the Greek cohort, 3GalNAc2Gal and 5GalNAc3Gal glycoforms were higher in G-IgANs than in G-HCs ($P = 0.008$ and 0.043, respectively) (**Figure 2**). Comparison between J-IgAN and G-IgAN showed that glycoforms with fewer GalNAc and Gal residues were higher in G-IgAN than in J-IgAN. Representative mass spectra of the desialylated tryptic fragments of IgA1 HR *O*-glycoforms acquired from Japanese and Greek patients with IgAN are shown in **Figure 3**. The relative abundance of the IgA1 HR glycoforms with 4GalNAc4Gal, 5GalNAc4Gal, 6GalNAc3Gal, 5GalNAc5Gal, 6GalNAc4Gal, and 6GalNAc5Gal was significantly higher in J-IgAN than in G-IgAN ($P = 0.013$, < 0.001, 0.029, 0.016, 0.003, and < 0.001, respectively). Conversely, the relative abundance of the IgA1 HR glycoforms with 3GalNAc2Gal, 4GalNAc2Gal, 4GalNAc3Gal, 5GalNAc2Gal, and 5GalNAc3Gal was significantly higher in G-IgAN than in J-IgAN ($P = 0.002$, < 0.001, < 0.001, < 0.001, and 0.001, respectively).

IgA1 HR glycopeptides possessed three to six *O*-glycans; >80% IgA1 HR glycopeptides from IgAN patients and HCs had four or five *O*-glycans per HR in IgA1 (**Figure 4a–d**). The relative abundance of IgA1 containing three *O*-glycans, referred to as 3GalNAc glycoform, differed significantly among the four groups ($P = 0.001$); this HR glycoform was higher in J-IgAN and G-IgAN than in the reference group (J-HC) based on Dunn's multiple comparison test (adjusted $P = 0.008$ and $P = 0.001$, respectively) (**Figure 4a**). The relative abundance of IgA1 containing six *O*-glycans per HR—the most *O*-glycan-rich glycoform referred to as 6GalNAc glycoform—differed among the four groups ($P < 0.001$) and was lower in G-IgAN than in J-HC (Dunnett's correction $P < 0.001$; **Figure 4d**).

The mean number of GalNAc residues per HR was the highest in J-HC, followed by J-IgAN, G-HC, and G-IgAN. The mean number of GalNAc residues per HR differed among the four groups ($P = 0.009$), and the levels were lower in G-IgAN than in the reference group, J-HC (Dunn's correction $P = 0.003$; **Figure 4e**).

Although there was a slight difference in the mean number of Gal residues per

HR and Gd-glycans per HR among the four groups (P = 0.021 and 0.042, respectively), Dunnett's and Dunn's multiple comparisons with the reference group (J-HC) did not show any difference (**Figure 4f–g**).

Multiple linear regression analyses showed that the diagnosis of IgAN and Greek ethnicity were associated with low mean number of GalNAc residues per HR when mutually adjusted for age, sex, races, and diagnosis of IgAN (**Table 4**). Furthermore, only diagnosis of IgAN was related to an increase in the relative abundance of 3GalNAc per IgA1 HR (**Table 5**). When multiple linear regression analyses were performed in patients with IgAN (n = 59), reduced mean number of GalNAc residues per HR was associated with the elevation of MAP, while high relative abundance of 3GalNAc glycoform was associated with a decrease in eGFR when age, sex, race, MAP, eGFR, urinary protein level, and antihypertensive medication use were mutually adjusted (**Tables 6 and 7**). However, the E or C scores were not associated with low mean number of GalNAc residues per HR or high relative abundance of 3GalNAc glycoform (**Tables S2–S5**).

In contrast, only Greek ethnicity was associated with a decrease in the mean number of Gal residues per HR when mutually adjusted for age, sex, race, and diagnosis of IgAN (**Table S6**). In a multiple regression analysis for patients with IgAN (n=59), only Greek ethnicity was found to be associated with low mean number of Gal residues per HR, although any other clinical confounders were not associated with the mean number of Gal residues per HR (**Table S7**). In order to confirm that MS analysis results reflect ELISA data, we comared serum Gd-IgA1 levels with the number of Gal residues per HR. A significant negative correlation was observed in both races of IgAN patients (J-IgAN, n = 36; G-IgAN, n = 23; r = -0.396, P = 0.017; r = -0.576, P = 0.004; **Figure S3**).

In summary, reduced number of GalNAc residues per HR and elevated relative abundance of IgA1 HR with 3 *O*-glycans was common in patients with IgAN. Moreover, an increase in MAP and decrease in eGFR, factors known to be associated with the progression of IgAN, were related to these two factors. As with Gd-IgA1, a reduction in the number of Gal residues per HR was associated with Greek ethnicity but not with any clinical parameter of IgAN.

DISCUSSION

Alteration in IgA1 HR *O*-glycosylation occurs in patients with IgAN (Dotz et al., 2021). We studied patient health and racial heterogeneity of IgA1 HR *O*-glycoforms using antibody-based Gd-IgA1 enzyme-linked immunosorbent assay (ELISA) and LC-MS. First, we confirmed that the serum level of Gd-IgA1 was elevated in IgAN patients and was higher in Greek than in Japanese patients. Second, using MS analysis, we found that Greek ethnicity was associated with a reduction in the mean number of Gal residues per HR, independent of the diagnosis of IgAN. Finally, we demonstrated that the decrease in the mean number of GalNAc residues per HR and increase in relative abundance of IgA1 with 3GalNAc residues per HR were a common feature found in both races of IgAN patients; these two variables were associated with elevated blood pressure and reduced eGFR, respectively.

In this study, using the two analytical methods, Gd-IgA1 ELISA and MS, Gal deficiency of IgA1 HR was found to be affected by racial differences. According to previous reports, common variations in *C1GALT1* are associated with increase in serum Gd-IgA levels, and the specific allele that leads to increases in serum Gd-IgA1 levels is more common in Europeans than in East Asians (Gale *et al.*, 2017; Kiryluk *et al.*, 2017). Furthermore, a recent study focusing on microRNA (miRNA) reported that the circulating levels of miR-148b, which negatively regulates the expression of *C1GALT1*, were higher in Caucasians than in Asians (Serino et al., 2016). These reports suggest that Gal deficiency is likely to be affected by racial differences in C1GalT1 activity. In contrast, the mean Gal number per HR was not associated with diagnosis of IgAN (**Table S6**), and neither serum Gd-IgA1 levels nor the mean number of Gal residues per HR were associated with clinical parameters of IgAN in this study (**Tables 3 and S7**).

A reduced number of *O*-glycans was observed in IgAN-derived IgA1 samples. Using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS, Inoue et al. found that the number of GalNAc residues per HR was significantly lower in patients with IgAN (as well as in those with Crohn's disease) than in patients with ulcerative colitis or other diseases, such as ischemic colitis, and in healthy volunteers (Inoue et al., 2012). Furthermore, Iwatani et al. (2012) reported that the reduction in the number of GalNAc residues per HR was reversed in IgAN patients who achieved remission after tonsillectomy combined with intravenous administration of

corticosteroids (Iwatani et al., 2012). In addition, upregulation of the expression of let-7b miRNA, which negatively regulates the expression of ppGalNAc-T2 (the key enzyme required for transferring GalNAc residues to the IgA1 HR, thereby playing an important role in the initiation of *O*-glycosylation and determination of the number and location of *O*-glycans) (**Figure S1b**) (Iwasaki et al., 2003), has been reported in the peripheral blood mononuclear cells of patients with IgAN (Serino et al., 2012; Serino et al., 2015). Let-7b miRNA levels were higher in the serum samples of patients with IgAN than in serum samples of healthy volunteers (Serino *et al.*, 2016). These findings are consistent with our results showing that a decrease in the mean number of GalNAc residues per HR and an increase in the relative abundance of IgA1 HR with 3 *O*-glycans are associated with diagnosis of IgAN (**Tables 4 and 5**).

Humans express 20 isoforms of ppGalNAc-Ts, among which ppGalNAc-T1, T2, T3, T4, T6, and T9 are expressed in IgA1-producing B cells (Gerken et al., 2013; Iwasaki *et al.*, 2003). Our *in vitro* studies demonstrated that pre-existing glycans affect sites of GalNAc attachment by GalNAc-Ts, as well as subsequent activity of other glycosyltransferases (Stewart et al., 2019; Stewart et al., 2020). This study suggested that the expression or activity of ppGalNAc-Ts may be altered in IgA1-producing cells of patients with IgAN, resulting in secretion of IgA1 with under-glycosylated HR.

The following question now arises: what is the origin of IgA1 with low GalNAc numbers in the HR? The association between IgAN and the mucosa has been recognized earlier (Floege and Feehally, 2016), and the increase in circulating nephritogenic IgA1 is thought to be derived from the displacement of mucosal associated lymphoid tissue (MALT)-derived B cells to systemic sites such as the bone marrow, which is designated as the “mucosal bone marrow axis” (Barratt et al., 2020; Suzuki and Tomino, 2007). A decreased mean number of GalNAc residues in the IgA1 HR was observed in inflammatory bowel disease, especially in Crohn’s disease, as well as in patients with IgAN (Inoue *et al.*, 2012). A breakdown of the mucosal barrier could lead to increased circulating levels of IgA1 with low GalNAc in the HR.

In this study, we show for the first time that a small number of GalNAc residues per HR and elevated relative abundance of IgA1 with 3GalNAc residues per HR is associated with high blood pressure and deterioration of kidney function, respectively.

High level of proteinuria, low eGFR, and hypertension are associated with high risk of kidney function loss in IgAN (Barbour and Reich, 2012). Patients with baseline hypertension (> 140/90 mmHg) and poorly controlled follow-up blood pressure showed a 20-year risk of death or dialysis (Barbour and Reich, 2012; Berthoux et al., 2011). However, the mechanism via which the change in IgA1 HR *O*-glycoform in patients with IgAN influences clinical parameters such as eGFR and blood pressure have not been elucidated in this study. Reduction in GalNAc content in IgA1 HR, i.e., defect in *O*-glycosylation in HR, may affect the tertiary structure of IgA1 and change the relative orientations of Fab and Fc (Narimatsu et al., 2010). Structural changes in IgA1 due to defect in *O*-glycosylation in HR might alter its solubility, antigenicity, or interaction with other proteins. These changes in the properties of IgA1 may promote the formation of immune complexes by antibody recognition and their glomerular deposition, leading to mesangio-proliferative injury (Suzuki *et al.*, 2011) that may develop into progressive loss of renal function and secondary elevation of blood pressure.

In conclusion, we showed in this study that i) serum levels of Gd-IgA1 were elevated in patients with IgAN and were more likely to be elevated in Caucasian Greeks than in Asian Japanese, although Gd-IgA1 level was not associated with clinical parameters at the time of renal biopsy in patients with IgAN; ii) low Gal content per HR was more pronounced in Caucasians than in Asians and was not associated with diagnosis of IgAN and clinical parameters at the time of renal biopsy in IgAN patients; iii) low GalNAc content per HR and high relative abundance of IgA1 HR with 3 *O*-glycans were the common features of patients with IgAN and were associated with high blood pressure and poor kidney function, respectively, in patients with IgAN. These results suggested that the defect in *O*-glycosylation in HR was associated with clinical parameters commonly observed in patients with IgAN and that the extent of *O*-galactosylation varied with ethnicity. The upstream pathways that result in reduced *O*-glycosylation of IgA1 in IgAN should be identified. Understanding the potential pathogenic roles of specific IgA1 *O*-glycoforms in IgAN will also help in the development of new biomarkers.

LIMITATIONS OF THE STUDY

There are three limitations of this study. First, candidates from only two countries were

analyzed in this study. To confirm the difference between Caucasians and Asians, we should include additional cohorts from other European and Asian countries and the US in the future. Second, the mechanisms through which the reduction in the number of GalNAc residues per HR influences the pathogenesis and clinical parameters of IgAN, such as eGFR and blood pressure, have not been elucidated. An abnormality in the IgA1 HR *O*-glycoform is not sufficient to explain the pathogenesis of IgAN, and the formation of high-molecular-mass IgA1-containing immune complexes that include autoantibodies to abnormally glycosylated IgA1 plays a crucial role in the pathogenesis of IgAN (Suzuki *et al.*, 2009; Suzuki *et al.*, 2011; Tomana *et al.*, 1997). In this study, the involvement of IgA1 with reduced *O*-glycan in HR in IgA1 immune complex formation was not clarified. Finally, the associations of the IgA1 HR *O*-glycoform with race and clinical parameters may be influenced by the difference in disease duration of IgAN at the time of renal biopsy. Further follow-up studies, including longitudinal characterization of IgA1 HR *O*-glycoforms, are required to establish the clinical significance of low number of GalNAc residues per HR in IgAN.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.P.S. and K.T.; Methodology, Y.O., S.N.C., N.M.K., M.J.S., K.N., H.H, D.I., M.H., Y. Y., N.T., M.B.R., J.N., A.A.P., F.P.S., and K.T.; Investigation, Y.O., H.Y., and S.C. Formal Analysis, Y.O. H.Y., S.O., S.C, and K.T.; Writing – Original Draft, Y.O.; Writing-Review & Editing F.P.S, K.T., J.N., and M.B.R.

DECLARATION OF INTERESTS

M.B.R. and J.N. are co-inventors on US patent application 14/318,082 (assigned to UAB Research Foundation). M.B.R. and J.N. are co-founders and co-owners of and consultants for Reliant Glycosciences, LLC. F.P.S. is the principal investigator of the Grant No. P8K5PA from Puglia Region and No. ARS01_00876 from Italian Ministry of University, Italy.

MAIN FIGURE TITLES AND LEGENDS

Figure 1. Comparison of serum Gd-IgA1 levels among four groups: J-HC, J-IgAN, G-HC, and G-IgAN. The medians (quartile range) of Gd-IgA levels were 0.40 (0.23–0.52), 0.86 (0.55–1.25), 0.73 (0.38–1.17), and 1.29 (0.85–1.96), respectively. Gd-IgA1 levels differed significantly among the four groups (Kruskal-Wallis test, $P < 0.001$). Gd-IgA1 levels were significantly higher in J-IgAN, G-HC, and G-IgAN than in the reference group (J-HC) (Dunn's correction, $P < 0.001$, $P = 0.005$, and $P < 0.001$, respectively). Gd-IgA1, galactose-deficient IgA1; J-HC, Japanese healthy control; J-IgAN, Japanese patients with IgAN; G-HC, Greek healthy control; G-IgAN, Greek patients with IgAN; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$

Figure 2. **Comparison of desialylated IgA1 HR *O*-glycoforms between healthy controls (HCs) and patients with IgA nephropathy (IgAN) in Japanese and Greek cohorts.** (a) IgA1 HR *O*-glycoforms of the Japanese cohort. (b) IgA1 HR *O*-glycoforms of the Greek cohort. The medians of relative abundance (%) in each *O*-glycoform are represented by black bars. Relative abundance of IgA1 HR with 3GalNAc3Gal increased significantly, whereas that of 5GalNAc4Gal decreased significantly in J-IgAN compared with that in J-HC (Mann-Whitney test, $P < 0.001$ and Student's *t*-test, $P = 0.040$, respectively). In the Greek cohort, the relative abundance of IgA1 HR with 3GalNAc2Gal and 5GalNAc3Gal was significantly higher (Student's *t*-test, $P = 0.008$ and Mann-Whitney test, $P = 0.043$, respectively) and that of 6GalNAc4Gal and 6GalNAc5Gal was significantly lower in G-IgAN than in G-HC (Student's *t*-test, $P = 0.006$ and $= 0.001$, respectively). J-HC, Japanese-HCs; J-IgAN, Japanese patients with IgAN; G-HC, Greek HCs; G-IgAN, Greek patients with IgAN; *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$

Figure 3. Representative mass spectra of the desialylated tryptic fragments of IgA1 HR *O*-glycoforms acquired from Japanese (a) and Greek (b) patients with IgAN. The monoisotopic *m/z* value of the HR *O*-glycopeptide ions and the number of sugar moieties assigned are shown above the individual peaks. The HR *O*-glycoforms, the levels of which were higher in Japanese patients than in Greek patients, are represented by upward arrows above the individual peaks in the mass spectra of Japanese patients. The HR *O*-glycoforms, the levels of which were elevated in Greek patients than in Japanese patients, are represented by upward arrows in the mass spectra of the Greek patients. Comparison of two groups was performed using Student's *t*-test or Mann-Whitney test depending on whether the variables were distributed normally. *, $0.01 < P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$

Figure 4. **Amount of a specific monosaccharide per HR.** (a) Relative abundance of IgA1 HR peptide with 3 GalNAc residues. This HR glycoform was higher in J-IgAN and G-IgAN than in the reference group (J-HC) based on Dunn's multiple comparison test ($P = 0.008$ and $P = 0.001$, respectively). (b) Relative abundance of IgA1 HR peptide with 4 GalNAc residues. This HR glycoform was higher in G-IgAN than in J-HC (Dunnett's correction $P=0.017$). (c) Relative abundance of IgA1 HR peptide with 5 GalNAc residues. (d) Relative abundance of IgA1 HR peptide with 6 GalNAc residues. This HR glycoform was lower in G-IgAN than in J-HC (Dunnett's correction $P < 0.001$). (e) Mean number of GalNAc per HR. The levels were lower in G-IgAN than in J-HC (Dunn's correction $P = 0.003$. (f) Mean number of Gal per HR. (g) Mean number of Gd-glycan per HR. The data are shown in the scatter dot plot (with line drawn at the median). GalNAc, *N*-acetylgalactosamine; Gal, galactose; Gd-glycan, galactose-deficient-glycan; HR, hinge region; J-HC, Japanese healthy controls; J-IgAN, Japanese patients with IgAN; G-HC, Greek healthy controls; G-IgAN, Greek patients with IgAN. *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***, $P < 0.001$

MAIN TABLES AND LEGENDS

Table 1. Demographic and clinical characteristics of Japanese and Greek patients with IgAN and their respective healthy controls.

	J-HC (n = 20)	J-IgAN (n = 60)	*P* value (J-HC vs. J-IgAN)	G-HC (n = 31)	G-IgAN (n = 63)	*P* value (G-HC vs. G-IgAN)	*P* value (J-IgAN vs. G-IgAN)
Age, years	33.0 (27.5–35.5)	36.0 (27.0–48.0)	0.016	48.0 (35.0–55.0)	45.0 (31.5–50.0)	0.336	0.059
Men (%)	10 (50.00)	30 (50.0)	1.000	17 (54.8)	36 (57.1)	0.832	0.427
sBP, mm Hg	n/a	120.0 (110.0–133.0)	-	n/a	135.0 (120.0–145.0)	-	0.001
dBP, mm Hg	n/a	76.5 (67.0–83.0)	-	n/a	80.0 (75.0–90.0)	-	0.003
MAP, mm Hg	n/a	92.7 (81.2–102.7)	-	n/a	100.0 (92.5–108.3)	-	0.001
Cr, mg/dl	n/a	0.81 (0.66–1.07)	-	n/a	1.40 (1.00–1.80)	-	<0.001
eGFR, ml/min/1.73 m^2	n/a	76.10 (51.5–96.9)	-	n/a	67.30 (44.45–83.85)	-	0.054
UP, g/gCr	n/a	1.17 (0.51–1.96)	-	n/a	1.50 (0.9–2.20)	-	0.178
Medication of antihypertensive agents, yes (%)	n/a	26 (43.3)	-	n/a	45 (71.4)	-	0.002

Presence of DL at the time of sample collection, yes (%)	n/a	29 (48.3)	-	n/a	22 (34.9)	-	0.131
Presence of DM at the time of sample collection, yes (%)	n/a	1 (1.7)	-	n/a	0 (0)	-	0.488
Past history of CVD events, yes (%)	n/a	1(1.7)	-	n/a	3 (4.8)	-	0.328
Past history of tonsillectomy, yes (%)	n/a	2 (3.3)	-	n/a	1 (1.6)	-	0.482
Past history of IS therapy, yes (%)	n/a	3 (5.0)	-	n/a	0 (0.0)	-	0.113
M1 score (%)	n/a	39 (67.2)	-	n/a	51 (81.0)	-	0.084
E1 score (%)	n/a	30 (51.7)	-	n/a	7 (11.1)	-	< 0.001
S1 score (%)	n/a	41 (70.7)	-	n/a	39 (61.9)	-	0.308
T(1,2) score (%)	n/a	18 (31.0)	-	n/a	23 (36.5)	-	0.525
C(1,2) score (%)	n/a	37 (63.8)	-	n/a	16 (25.4)	-	< 0.001

Data are presented as median (interquartile range) or number (%).
J-IgAN, Japanese IgAN patients; J-HC, Japanese healthy controls; G-IgAN, Greek IgAN patients; G-HC, Greek healthy controls; sBP, systolic blood pressure; dBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; Cr, serum creatinine concentration; UP, urinary protein-to-creatinine ratio; DL, dyslipidemia; DM, diabetes mellitus; CVD, cardiovascular disease; IS, immunosuppressive therapy; M, mesangial proliferation; S, segmental glomerulosclerosis; E, endocapillary proliferation; T, interstitial fibrosis/tubular atrophy; C, crescents.

Table 2. Multiple linear regressions of Gd-IgA1 (mg/dl) on age, sex, race, and diagnosis of IgAN (n = 174).

Independent variable	Regression coefficient (95% confidence interval)	*P* value
Age (years)	0.010 (0.002 to 0.019)	0.018
Male (Ref: female)	0.064 (-0.155 to 0.284)	0.564
Greek (Ref: Japanese)	0.550 (0.323 to 0.778)	< 0.001
IgAN (Ref: HC)	0.550 (0.308 to 0.791)	< 0.001

Gd-IgA1, galactose-deficient IgA1; IgAN, IgA nephropathy; HC, healthy control.

Table 3. Multiple linear regressions of Gd-IgA1 (mg/dl) on age, sex, race, MAP, eGFR, urinary protein level, and antihypertensive medication use (n = 123; consists of only patients with IgAN).

Independent variable	Regression coefficient (95% confidence interval)	*P* value
Age (years)	0.012 (-0.001 to 0.025)	0.073
Male (Ref: female)	0.127 (-0.170 to 0.423)	0.399
Greek (Ref: Japanese)	0.544 (0.232 to 0.857)	0.001
MAP (mmHg)	0.007 (-0.007 to 0.020)	0.329

eGFR (ml/min/1.73 m^2)	0.004 (-0.003 to 0.011)	0.239
Urinary protein (g/gCr)	0.000 (-0.071 to 0.071)	0.994
Antihypertensive medication use, yes (Ref: no)	0.158 (-0.162 to 0.477)	0.331

Gd-IgA1, galactose-deficient IgA1; IgAN, IgA nephropathy; HC, healthy control; MAP, mean arterial pressure; eGFR, estimated glomerular filtration rate.

Table 4. Multiple linear regressions of mean number of GalNAc per HR on age, sex, race, diagnosis of IgAN (n = 85).

Independent variable	Regression coefficient (95% confidence interval)	*P* value
Age (years)	-0.001 (-0.002 to 0.000)	0.081
Male (Ref: female)	-0.013(-0.040 to 0.015)	0.357
Greek (Ref: Japanese)	-0.034 (-0.063 to -0.005)	0.022
IgAN (Ref: HC)	-0.045 (-0.075 to -0.015)	0.004

GalNAc, *N*-acetylgalactosamine; HR, hinge region; IgAN, IgA nephropathy; HC, healthy control.

Table 5. Multiple linear regressions of relative abundance of 3GalNAc on age, sex, race, diagnosis of IgAN (n = 85).

Independent variable	Regression coefficient (95% confidence interval)	*P* value
Age (years)	0.018 (-0.011 to 0.047)	0.214
Male (Ref: female)	0.453 (-0.277 to 1.182)	0.220
Greek (Ref: Japanese)	-0.248 (-0.522 to 1.018)	0.524
IgAN (Ref: HC)	1.674 (0.865 to 2.482)	< 0.001

GalNAc, *N*-acetylgalactosamine; IgAN, IgA nephropathy; HC, healthy control.

Table 6. Multiple linear regressions of mean number of GalNAc residues per HR on age, sex, race, MAP, eGFR, urinary protein level, and antihypertensive medication use (n = 59; consists of only patients with IgAN).

Independent variable	Regression coefficient (95% confidence interval)	*P* value
Age (years)	0.000 (-0.001 to 0.002)	0.687
Male (Ref: female)	-0.002 (-0.037 to 0.032)	0.905
Greek (Ref: Japanese)	-0.008 (-0.048 to 0.031)	0.675
MAP (mm Hg)	-0.002 (-0.003 to 0.000)	0.021
eGFR (ml/min/1.73 m^2)	0.001 (0.000 to 0.002)	0.074
Urinary protein (g/gCr)	-0.002 (-0.008 to 0.005)	0.613
Antihypertensive medication use, yes (Ref: no)	-0.023 (-0.062 to 0.016)	0.239

GalNAc, *N*-acetylgalactosamine; HR, hinge region; IgAN, IgA nephropathy; HC, healthy control; MAP, mean arterial pressure; eGFR, estimated glomerular filtration rate.

Table 7. Multiple linear regression of relative abundance of 3GalNAc glycoform with age, sex, race, MAP, eGFR, urinary protein, antihypertensive medication use (n = 59; consists of only patients with IgAN).

Independent variable	Regression coefficient (95% confidence interval)	*P* value
Age (years)	-0.016 (-0.059 to 0.028)	0.469
Male (Ref: female)	-0.059 (-1.004 to 0.885)	0.900
Greek (Ref: Japanese)	-0.462 (-1.545 to 0.620)	0.395
MAP (mm Hg)	0.037 (-0.005 to 0.080)	0.084
eGFR (ml/min/1.73 m^2)	-0.026 (-0.048 to -0.003)	0.029
Urinary protein (g/gCr)	-0.029 (-0.207 to 0.149)	0.744

Antihypertensive medication use, yes (Ref: no)	0.575 (-0.485 to 1.635)	0.281

GalNAc, *N*-acetylgalactosamine; HR, hinge region; IgAN, IgA nephropathy; HC, healthy control; MAP, mean arterial pressure; eGFR, estimated glomerular filtration rate.

1

STAR METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kazuo Takahashi (kazuot@fujita-hu.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The data set supporting the findings of this study have been deposited at Mendeley and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethical approval

All experimental protocols were approved by the Institutional Review Board (IRB) of the Fujita Health University (approved number HM18-421). Informed consent was obtained from all subjects, and all the methods were performed in accordance with the relevant guidelines and regulations. The protocol approval number 17/2.3.2010 of the Ethics Committee of the Aristotle University of Thessaloniki (Greece) was used to collect and store blood and urine samples of patients with IgAN.

Subjects

Four well-characterized cohorts were included in this study: Asians (Japanese healthy controls [J-HC], n = 20; Japanese IgAN patients [J-IgAN], n = 60) and Caucasians (Greek healthy controls [G-HC], n = 31; Greek IgAN patients [G-IgAN], n = 63). The patients were diagnosed as having IgAN via kidney biopsy at the Department of Nephrology, Fujita Health University School of Medicine (Toyoake, Japan) and at the Renal Unit, Aristotle University of Thessaloniki (Greece). Serum samples were collected at the time

of biopsy. Patients and controls in each group were randomly sampled from the pool of available individuals. Patients aged > 70 years at the time of renal biopsy or with secondary IgAN were excluded. MS analysis of *O*-glycoform was performed in a subset of Caucasians and Asians, using samples randomly selected from the total cohort.

Clinical information at renal biopsy, such as age, sBP, dBP, and MAP (mmHg), sCr, assessed using the enzymatic method (mg/dl), and proteinuria (g/gCr), information on comorbidities, such as DM and DL, medication with antihypertensive agents, and past history of CVD, tonsillectomy, and use of immunosuppressive agents, was collected from medical records. eGFR (ml/min/1.73 m^2) was calculated using the following equation: Japanese equation: eGFR= 194 × serum creatinine$^{-1.094}$ × age$^{-0.287}$ × 0.739 [if female]; the CKD-EPI formula was used for Greek patients. Pathological characteristics were assessed by two nephrologists according to the Oxford classification (Trimarchi et al., 2017). The personal information of patients can be downloaded from DOI: 10.17632/mm4d3rj7xk.1.

METHOD DETAILS

Measurement of Gd-IgA1 levels

Total serum Gd-IgA1 levels were measured using an ELISA kit (Code Number 27600, Immuno-Biological Laboratories, Fujioka, Gunma, Japan). Data are expressed in mg/dl based on the standard curve.

Purification of IgA1 and sample preparation for MS

IgA1 was purified from 100 µl serum of patients with IgAN and HCs using affinity chromatography with anti-human IgA (0855068, MP Biomedicals, Irvine, CA, USA) coupled to a HiTrap NHS-activated HP column (17071601, GE Healthcare, Chicago, IL, USA). The purified samples were aliquoted and stored at −80 °C. For IgA1 HR *O*-glycoform profiling, 5 µg of purified IgA1 proteins was treated with 2.5 mU neuraminidase (GK80040, ProZyme, Hayward, CA, USA) in 50mM sodium phosphate, pH 6.0, at 37°C, overnight. The digests were then reduced by incubating with 20mM dithiothreitol (D5545, Sigma, St. Louis, MO, USA) for 15 min at room temperature, and sequentially digested using trypsin (V528A, Promega, Madison, WI, USA)(enzyme-to-substrate ration of 1:50) in 100mM NH_4HCO_3, pH 8.3, at 37 °C, overnight (Ohyama *et*

al., 2020) (**Figure S2**).

LC-MS analysis for profiling of IgA HR *O*-glycoforms

Online LC was performed using an EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a trap (Acclaim PepMap 100 C18 LC column, 3μm, 75μm ID × 20mm; Thermo Fisher Scientific) (Ohyama *et al.*, 2020). For the analysis, 500 ng desialylated and trypsin-digested IgA1 were loaded onto a C18 EASY-Spray column (75 μm × 15 cm, 2.1 μm, 100 Å; Thermo Fisher Scientific). Hybrid quadrupole mass filter/linear ion trap/orbitrap MS (Orbitrap Fusion, Thermo Fisher Scientific) was alternated between a full orbitrap MS scan (*m/z* 500–1,700) at a resolving power of 120 000, S-lens radio frequency of 60%, and subsequent MS/MS scan of the abundant precursor ions.

Data analysis for IgA1 HR *O*-glycoform profiling

All spectra were analyzed using the Xcalibur Qual Browser 2.2 (Thermo Fisher Scientific) software. Individual IgA1 *O*-glycopeptides were identified by referencing the theoretical monoisotopic mass list, which was created based on mass values of trypsin-digested IgA1 HR amino acid sequences using the GlycoMod tool (http://www.expasy.org) (Ohyama *et al.*, 2020; Renfrow et al., 2007; Takahashi et al., 2014; Takahashi et al., 2012; Takahashi et al., 2010). The ion chromatogram was extracted from five isotopic peaks of each glycopeptide ion, and the area under the curve (AUC) was obtained. To increase the throughput of the analysis, the in-house automated program Glycan Analyzer (MKI, Tokyo, Japan) was used for spectra identification and AUC acquisition of each glycopeptide. Relative abundance (RA, %) of each glycopeptide was obtained by dividing the AUC of each glycopeptide extracted ion chromatogram (XIC) by the total AUC for all glycopeptide XIC. The amounts of GalNAc and Gal per HR were calculated according to the following equation (Wada et al., 2010):

Amount of GalNAc (or Gal) = $\sum$\{glycopeptide relative abundance % × 10^{-2} × number of GalNAc (or Gal) in the glycopeptide\}

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis and graphing were performed using Statistical Package for the Social Sciences (SPSS) Statistics (version 22.0) and GraphPad Prism 9. Data for continuous variables are expressed as median (interquartile range). Comparison of two groups was performed using Student's *t*-test or Mann-Whitney test depending on whether the variables were distributed normally. For comparing the four groups, one-way analysis of variance was performed if the variables were distributed normally, followed by Dunnett's test for multiple comparisons against the reference group (J-HC). Kruskal-Wallis test, followed by Dunn's test for multiple comparisons against the reference group, was performed if the variables were not normally distributed. Categorical variables were expressed in percentages and compared using the χ^2 test. A multiple linear regression analysis was performed with Gd-IgA1, mean number of GalNAc residues per HR, relative abundance of IgA1 containing 3 *O*-glycans, and mean number of Gal residues per HR as dependent variable, and age, sex, race, and presence of IgAN as the independent variables for the cohort that included HCs and IgAN patients. Age, sex, race, MAP, eGFR, proteinuria, medication with antihypertensive agents, and Oxford classification score (C1, C2, E1 score) were included as the independent variables for the cohort including only IgAN patients. P values < 0.05 were considered statistically significant.

ADDITIONAL RESOURCES

No additional resources are available.

SUPPLEMENTAL INFORMATION TITLES AND LEGENDS

Table S1. Demographic and clinical characteristics of a sub-cohort used for analysis of the IgA1 HR *O*-glycoform in Japanese and Greek IgAN patients and control groups, related to Figure 2-4 and Table 4-7.

Table S2. Multiple linear regressions of mean number of GalNAc per HR on age, sex, race, MAP, eGFR, urinary protein, antihypertensive medication use, and E score (n = 59; consists of only patients with IgAN), related to Table 6.

Table S3. Multiple linear regressions of mean number of GalNAc per HR on age, sex, race, MAP, eGFR, urinary protein, antihypertensive medication use, and C score (n = 59; consists of only patients with IgAN), related to Table 6.

Table S4. Multiple linear regressions of relative abundance of 3GalNAc glycoform on age, sex, race, MAP, eGFR, urinary protein, antihypertensive medication use, and E score (n = 59; consists of only patients with IgAN), related to Table7.

Table S5. Multiple linear regressions of relative abundance of 3GalNAc glycoform on age, sex, race, MAP, eGFR, urinary protein, antihypertensive medication use, and C score (n = 59; consists of only patients with IgAN), related to Table 7.

Table S6. Multiple linear regressions of mean number of Gal per HR on age, sex, race, and presence of IgAN (n = 85), related to Table4.

Table S7. Multiple linear regressions of mean number of Gal per HR on age, sex, race, MAP, eGFR, urinary protein level, and antihypertensive medication use (n = 59; consists of only patients with IgAN), related to Table 6.

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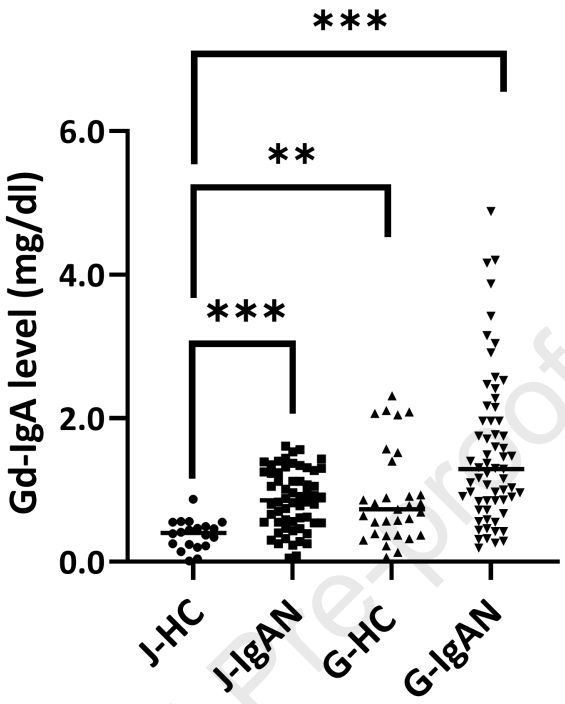

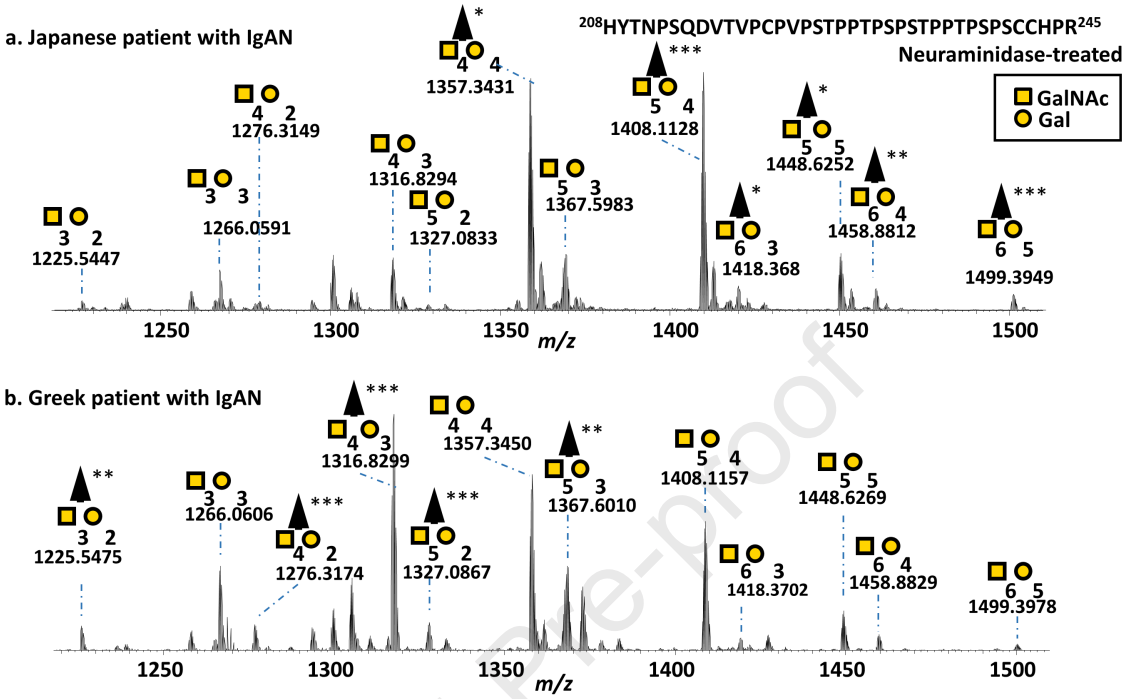

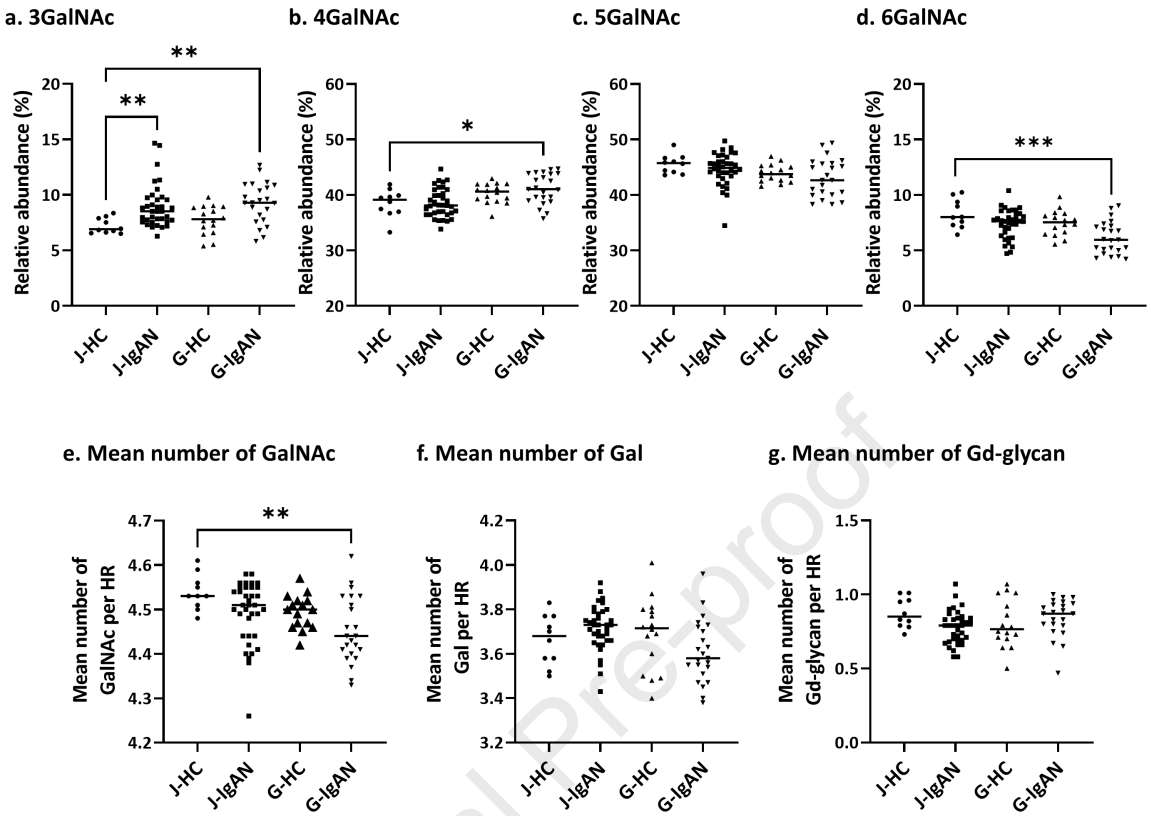

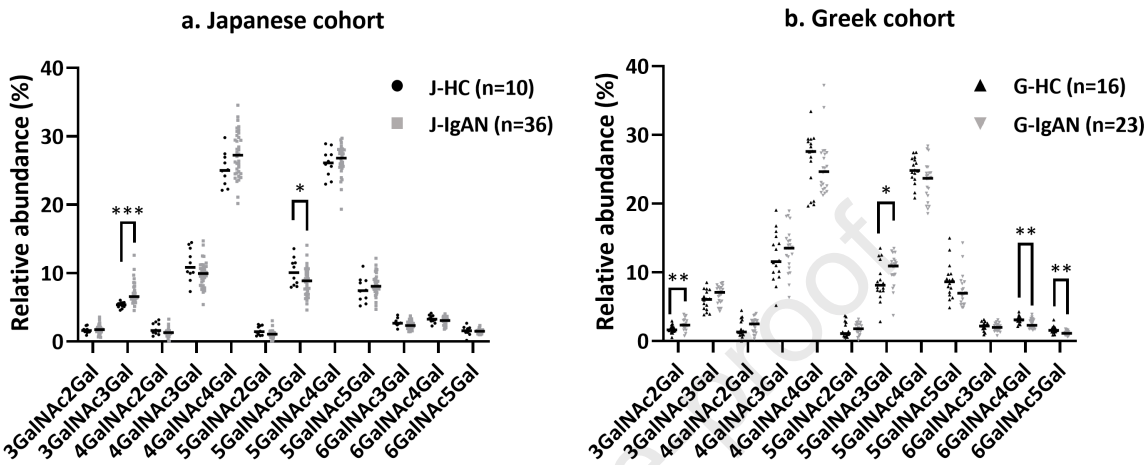

- Elevated serum Gd-IgA1 is more pronounced in Caucasians than in Asians
- Reduced number of IgA1 HR *O*-glycans is common in IgAN
- This feature is associated with reduced kidney function and high BP in IgAN
- Specific IgA1 *O*-glycoforms in IgAN will inform development of new biomarkers

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human IgA antibody	MP Bio	Cat#0855068, RRID:AB_2334295
Critical commercial assays		
Gd-IgA1 Assay Kit	IBL	Cat#27600
HiTrap NHS-activated HP Column	GE Healthcare	Cat#17071601
Neuraminidase	Prozyme	Cat#GK80040
DL-Dithiothreitol	Sigma	Cat#D5545
Trypsin	Promega	Cat#V528A
Deposited data		
Dataset of Greek and Japanese cohort	Mendeley	DOI: 10.17632/mm4d3rj7xk.1.
Software and algorithms		
Xcalibur Qual Browser 2.2	Ohyama et al., 2020	https://www.thermofisher.com/order/catalog/product/OPTON-30965
Glycan Analyzer	Ohyama et al., 2020	N/A