Glycosylation Profile of IgG in Moderate Kidney Dysfunction

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ABSTRACT

Glycans constitute the most abundant and diverse form of the post-translational modifications, and animal studies have suggested the involvement of IgG glycosylation in mechanisms of renal damage. Here, we explored the associations between IgG glycans and renal function in 3274 individuals from the TwinsUK registry. We analyzed the correlation between renal function measured as eGFR and 76 N-glycan traits using linear regressions adjusted for covariates and multiple testing in the larger population. We replicated our results in 31 monozygotic twin pairs discordant for renal function. Results from both analyses were then meta-analyzed. Fourteen glycan traits were associated with renal function in the discovery sample (P < 6.5 × 10⁻⁴) and remained significant after validation. Those glycan traits belong to three main glycosylation features: galactosylation, sialylation, and level of bisecting N-acetylglucosamine of the IgG glycans. These results show the role of IgG glycosylation in kidney function and provide novel insight into the pathophysiology of CKD and potential diagnostic and therapeutic targets.


Chronic kidney disease affects 13% of the adult population in developed countries and it is associated with increased cardiovascular morbidity and mortality.¹,² Though many genetic³–⁴ and environmental factors (such as diabetes, hypertension and ageing)⁵ are implicated in the development of kidney damage, its physiopathology is still not fully understood. Heritability estimates for CKD range between 0.33 and 0.41⁷,⁸ and despite the discovery of several important genetic associations, these loci collectively account for only 1.4% of the variation in eGFR.⁵ This suggests that epigenetic or post-transcriptional factors may be playing an important role in renal damage.

Glycosylation is the most abundant and diverse form of post-transcriptional modification and participates in every physiologic process.⁹ Immunoglobulin G is an excellent glycoprotein model as its glycosylation is well defined and many important functional effects of alternative IgG glycosylation have been described.¹⁰ N-glycans attached to the conserved asparagine 297 in the Fc part of IgG are important modulators of IgG effector functions.¹¹ For example, glycosylation acts as a switch between pro- and anti-inflammatory IgG functionality. Malfunction of this system is associated with different inflammatory and autoimmune
diseases such as SLE,12 rheumatoid arthritis, inflammatory bowel diseases,13,14 cancer15,16 and AIDS.17 Furthermore, it has been shown that inflammation pathways play a key role in endothelial and kidney damage.18,19 Indeed, the activation of inflammatory pathways and subsequent fibrosis are hallmark of renal injury.20,21 Different IgG glycosylation profiles may provide an at-risk phenotype to the development of renal damage.

Animal models highlighted the potential role of IgG glycosylation in the pathophysiologic mechanism involved in renal damage. Indeed studies have shown that modulation of ANCA IgG glycosylation reduces its pathogenicity in mouse ANCA-associated GN.21 Also, IgG Fcγ receptor deficiency was found to be renoprotective in a mouse model of diabetic nephropathy.20 Human studies suggest that aberrant glycosylation of the IgA1 is implicated in the deposit and formation of the immunocomplex IgA–IgG in patients with IgA nephropathy.22,23

However, no human studies investigated the role of the IgG glycosylation profiles in the onset of CKD.

The aim of this study is to investigate the potential role of IgG glycosylation in kidney function, by analyzing IgG glycome composition in a large population-based cohort from the UK. As glycans are associated with many factors including genes,24 we validate our significant results in an independent population of identical twins discordant for renal diseases.

RESULTS

Levels of 76 IgG glycans (24 directly measured and 52 derived traits) (Supplemental Figure 1) were obtained in 3274 individuals with different eGFR from the TwinsUK population (age range: 18–87 years). The demographic characteristics of the study populations are presented in Table 1. We identified 31 monozygotic (MZ) twin pairs discordant for the renal phenotype (difference in eGFR >15 mL/min per 1.73 m²).

We first ran the linear regressions in the discovery population adjusting for age, sex, body mass index (BMI), diabetes, hypertension, glycan analysis batch and family relatedness, excluding the MZ discordant twins. We controlled for multiple testing using Bonferroni correction (P<0.05/76 glycan traits). This identified 14 glycans significantly associated with eGFR; six glycans were positively associated with eGFR, while eight were negatively associated (Table 2, Supplemental Table 1). To ensure that sexual hormones did not affect our results, we ran the same linear regression analysis including menopause as a covariate and our results were unchanged.

We then assessed whether these associations with renal function were robust by testing an independent group of MZ twins discordant for renal disease. The regression coefficients were in the same direction in both analyses (discordant identical twins and the rest of the population). We then combined the results using inverse-variance fixed effect meta-analysis. All 14 glycans remained Bonferroni significant (Table 2). As depicted in Figure 1 and Table 2, the 14 significant glycan traits fell into three particular glycosylation features: galactosylation, sialylation and the level of bisecting N-acetylgalactosamine (GlcNAc) of the IgG glycans.

We observed a decrease in agalactosylated glycans: A2 (GP2 and GP2n) and FA2B (GP6 and GP6n) glycan structures and derived trait G0n, which combines all agalactosylated structures. Conversely, glycan with galactose on both antennae, FA2G2 (GP14 and GP14n), and the G2n derived trait, representing the percentage of digalactosylated structures in neutral IgG glycans, increased in parallel with the eGFR. The same pattern was observed in the MZ discordant pairs. As for sialylation, the major sialylated glycan, FA2G2S1 (GP18) and the percentage of sialylated structures without bisecting GlcNAc (represented by the ratio FGS/[F+FG+FGS]) increased with eGFR.

The level of bisecting GlcNAc in sialylated IgG glycans represented by three ratios, FBS1 total/FStotal, FB1/F1 and FBS1/F1 + FBS1, as well as in digalactosylated neutral gG glycans (FG2n/[BG2n+FBG2n]) were found to be inversely associated with eGFR.

To reinforce our findings we searched for associations in an independent population with more severe renal phenotype (eGFR < 30 mL/min per 1.73 m²). Eight twins, mean aged 65.0 (range 42.2–75.5 years) with CKD stage 4/5 (mean eGFR 24.7 [range 8.0–27.3]) were compared with their age-matched cotwin with eGFR > 30 ml/min per 1.73 m². As depicted in Figure 1, IgG glycans profiles follow the same patterns as were observed in the discovery population with the worsening of the renal function.

To determine whether the findings were restricted to IgG or to a more general change in glycosylation of multiple proteins, we searched for association between total plasma glycome25,26 and eGFR in a subset 426 individuals (eGFR, mL/min/1.73 m²: 78.95 ± 16.00). We found no difference in plasma glycosylation, suggesting that the effects we see here are likely direct effects of IgG glycosylation. However, the lack of association might also be due to power issues and so further study on larger sample size is needed to test this (Supplementary Table 2).

Table 1. General characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>Discovery Population</th>
<th>MZ Discordant Twins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size, n</td>
<td>3212</td>
<td>62</td>
</tr>
<tr>
<td>Age, years</td>
<td>52.67±14.15</td>
<td>55.45±12.2</td>
</tr>
<tr>
<td>MZ: DZ: singletons</td>
<td>506/172/934</td>
<td>62/0:0</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>3050 (94.9)</td>
<td>60 (96.7)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.95±4.65</td>
<td>25.64±5.65</td>
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<tr>
<td>Creatinine, mg/ml</td>
<td>0.83±0.15</td>
<td>0.75±0.10</td>
</tr>
<tr>
<td>eGFR, mL/min per 1.73 m²</td>
<td>84.15±17.02</td>
<td>88.52±9.91</td>
</tr>
<tr>
<td>CKD (eGFR&lt;60), n (%)</td>
<td>294 (94.15)</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>Type II diabetes, n (%)</td>
<td>72 (2.2)</td>
<td>4 (6.4)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>705 (21.9)</td>
<td>18 (29.0)</td>
</tr>
</tbody>
</table>

CKD eGFR estimated using Chronic Kidney Disease Epidemiology Collaboration equation. Values for categorical variables are given as n (%); values for continuous variable as mean (±SD). MZ: DZ, monozygotic: dizygotic.
Table 2. Glycan traits significantly associated with eGFR in the discovery, validation, and meta-analysis

<table>
<thead>
<tr>
<th>Glycan</th>
<th>Description</th>
<th>$h^2$</th>
<th>Discovery $\beta$ (95% CI)</th>
<th>P value</th>
<th>MZ Discordant $\beta$ (95% CI)</th>
<th>P value</th>
<th>Fixed effect meta-analysis $\beta$ (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP18</td>
<td>The percentage of FA2G2S1 glycan in total IgG glycans</td>
<td>0.73</td>
<td>1.48 (0.89 to 2.07)</td>
<td>$8.60 \times 10^{-7}$</td>
<td>0.59 (–2.23 to 3.41)</td>
<td>$4.23 (2.38 to 7.52)$</td>
<td>$9.51 \times 10^{-7}$</td>
<td></td>
</tr>
<tr>
<td>GP14</td>
<td>The percentage of FA2G2 glycan in total IgG glycans</td>
<td>0.36</td>
<td>1.46 (0.85 to 2.07)</td>
<td>$2.92 \times 10^{-6}$</td>
<td>1.33 (–1.81 to 4.48)</td>
<td>$4.29 (2.35 to 7.81)$</td>
<td>$2.04 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>GP6</td>
<td>The percentage of FA2B glycan in total neutral IgG glycans (GP&quot;)</td>
<td>0.75</td>
<td>–1.39 (–1.98 to –0.80)</td>
<td>$3.56 \times 10^{-6}$</td>
<td>–0.84 (–3.44 to 1.76)</td>
<td>$0.26 (0.14 to 0.45)$</td>
<td>$3.16 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>GP14&quot;</td>
<td>The percentage of FA2G2 glycan in total neutral IgG glycans (GP&quot;)</td>
<td>0.47</td>
<td>1.29 (0.68 to 1.90)</td>
<td>$3.06 \times 10^{-5}$</td>
<td>1.99 (–1.70 to 5.67)</td>
<td>$3.70 (2.03 to 7.63)$</td>
<td>$1.82 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>FBS1/FS1</td>
<td>Ratio of fucosylated monosialylated structures with and without bisecting GlcNAc</td>
<td>0.39</td>
<td>–1.12 (–1.65 to –0.59)</td>
<td>$3.43 \times 10^{-5}$</td>
<td>–0.58 (–3.16 to 1.99)</td>
<td>$0.30 (0.20 to 0.56)$</td>
<td>$3.42 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>FBS1/(FS1+FBS1)</td>
<td>The incidence of bisecting GlcNAc in all fucosylated monosialylated structures in total IgG glycans</td>
<td>0.42</td>
<td>–1.10 (–1.63 to –0.57)</td>
<td>$4.33 \times 10^{-5}$</td>
<td>–0.60 (–3.14 to 1.95)</td>
<td>$0.30 (0.20 to 0.57)$</td>
<td>$4.40 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>G2&quot;</td>
<td>The percentage of digalactosylated structures in total neutral IgG glycans</td>
<td>0.41</td>
<td>1.20 (0.60 to 1.80)</td>
<td>$8.81 \times 10^{-5}$</td>
<td>1.98 (–1.83 to 5.78)</td>
<td>$3.38 (1.87 to 6.10)$</td>
<td>$5.53 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>GP6&quot;</td>
<td>The percentage of FA2B glycan in total IgG glycans</td>
<td>0.75</td>
<td>–1.14 (–1.71 to –0.57)</td>
<td>$8.90 \times 10^{-5}$</td>
<td>–1.01 (–3.78 to 1.76)</td>
<td>$0.32 (0.18 to 0.56)$</td>
<td>$6.84 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>FBS\text{total} / FS\text{total}</td>
<td>Ratio of all fucosylated sialylated structures with and without bisecting GlcNAc</td>
<td>0.23</td>
<td>–1.07 (–1.60 to –0.54)</td>
<td>$8.23 \times 10^{-5}$</td>
<td>–0.30 (–2.84 to 2.23)</td>
<td>$0.36 (0.21 to 0.60)$</td>
<td>$9.52 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>G0&quot;</td>
<td>The percentage of agalactosylated structures in total neutral IgG glycans</td>
<td>0.72</td>
<td>–1.16 (–1.76 to –0.56)</td>
<td>$1.52 \times 10^{-4}$</td>
<td>–1.20 (–4.71 to 2.31)</td>
<td>$0.31 (0.17 to 0.57)$</td>
<td>$1.20 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>GP2&quot;</td>
<td>The percentage of A2 glycan in total neutral IgG glycans (GP&quot;)</td>
<td>0.71</td>
<td>–0.91 (–1.42 to –0.40)</td>
<td>$5.02 \times 10^{-4}$</td>
<td>–2.00 (–4.66 to 0.67)</td>
<td>$0.39 (0.23 to 0.64)$</td>
<td>$2.20 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>GP2</td>
<td>The percentage of A2 glycan in total IgG glycans</td>
<td>0.72</td>
<td>–0.90 (–1.42 to –0.38)</td>
<td>$6.28 \times 10^{-4}$</td>
<td>–2.33 (–5.13 to 0.47)</td>
<td>$0.39 (0.23 to 0.64)$</td>
<td>$2.55 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>FGS/FS + FG + FG2S</td>
<td>The percentage of sialylation of all fucosylated structures without bisecting GlcNAc in total IgG glycans</td>
<td>0.69</td>
<td>1.01 (0.46 to 1.56)</td>
<td>$2.96 \times 10^{-4}$</td>
<td>0.57 (–2.21 to 3.35)</td>
<td>$2.71 (1.58 to 4.64)$</td>
<td>$2.85 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>FG2&quot;/(BG2&quot; + FBG2&quot;)</td>
<td>Ratio of fucosylated digalactosylated nonbisecting GlcNAc structures and all digalactosylated structures with bisecting GlcNAc</td>
<td>0.66</td>
<td>0.91 (0.38 to 1.44)</td>
<td>$7.32 \times 10^{-4}$</td>
<td>0.93 (–1.59 to 3.44)</td>
<td>$2.49 (1.48 to 4.19)$</td>
<td>$5.54 \times 10^{-4}$</td>
<td></td>
</tr>
</tbody>
</table>

*Estimates of heritability ($h^2$) come from Menni et al. Plos One 2013.24
Finally, we assessed whether glycan profiles could improve the prediction of the CKD status (as per Guidelines, CKD cases have eGFR \( \leq 60 \text{ mL/min per } 1.73 \text{ m}^2 \)) beyond that achieved with age and sex. In the discriminative model only the four main glycans (GP2, GP6, GP14, and GP18) were included. The predictive ability for CKD status, as measured by the area under the curve was 0.87 (95% confidence interval [95% CI], 0.85 to 0.89) for clinical parameters alone, 0.81 (95% CI, 0.78 to 0.84) for glycans alone, and 0.88 (95% CI, 0.86 to 0.90) for the model incorporating a combination of glycans and clinical parameters (\( P = 0.23 \)) (Supplemental Figure 2).

DISCUSSION

This is the first study to investigate the potential role of IgG glycosylation in kidney function. We identified 14 IgG glycan traits with high statistical significance associated with eGFR and validated them in an independent subset of MZ twins discordant for renal disease. Moreover we see the same pattern in a small independent sample with a more extreme renal dysfunction.

The glycans identified fall into three principal glycan traits.

Galactosylation of IgG

Decreased IgG galactosylation has been found to be associated with rheumatoid arthritis\(^{27}\) as well as with several autoimmune and inflammatory diseases\(^{16}\) and with chronologic and biologic age.\(^{28}\) The decrease in galactosylation is not disease-specific, but a general phenomenon that is associated with decreased immunosuppressive and anti-inflammatory potential of circulating IgG. We observed a higher risk of CKD in subjects with agalactosylated glycans (GP2, GP6, and G0n) and lower in those with galactosylated IgG (GP14 and G2n). Lack of terminal galactose activates complement cascade and makes IgG pro-inflammatory, whereas the addition of galactose decreases its inflammatory potential.\(^{29,30}\) Hence, the IgG galactosylation pattern observed in our population supports the theory that complement activation/dysregulation is crucial in renal damage.\(^{31}\) It is not clear whether IgG galactosylation is a consequence or an individual predisposition for a disease. The heritability of galactosylated glycans was very high,\(^{24}\) indicating that galactosylation could partly be genetically predetermined. This hypothesis is further supported by the fact that in rheumatoid arthritis, the decrease in IgG galactosylation was observed up to several years before the onset of the disease.\(^ {32-35}\)

Sialylation

Further extension of IgG glycans by the addition of sialic acid dramatically changes the physiologic role of IgG, converting it from a proinflammatory into an anti-inflammatory agent.\(^ {36,37}\) This relatively small fraction of sialylated IgG is believed to be responsible for the immunosuppressive activity of intravenously administered immunoglobulins.\(^ {38}\) Approximately 50% of IgG glycans are not sialylated and are proinflammatory.\(^ {39}\) However, the terminal α2,6-sialylation of IgG glycans decreases the ability of IgG to bind Fcy receptors (FcyRs), which increases expression of inhibitory FcyRIIB and is anti-inflammatory.\(^ {40}\) Contrary to changes in galactosylation, the significant changes in sialylation have not been associated

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Figure 1. Correlation of IgG glycosylation and eGFR in the discovery and MZ discordant populations. (A) Directly measured glycan structures. (B) Derived traits that measure sialylation, galactosylation, and bisecting GlcNAc.
with other diseases. Recently, some of us found that major sialylated glycans (GP16, GP18, and GP23) were significantly decreased in patients with SLE (F. Vučković et al., submitted for publication). In our population, the major sialylated glycan, FA2G2S1 (GP18), and the ratio FGS/(F+FG+FGS), which represents the percentage of sialylated structures without bisecting GlcNAc in total IgG glycans, were decreased in patients with CKD (green dots in Figure 1). These sialylated glycan traits displayed a protective independent risk for CKD.

Bisecting N-Acetylglicosamine and Core Fucosylation of IgG

Another feature is the role of core fucose in the modulation of antibody-dependent cellular cytotoxicity. On average, 95% of the IgG population is core fucosylated; hence, most of the immunoglobulins have a "safety switch", which prevents them from antibody-dependent cellular cytotoxicity. IgG-containing glycans that lack core fucose have 100-fold higher affinity to the FcγRIIIa and are therefore much more efficient than fucosylated glycoforms. We have observed a significant and independent decreased risk of CKD when sialylated and core fucosylated glycans did not have bisecting GlcNAc; and in contrast, lower eGFR if those glycans contained bisecting GlcNAc (FBStotal/FStotal, FBS1/FS1, and FBS1/[FS1+FBS1]). Also for neutral digalactosylated glycans, when there is less of these glycans with bisecting GlcNAc, the ratio FG2n/(BG2n+FBG2n) is higher and this is positively associated with eGFR. The presence of bisecting GlcNAc was always associated with a higher risk of CKD.

It is not clear how the modulation of antibody-dependent cellular cytotoxicity could affect the renal damage in the onset of a nonautoimmune CKD. Studies in experimental animals have reported that modifications in the Fcγ receptor can diminish renal damage in a well known autoimmune disease, ANCA-related GN, as well as in diabetic nephropathy. On the other hand, renal fibrosis is the common pathway of many kidney diseases and leads to progressive renal failure; natural killer cells have been linked with this process in different organ systems.

Notably, glycan traits associated with lower eGFR have on average a higher heritability (Table 2). For example, the agalactosylated IgG glycans we found associated with lower eGFR, have a high heritability, ranging from 0.72 to 0.75, whereas galactosylated glycans GP14 and G2n derived trait have a low heritability (0.36 and 0.41, respectively). The highly heritable glycans associated with eGFR, have been previously associated with different genes. However, there is as yet no overlap with genes previously reported in CKD genome-wide association studies. Our findings may indicate a new approach to deeper understanding of the contribution of genetics in IgG glycosylation and kidney damage.

Although the identified glycans do not predict incident CKD (defined as eGFR<60 mL/min per 1.73 m²) more accurately than clinical parameter, their inclusion in the models improves the incident CKD risk prediction. These glycans may be more sensitive to earlier stages of reduced renal function, as the eGFR-defined onset of CKD occurs only after half of the
kidneys’ filtration ability has been lost. Longitudinal studies could help to address this hypothesis.

The present study has several strengths. First, we employed a two-stage design (discovery and independent replication with stringent P values), so minimizing the risk of false positive findings. Second, we used identical twins discordant for renal function in the validation analysis. Glycan levels may be influenced by many factors including genetics, age and environment.\(^2\) As identical twins share 100% of their genetic makeup, and are matched perfectly for age, gender, social class, etc., we were able to validate the role of IgG on renal function; isolating the nongenetic contribution. These data help us to understand the complex interplay between genetic and nongenetic influences that determine renal function.

We note some study limitations. First, there is a female predominance in our study sample (95% of the individuals are, for historical reasons, women). Second, our population being volunteers is slightly healthier than average with a lower rate of diabetes and results might not be generalizable to more severe diabetes populations. Third, the cross-sectional nature of our data does not allow us to draw conclusions as to whether the glycans identified are causative of kidney function decline or merely correlated with it. Finally, we cannot provide reliable estimates as to what proportions of the identified glycans were from Fc and from Fab, respectively. However, in a small pilot of Fc-glycopeptides by nano-liquid chromatography tandem mass spectrometry\(^3\) on 96 representative age-matched individuals from the extremes of the eGFR distribution, we find the same direction of effect with renal function for all but one. This suggests that our initial observations mostly come from the Fc glycans (Supplemental Table 3).

Our results highlight the promising role of glycomics in renal studies. Uncovering this relationship by extending the research with clinical subsets and longitudinal data would help to identify further novel markers that would be potentially useful to detect at-risk patients, in the early stages of CKD. These results open new avenues to our understanding of renal damage and encourage further studies in populations with more severe CKD and proteinuria information, as well as studies comparing patients with autoimmune CKD with patients whose CKD is due to other etiologies. Moreover, this would help to gain additional insights into the pathophysiology of CKD and potential therapeutic targets.

**CONCISE METHODS**

**Study Subjects**

Study subjects were twins enrolled in the TwinsUK registry, a national register of adult twins. Twins were recruited as volunteers by successive media campaigns without selecting for particular diseases or traits.\(^4\) In this study we analyzed data from 3274 individuals with glycomics and creatinine data available. The study was approved by St. Thomas’ Hospital Research Ethics Committee, and all twins provided informed written consent.

**Phenotype Definitions**

Data relevant to the present study included BMI (body weight in kilograms divided by the square of height in square meters), type II diabetes (defined if fasting glucose $\geq 7$ mmol/L or physician’s letter confirming diagnosis) and hypertension. Renal parameters; eGFR was calculated from standard creatinine using the Chronic Kidney Disease Epidemiology Collaboration equation.\(^5\) CKD was defined as an eGFR $<60$ ml/min per 1.73 m$^2$ according to the current Kidney Disease Outcome Quality Initiative (K/DOQI) guidelines.\(^6\) MZ pairs were considered discordant for renal function if one twin had an eGFR $\geq 90$ and the other had eGFR $\leq 90$ mL/min per 1.73 m$^2$ and the difference between their eGFR levels was $>15$ ml/min per 1.73 m$^2$.

**Analysis of IgG Glycans**

**Isolation of IgG from Human Plasma**

The IgG was isolated using protein G monolithic plates (BIA Separations, Ajdovščina, Slovenia) as described previously.\(^2\)

**Glycan Release and Labeling**

Glycan release and labeling were performed essentially as previously described.\(^2,4,2\) Briefly, dried IgG was denatured with 2% SDS (wt/vol) and N-glycans were released by digestion with PNGase F (ProZyme, Hayward, CA). After deglycosylation, N-glycans were labeled with 2-AB fluorescent dye. Free label and reducing agent were removed from the samples using hydrophilic interaction chromatography–solid-phase extraction.

**Hydrophilic Interaction Chromatography-UPLC**

Fluorescently labeled N-glycans were separated by hydrophilic interaction chromatography on a Waters Acquity UPLC instrument (Waters, Milford, MA) as described previously.\(^2\) Data processing was performed using an automatic processing method with a traditional integration algorithm after which each chromatogram was manually corrected to maintain the same intervals of integration for all the samples. The chromatograms were all separated in the same manner into 24 peaks and the amount of glycans in each peak was expressed as a percentage of the total integrated area. In addition to 24 directly measured glycan structures, 52 derived traits were calculated. These derived traits average particular glycosylation features (galactosylation, fucosylation, bisecting GlcNAc, and sialylation) (Supplemental Figure 1, Table 1).

**Statistical Analysis**

Statistical analysis was carried out using Stata version 12 and R (version 3.1.2) and visualized using the ggplot2 package.

Glycans were globally normalized and log transformed using the right-skewness of their distributions. To remove experimental biases, all measurements were adjusted for batch and run-day effects using ComBat (R-package sva). Derived glycan traits were calculated using normalized and batch-corrected glycan measurements (exponential of batch corrected measurements). All variables were centered and scaled to have mean 0 and standard deviation 1. Outliers (more than 6SD from the mean) were excluded from the analysis.

Association analyses between eGFR and glycan traits were performed using random intercept linear regressions adjusting for age, sex, BMI, diabetes, hypertension, and family relatedness as
random effect. We used a conservative Bonferroni correction to account for multiple testing assuming 76 independent tests as suggested by Pucic et al.,\textsuperscript{42} so giving a significant threshold of ($P<6.5\times10^{-5}$; 0.05/76). The Bonferroni-significant eGFR glycan associations were replicated in the previously excluded group of MZ discordant twins using the same model. Paired $t$-tests were used to evaluate the association with incident CKD in an independent subset of twins where one co-twin had a significant decline in renal function.

To assess how glycans can improve the prediction of CKD (eGFR<60 ml/min per 1.73 m$^2$), three Least Absolute Shrinkage and Selection Operator regression models were created (R package glmnet): The first one using only clinical parameters; age, sex, type II diabetes, and hypertension, to predict CKD; the second using the set of original glycan traits, which were found to be Bonferroni significant before (GP2, GP6, GP14, GP18), and the last one using both glycans and clinical parameters. The quality of all three models was assessed using a ten-fold cross-validation. The regularization parameter $\lambda$ was trained separately for each fold using a nested cross-validation. Receiver operating characteristic curves (and particularly the area under the curves) were calculated for each fold and averages and confidence intervals were reported.

ACKNOWLEDGMENTS

TwinsUK was funded by the Wellcome Trust, European Community’s Seventh Framework Programme (FP7/2007–2013). The study also receives support from the National Institute for Health Research (NIHR) Clinical Research Facility at Guy’s & St. Thomas’ NHS Foundation Trust and NIHR Biomedical Research Centre based at Guy’s and St. Thomas’ NHS Foundation Trust and King’s College London.

Tim Spector is holder of an ERC Advanced Principal Investigator award.

Clara Barrios is supported by a grant from the Spanish Society of Nephrology and RedinRen RD12/0021/0024.

Julio Pascual, María José Soler, and Eva Rodríguez are supported by grants from Institute Carlos III and RedinRen.

Glycan analysis was partly funded by European Commission FP7 projects IBD-BIOM (contract #305479), HighGlycan (contract #278353), MIMOmics (contract #305280), HTTP-GlycoMet (contract #324400), and IntegrAlife (contract #315997) grants.

DISCLOSURES

Gordan Lauc is founder and owner of Genos, a private research organization that specializes in high-throughput glycomic analysis and has several patents in this field.

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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2015010109/-/DCSupplemental.