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# N-glycosylation of immunoglobulin G predicts incident hypertension

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**Objectives:** Glycosylation of immunoglobulin G (IgG) is an important regulator of the immune system and has been implicated in prevalent hypertension.The aim of this study is to investigate whether the IgG glycome begins to change prior to hypertension diagnosis by analysing the IgG glycome composition in a large population-based female cohort with two independent replication samples.

**Methods:** We included 989 unrelated cases with incident hypertension and 1628 controls from the TwinsUK cohort (mean follow-up time of 6.3 years) with IgG measured at baseline by ultra-performance liquid chromatography and longitudinal BP measurement available. We replicated our findings in 106 individuals from the 10 001 Dalmatians and 729 from KORA S4. Cox regression mixed models were applied to identify changes in glycan traits preincident hypertension, after adjusting for age, mean arterial pressure, BMI, family relatedness and multiple testing (FDR $< 0.1$). Significant IgG-incident hypertension associations were replicated in the two independent cohorts by leveraging Cox regression mixed models in the 10 001 Dalmatians and logistic regression models in the KORA cohort.

**Results:** We identified and replicated four glycan traits, incidence of bisecting GlcNAc, GP4, GP9 and GP21, that are predictive of incident hypertension after adjusting for confoundes and multiple testing [hazard ratio (95% CI) ranging from 0.45 (0.24–0.84) for GP21 to 2.9 (1.5–5.68) for GP4]. We then linearly combined the four replicated glycans and found that the glycan score correlated with incident hypertension, SBP and DBP.

**Conclusion:** Our results suggest that the IgG glycome changes prior to the development of hypertension.

**Keywords:** basic science research, biomarkers, glycomics, incident hypertension, risk factors

**Abbreviations:** BP, blood pressure; CVD, cardiovascular diseases; IgG, immunoglobulin G; MAP, mean arterial pressure

## INTRODUCTION

Hypertension is the most prevalent modifiable risk factor for cardiovascular morbidity and mortality worldwide [1]. The risk factors for hypertension are multifactorial and include both genetic predisposition and environmental or lifestyle factors, such as salt intake, diet, alcohol use and sedentary behaviour [1,2].

N-glycans are complex carbohydrate structures added to the protein backbone via the process of N-glycosylation and are one of the main contributors to protein structural and functional properties [3]. Most of the known proteome contains glycans [4], which are highly responsive to both environmental and genetic stimuli and frequently change in response to various pathophysiological conditions, including cardiovascular diseases (CVDs). We recently reported that certain plasma N-glycan traits can be leveraged to improve the accuracy of established CVD risk prediction models, thereby suggesting that N-glycans are sensitive to processes involved in early disease development [5]. Animal and cross-sectional human studies also suggest that alterations of the immunoglobulin G (IgG) N-glycome, which is known to influence inflammatory response [6], may be involved in both hypertension and blood pressure (BP) regulation [7], subclinical atherosclerosis and atherosclerosis risk factors [8]. In our recent article measuring 76 IgG glycan traits as well as GlycA in two independent cohorts in the UK [8], we described the association of four IgG traits, in addition to the previously

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reported GlycA, with measures of atherosclerosis. In the same study, we also presented IgG N-glycans' incremental predicitive value when combined with the ACC/AHA risk score [8]. These findings suggest that changes to the IgG glycome could be a novel biomarker to predict incident hypertension.

Here, we aimed to investigate changes in IgG glycans and incident hypertension by analysing the IgG glycome composition in a large population-based female cohort from TwinsUK with longitudinal glycomics and BP data. We then replicated the glycan-associated traits in two independent samples from the 10 001 Dalmatians study and KORA S4 cohort.

## MATERIALS AND METHODS

A flowchart of the study design is presented in Fig. 1.

### Study population

#### Discovery cohort

Study participants were individuals enrolled in the TwinsUK registry, a national register of adult twins recruited as volunteers without selecting for any particular disease or traits [9]. Here, we analysed data from 2617 women (989 incident hypertension cases, 1628 controls) (mean follow-up time of 6.3 years). Data relevant to the present study include BP (at baseline and follow-up), antihypertensive drug use, BMI, mean arterial pressure (MAP), age and IgG glycans assessed using HILIC-UPLC-FLR, as described in the following section.

#### Replication cohorts

We replicated our results in two independent samples, the 10 001 Dalmatians study [10] and the KORA cohort [11].

*10 001 Dalmatians*: We included 106 individuals from the '10 001' Dalmatians study, a study designed to investigate the health of the isolated island communities, including the Croatian islands of Vis, Korčula and Split [10]. Included individuals had all relevant measures, including age, sex, BMI and IgG glycans quantified by HILIC-UPLC-FLR, with an average follow-up time of 5.4 years.

*KORA*: We further replicated in 729 individuals with relevant measures and IgG glycans measured using LC-ESI-MS/MS analysis from KORA F4, which is the first follow-up study of the population-based KORA S4 study (Cooperative Health Research in the Region of Augsburg) [11]. The covariates included were age, sex, BMI and MAP at the time

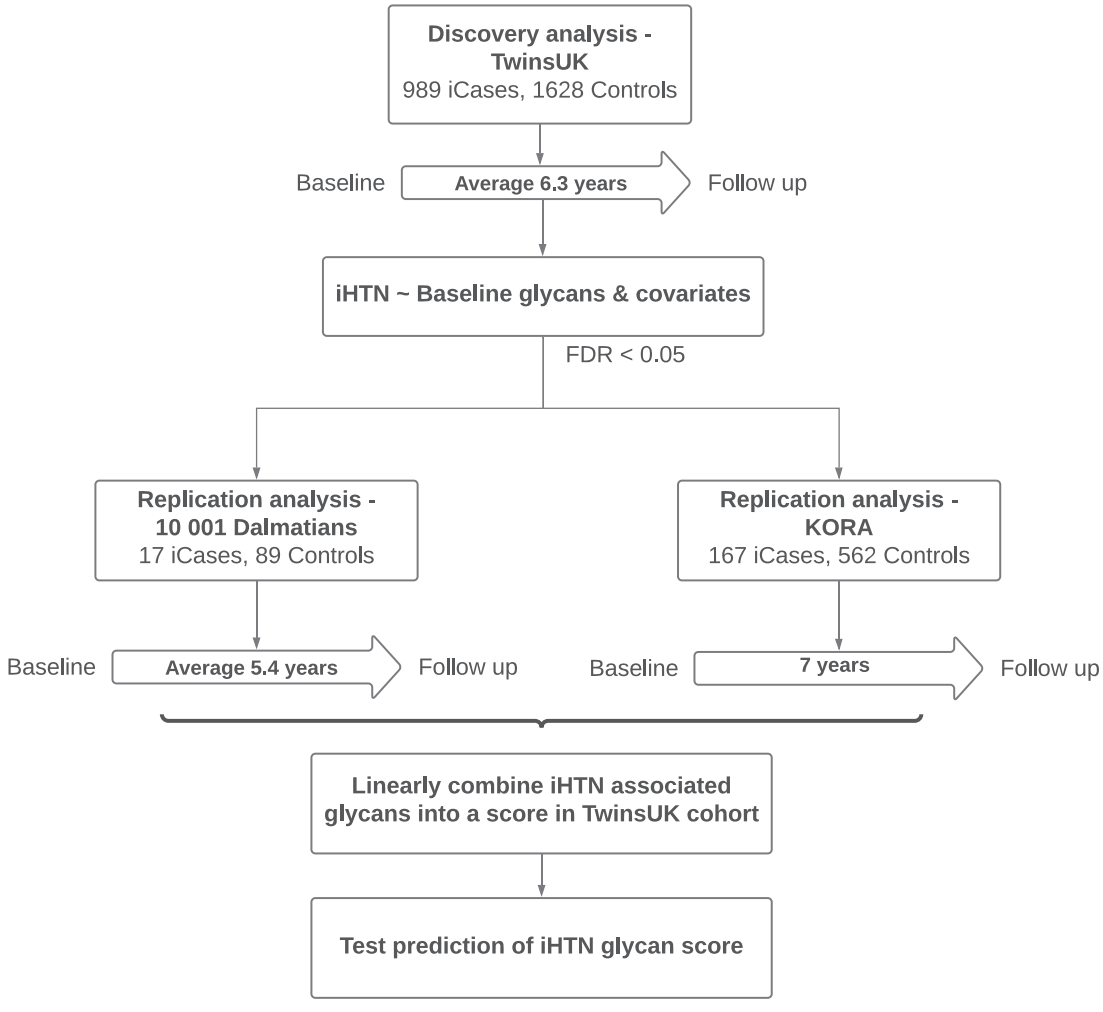


**FIGURE 1** Flow chart of study design. iCases, incident hypertension cases.

of KORA F4. BP is measured in KORA FF4, which is the second follow-up study of KORA S4 carried out 7 years after KORA F4.

## Phenotype definitions

BP was measured by a trained nurse using the Marshall mb02, the Omron Mx3 or the Omron HEM713C Digital Blood Pressure Monitor (Omron Healthcare, Hoofddorp, The Netherlands) (coefficient of variation = 8.4%). Measurements were performed with the patient in the sitting position for three minutes. At each visit, the cuff was placed on the individual's arm so that it was approximately 2–3 cm above the elbow joint of the inner arm. Measurements were carried out with the individual's arm resting on a table ensuring that the cuff was placed at the same level of the heart. Three measurements were taken with an interval of approximately 1 min between each reading, with the mean of the second and third measurements recorded to mitigate white-coat effect.

In the three cohorts, BP was measured at baseline and longitudinally [average follow-up time 6.3 (± 3.7) years in TwinsUK]. Individuals were classified as hypertensive cases based on their BP level (SBP >140 mmHg OR DBP >90 mmHg), use of BP-lowering drugs or a recorded diagnosis of hypertension by the doctor. On the basis of the above definition, hypertension was defined as prevalent or incident and all individuals with prevalent hypertension at baseline excluded from the analysis.

## Ethical statement

TwinsUK, 10 001 Dalmatians and KORA cohorts were designed following the Declaration of Helsinki and were given approval by their local ethical committees. For TwinsUK, volunteers provided informed written consent and the study was approved by St. Thomas' Hospital Research Ethics Committee (REC Ref: EC04/015). For KORA, all study participants provided written informed consent and approval was granted from the Bavarian Medical association Ethics committee (Bayerische Landesärzte-kammer) and the Bavarian commissioner for data protection and privacy (Bayerischer Datenschutzbeauftragter). The 10 001 Dalmatians study received ethical approval from the ethics committee of the Sisters of Mercy University Hospital in Zagreb and all participants provided informed consent.

## Analysis of the IgG N-glycoprofile

IgG Isolation was performed on protein G monolithic 96-well plates as previously described [12]. Detailed description of the isolation protocol is available in the Supplementary Material (Supplementary Material: EXPERIMENTAL PROTOCOL DESCRIPTION, http://links.lww.com/HJH/B733).

### IgG N-glycoprofiling of the TwinsUK cohort

Deglycosylation, RapiFluor-MS labelling and purification step of IgG N-glycans was performed using GlycoWorks RapiFluor-MS N-Glycan Kit obtained from Waters Corporation (Milford, Massachusetts, USA), in compliance with the manufacturer's protocol [13]. All samples containing eluted and labelled glycans were stored at −20°C until further use. RapiFluor-MS labelled IgG N-glycans were analysed using HILIC-UPLC-FLR on Water Acquity UPLC H-class instruments. Detailed description of the analysis is available in the Supplementary Material (Supplementary Material: EXPERIMENTAL PROTOCOL DESCRIPTION, http://links.lww.com/HJH/B733).

### IgG N-glycoprofiling of the 10 001 Dalmatians cohort

Deglycosylation, 2-AB (Merck, Germany) labelling and purification step of IgG N-glycans was performed following our standardized protocol as described in detail by Jurić *et al*. [14]. At the end of the protocol, the samples containing eluted and labelled glycans were stored at -20°C until further use. 2-AB labelled IgG N-glycans were analysed using HILIC-UPLC-FLR on Water Acquity UPLC H-class instruments. Detailed description of the analysis is available in the Supplementary Material (Supplementary Material: EXPERIMENTAL PROTOCOL DESCRIPTION, http://links.lww.com/HJH/B733).

### IgG N-glycoprofiling of the KORA cohort

Digestion of IgG to tryptic glycopeptides and their following purification was performed as described previously [15]. Purified tryptic IgG glycopeptides-containing eluates were dried in a vacuum centrifuge and then dissolved with a volume of 20 μl of ultrapure water. Detailed description of LC-ESI-MS/MS analysis of the IgG glycopeptides from the KORA cohort is available in the Supplementary Material (Supplementary Material: EXPERIMENTAL PROTOCOL DESCRIPTION, http://links.lww.com/HJH/B733).

The chromatograms were all separated in the same manner into peaks (Supplementary Figure 1, http://links.lww.com/HJH/B733) and the amount of glycans in each peak was expressed as percentage of total integrated area (Supplementary Table 1, http://links.lww.com/HJH/B733). In addition to directly measured glycan structures, derived traits were calculated as described in Supplementary Table 2, http://links.lww.com/HJH/B733. These derived traits average particular glycosylation features such as, galactosylation, fucosylation and sialylation, across different individual glycan structures. Consequently, they are more closely related to individual enzymatic activities and underlying genetic polymorphisms [16,17].

## Statistical analysis

Statistical analysis was performed using R version 3.6.3 [18]. Glycans were global normalised and log transformed because of a right-skewed distribution. To adjust for technical biases, all measurements were adjusted for batch and run-day effects using the R-package sva [19]. Derived glycan traits were calculated using normalized and batch-corrected glycan measurements (exponential of batch-corrected measurements). Glycan variables were transformed to standard normal distribution by inversion transformation of ranks (R package 'GenABEL') [20].

In the discovery cohort, Cox regression mixed models (R package 'coxme' [21]) were used to identify changes in glycans before incident hypertension, with an average follow-up time of 6.3 (±3.7) years, after adjusting for age, MAP, BMI, family relatedness and multiple testing (FDR < 0.1) (Fig. 1).

Cox regression mixed models were further employed to replicate the hypertension-associated glycans in the 10 001

**TABLE 1. Descriptive characteristics of the study population**

| Cohort | TwinsUK | | | 10 001 Dalmatians | | | KORA | | |
|---|---|---|---|---|---|---|---|---|---|
| Timepoint | Baseline | Follow up | | Baseline | Follow up | | Baseline | Follow up | |
| Hypertension status | All | Controls | iCases | All | Controls | iCases | All | Controls | iCases |
| *N* | 2617 | 1628 | 989 | 106 | 89 | 17 | 729 | 562 | 167 |
| Females, *N* (%) | 2617 (100%) | 1628 (100%) | 989 (100%) | 72 (68%) | 63 (71%) | 9 (53%) | 415 (57%) | 330 (59%) | 85 (51%) |
| Age (years) Mean (SD) | 54 (12) | 58 (11) | 63 (9) | 57 (11) | 62 (12) | 62 (7) | 58 (8) | 64 (8) | 67 (8) |
| BMI (kg/m$^2$) Mean (SD) | 25.7 (4.5) | 25.3 (4.4) | 27.4 (5.2) | 27.7 (3.8) | *NA* | *NA* | 26.7 (4.0) | 26.7 (4.1) | 28.5 (5.1) |
| SBP (mmHg) Mean (SD) | 122 (14) | 119 (11) | 137 (13) | 132 (17) | 127 (10) | 152 (7) | 117 (13) | 114 (13) | 125 (19) |
| DBP (mmHg) Mean (SD) | 76 (9) | 72 (8) | 81 (10) | 79 (8) | 78 (6) | 85 (10) | 74 (7) | 71 (7) | 75 (11) |
| MAP (mmHg) Mean (SD) | 91 (10) | 88 (8) | 100 (9) | 97 (10) | 94 (6) | 107 (8) | 88 (9) | 86 (8) | 91 (13) |

iCases, incident hypertension cases; MAP, mean arterial pressure.

Dalmatians cohort. In the KORA cohort, all study participants had the same follow-up time, hence we used logistic regressions to replicate the associated glycans. IgG glycans were considered replicated if *P* value was less than 0.05 and direction of effects were consistent (Fig. 1).

To assess the combined effects of the incident-hypertension associated glycans on BP phenotypes, we generated a score by linearly combining the transformed glycan traits from the TwinsUK cohort alongside covartiates (Fig. 1).

## RESULTS

The demographic characteristics of the study populations are presented in Table 1. Here, we measured baseline and follow-up (1–19 years) levels of 20 directly measured and 10 derived glycan traits in 2617 individuals from the TwinsUK cohort, 106 individuals from the Dalmatians study and 729 participants from KORA. Description of the glycan structures and formulas used for the calculation of glycan traits are available in Supplementary Table 1, http://links.lww.com/HJH/B733 and Supplementary Table 2, http://links.lww.com/HJH/B733 respectively.

In the discovery cohort, we identify six IgG glycan traits (B, GP4, GP9, GP10, GP15, GP21) associated with an increased risk of incident hypertension after adjusting for covariates and multiple testing (FDR < 0.1) (Fig. 2 and Supplementary Table 3, http://links.lww.com/HJH/B733). Results are consistent when further adjusting for diet (as measured by the Healthy Eating Index), physical activity, type 2 diabetes and rheumatoid arthritis (at $P < 0.5$ for B, GP9, GP15, GP21, $P < 0.1$ for GP4 and GP10).

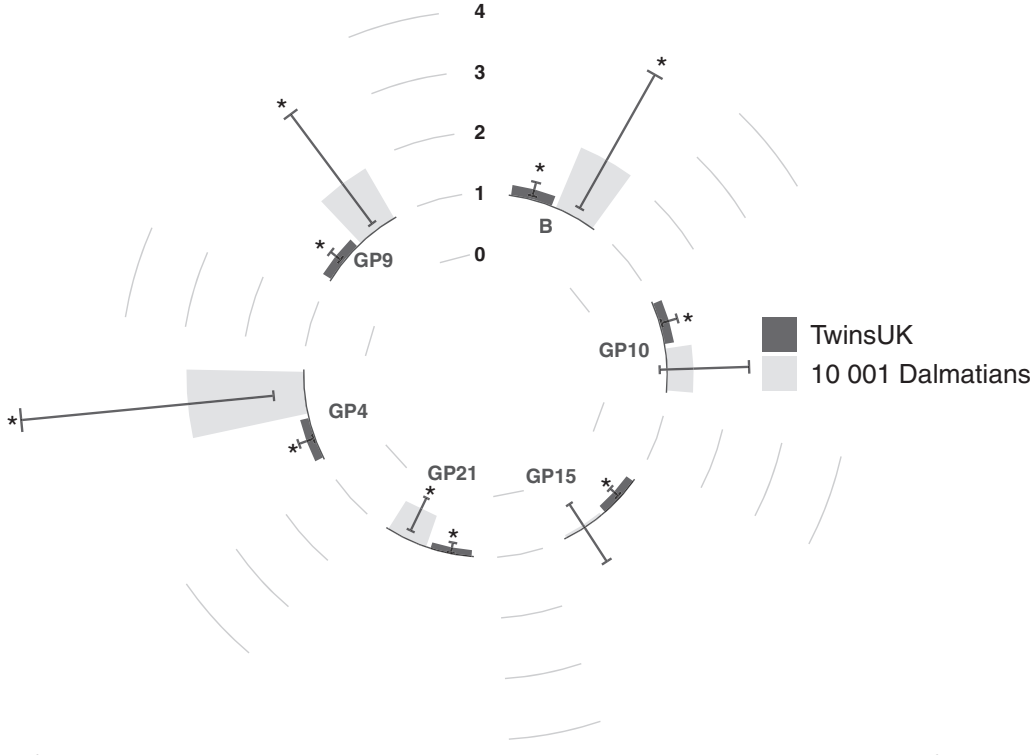


**FIGURE 2** Hazard ratios for each glycan trait in incident hypertension. Bars represent hazard ratios and error bars represent 95% confidence interval as represented by each sample. [a] FDR < 0.1 in TwinsUK and a $P < 0.05$ and same direction in Dalmatians 10 001. Odds ratios for KORA S4 validation can be found in Supplementary Table 3, http://links.lww.com/HJH/B733.

We then validated the hypertension associated glycan traits in both the Dalmatians and KORA cohort. Four of the six glycan traits (B, GP4, GP9 and GP21) were replicated ($P < 0.05$) in the Dalmatians cohort using Cox models after adjusting for covariates in the overall cohort [hazard ratio (95% confidence interval, 95% CI); B = 2.07 (1.16–3.68), $P = 0.014$; GP4 = 2.92 (1.50–5.68), $P = 0.002$; GP9 = 1.94 (1.12–3.36), $P = 0.019$; GP21 = 0.45 (0.24–0.84), $P = 0.012$] and in the female-only sample (Fig. 2 and Supplementary Table 3, http://links.lww.com/HJH/B733, Supplementary Table 4, http://links.lww.com/HJH/B733). Due to technical differences in the quantification of the glycome, of the four previously replicated glycan traits, only B and GP4 were measured in KORA and results showed consistent effects [odds ratio (OR) (95% CI); B = 1.20 (0.99–1.46), $P = 0.06$; GP4 = 1.28 (1.04–1. 58), $P = 0.02$] (Supplementary Table 3, http://links.lww.com/HJH/B733). Although the association with B was borderline significant in the KORA cohort, we found loss of information due to the unnecessary rank transformation we used in KORA for consistency. Indeed, when we run the analysis on the untransformed bisecting glycan (B), the association was nomincally significant [2.15 (1.07–4.29); $P = 0.03$].

To assess the combined effect of the four associated glycans on incident hypertension, we linearly combined the glycan traits and covariates to compute a glycan score as illustrated below in TwinsUK.

$$\begin{aligned} \textit{Glycan score} = {} & (0.15339 \times B) + (0.12235 \times GP4) \\ & + (0.13814 \times \mathrm{GP9}) \\ & - (0.11118 \times \mathrm{GP21}) \\ & + (0.11636 \times \mathrm{age}) \\ & + (0.02476 \times \mathrm{BMI}) \\ & + (0.06395 \times \mathrm{Ma\,p}) \end{aligned}$$

This linear combination was associated with incident hypertension in a binomical mixed effect logistic regression model (BETA[SE], $P = 0.85$[0.06], $2 \times 10^{-16}$), and with SBP at follow up (BETA[SE], $P = 3.88$[0.16], $2 \times 10^{-16}$), DBP at follow up (BETA[SE], $P = 1.1$[0.12], $2 \times 10^{-16}$) in a linear mixed effect regression model.

Likelihood ratio tests between the full model (including the four glycans and covariates, and the null model showed that the inclusion of the four replicated glycans significantly improves the quality of the model (loglik; FULL = −7644.2, NULL = −7440.4, $P = 2.2 \times 10^{-16}$).

Using a time-dependent ROC curve, we also see a marginal improvement of 0.4% in the predictive value through the inclusion of our hypertension-associated glycan traits in comparison to our NULL model (AIC; FULL = 0.983, NULL = 0.979).

## DISCUSSION

Here, we report that the baseline IgG N-glycome is predictive of incident hypertension. We identified six incident hypertension associated glycan traits in TwinsUK and independently replicated four to contribute to hypertension prediction, namely, B (incidence of bisecting GlcNAc), GP4, GP9 and GP21 (Fig. 2). B and GP4 were validated also in KORA, wherein glycans were measured using a different analytical method and report concordant direction of effect for both glycans (Supplementary Table 3, http://links.lww.com/HJH/B733).

We find three of the four replicated glycan traits (B, GP4 and GP9) were significantly increased in individuals who developed hypertension during follow-up when compared with those defined as controls, whereas only GP21 was decreased. B, GP4 and GP9 represent simple glycan structures containing core fucose with only one or no galactose residues attached to the bisecting GlcNAc. Alternatively, GP21 is a more complex digalactosylated structure with two sialic acid residues attached to the galactose molecules, but without the bisection [12]. Furthermore, the linear combination of these four glycan traits correlated with incident hypertension, SBP and DBP and showed a small but significant improvement in AUC in predicting hypertension.

Our results are consistent and supported by the current literatature. Indeed, in a cross-sectional study of an ethnically diverse sample from China, Croatia and Scotland, Wang *et al.* [4] reported positive correlations between GP4 and cross-sectional hypertension. In addition, incidence of bisecting GlcNAc, GP4, GP9 and GP21 have previously been associated with metabolic phenotypes, including dyslipidaemia [22,23], BMI [23], inflammation [24], obesity [25] and ageing [26,27]. This suggests the glycomes involvement in contributing to a dyshomeostatic, pro-inflammatory environment, a recognized state involved in hypertension [28]. Moreover, in hypertensive mice (via an infusion of angiotensin II), Chan *et al.* [29] report an increase in circulating IgG, which accumulates in the aortic adventia. This increase was further linked to B cell activation [29]. B cells are a component of the adaptive immune system, an important player in renal and vascular inflammation [30]. Mice deficient in B cell-activating receptor showed no increase in circulating IgG or SBP [29].

Our study benefits from a large sample size, the longitudinal nature of our data that allowed us to investigate longitudinal changes and the presense of multiple independent replication samples of diverse European origins. Nevertheless, our study also has some limitations. Firstly, our discovery cohort was solely female and both replication samples were also female dominant (Dalmatians, 68%; KORA 57%, Table 1). Secondly, given the technical differences in glycome quantification in the KORA cohort (MS compared with UPLC in Twins and Dalmatians), both GP9 and GP21 were not quantified and therefore we were unable to provide a second replication for these associations. Specifically, UPLC measures glycans from both the Fab and the Fc portion of the IgG, while MS analysis (used only on KORA samples) measures only the Fc portion. Moreover, MS analysis facilitates the differentiation of glycopeptide structures based on their *m/z* ratio; however, it is unable to differentiate structures of the same *m/z* ratio but with opposed orientation of the antennae. Whereas the separation of conversely oriented antennae in glycan structures is possible with UPLC analysis, as different orientation results in different retention time. Finally, hypertensive

subjects were characterized only by higher systolic values, but we did not collect information on obstructive sleep apnoea syndrome.

In conclusion, our results indicate that the IgG glycoprofile of individuals who will develop incident hypertension during follow-up deteriorates in a proinflammatory, obesity-related pattern years before the actual diagnosis, supporting the role of IgG glycosylation in incident hypertension and warrants further exploration.

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The data used in this study are held by the department of Twin Research at King's College London. The data can be released to bona fide researchers using our normal procedures overseen by the Wellcome Trust and its guidelines as part of our core funding (https://twinsuk.ac.uk/resources-for-researchers/access-our-data/).

G.L., A.M.V. and C.M. conceived and designed the experiment; H.D. and A.Ci. performed the experiment; D.K., P.L., C.G. and C.M. analysed the data; H.G., A.P., O.P., O.G., M.M., T.D.S., S.P. contributed reagents, materials and/or analysis tool. D.K., A.Cv., P.L., G.L., C.M. wrote the original manuscript. All authors revised the manuscript.

### Conflicts of interest

G.L. .is a founder and owner, H.D. and A.Ci. are employees of Genos Ltd, which offers commercial service of glycomic analysis and has several patents in this field. T.D.S. is a cofounder and A.M.V. is a consultant for Zoe Global Ltd. As founder and employees of companies with invested interest, G.L. and A.M.V. conceived the analyses. H.D. and A.Ci. performed glycan quantification. No individual with potential conflicting interests had any involvement with the analysis of the data or the direction of the manuscript text. All other authors declare no competing financial interests.

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