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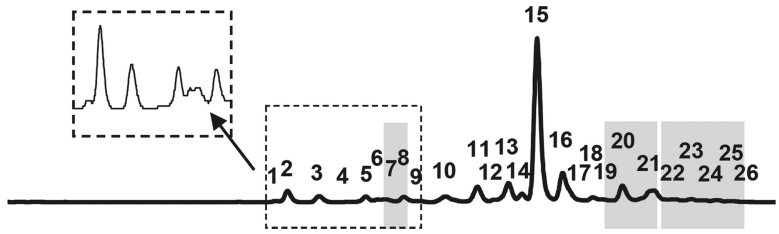

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Variability, Heritability and Environmental Determinants of Human Plasma N-Glycome

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Plasma glycans were analyzed in 1008 individuals to evaluate variability and heritability, as well as the main environmental determinants that affect glycan structures. By combining HPLC analysis of fluorescently labeled glycans with sialidase digestion, glycans were separated into 33 chromatographic peaks and quantified. A high level of variability was observed with the median ratio of minimal to maximal values of 6.17 and significant age- and gender-specific differences. Heritability estimates for individual glycans varied widely, ranging from very low to very high. Glycome-wide environmental determinants were also detected with statistically significant effects of different variables including diet, smoking and cholesterol levels.

Keywords: Human plasma glycome • N-glycosylation • Glycan analysis • Variability of glycans • Heritability of glycans • Age-dependent changes

Introduction

Despite glycosylation being the most complex and abundant post-translational modification, we have only recently started to understand the importance of these complex oligosaccharide structures (glycans) attached to protein backbones.[1] This is not surprising since the branched structures of sugars make the analysis of glycoconjugates significantly more challenging than the analysis of linear DNA and protein sequences. However, a significant part of more than a half of all proteins, and of nearly all membrane and extracellular proteins, are glycans,[2] and to be able to understand the function of these proteins, we also have to understand their glycan moieties.

Glycan synthesis is an extremely complex process that involves hundreds of different enzymes.[3] Some of these enzymes are very specific and contribute to the synthesis of a limited number of structures on a small number of proteins, while others (like ER-glycosyltransferases involved in synthesis of N-glycans, or pathways that produce sugar nucleotides) affect thousands of different proteins. The intricate mechanisms by which the interplay of gene expression and intracellular localization of their products give rise to specific glycan structures is only starting to be understood.[4]

N-glycosylation is essential for multicellular life and its complete absence is embryonically lethal.[5] Mutations that significantly reduce the potential to synthesize the N-glycan core result in a set of severe diseases named "congenital disorders of glycosylation".[6] Mutations that result in the creation of a new glycosylation site can also be deleterious. A number of such mutations have been identified and it is predicted that up to 1.4% of all known disease-causing missense mutations result in gains of glycosylation.[7,8] On the other hand, terminal variability in glycans is common (e.g., ABO blood groups) and contribute to the protein heterogeneity in a population that is advantageous for evading pathogens and adapting to changing environment.[9]

Glycans display a much higher interspecies variability than proteins,[10–12] but glycome variability within a species has never been thoroughly examined. Structural and conformational aspects of glycans are very complex[13] and a small change in a glycan structure can have important functional consequences, indicating that variability in glycans might explain an important part of human phenotypic variability. The heritability of glycans

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is also an important question that has not been addressed previously. The fact that there are hundreds of genes involved in the complex glycan metabolic pathways argues in favor of a strong genetic regulation, but environmental influences on glycan structures have also been reported.[14–16] In this report, we have quantified 33 glycan pools and glycosylation features in 1008 individuals and used these data to evaluate the variability and heritability of glycans and report associations with environmental variables that may affect the N-glycan levels of human plasma proteins.

Experimental Procedures

Human Samples. This study was based on samples from respondents who were residents of the Croatian Adriatic island of Vis and who were recruited within a larger genetic epidemiology program which sought to investigate genetic variability and map genes influencing common complex diseases and disease traits in genetically isolated populations.[17,18] Island Vis is one of the Dalmatian islands that is 45 km away from the mainland, and managed to retain a fair amount of isolation throughout the history. The genetic-epidemiology program on the island began in 2002, and continues on today. The sampling framework was based on the voting register which was used to send postal invitations to all adult inhabitants (over 18 years of age). Furthermore, the sampling scheme was supplemented with numerous efforts to increase the participation rate, including contacts with local physicians and other prominent community members. All these actions led to the creation of the very interesting scientific resource for complex human diseases investigations.

The sample for this study consisted of a total of 1008 individuals aged 18−93 years (median age 56, interquartile range 24 years). There were 415 men (41.2%) and 593 women (58.8%). Genealogical records were reconstructed based on the Church Parish records and information provided by the respondents, and then checked against genetic data on allele sharing between relatives as a quality control measure to exclude data errors. The sample contained a total of 809 genealogical relationships (including 205 parent-child, 123 sibling, and 481 other relationships).

All of the examinees were interviewed by one of the trained surveyors, based on an extensive questionnaire.[19] The questionnaire collected data on personal characteristics (name, date and place of birth, gender, marital status, education level and occupation), selected health-related lifestyle variables (such as diet and smoking status), health complaints, drug intake and hospitalization records. A socio-economic status score was defined by the number of positive answers to a set of four questions on ownership of household goods chosen to discriminate between groups within the study population. SBP and DBP (in mmHg), lung function parameters, height (mm) and weight (kg) were each measured in local health centers and dispensaries between 8 and 11 a.m. All physiological measurements were made by a survey team with many years of experience in similar surveys and using standard methods. Biochemical analyses of creatinine, uric acid, HDL, LDL, total cholesterol, triglycerides, insulin, fibrinogen, CRP and blood glucose were performed on fasting blood samples collected between 8:30 and 9:30 a.m. Serum samples were aliquoted, stored in a −20 °C freezer without delay and then transported frozen to a single biochemical laboratory based in Zagreb. The laboratory employed stringent internal quality control procedures and was accredited for performing the analyses under study by an international external quality assurance (RIQAS) group. Indexes of smoking and alcohol consumption were obtained from standard questionnaires.

Plasma samples were centrifuged at the time of collection and stored at −70 °C until analysis. This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All respondents signed informed consent before participating in the study and the study was approved by the appropriate Ethics Board of the University of Zagreb Medical School.

Glycan Release and Labeling. The N-glycans from plasma samples (5 μL) proteins were released and labeled with 2-aminobenzamide (LudgerTag 2-AB labeling kit Ludger Ltd., Abingdon, U.K.) as described previously.[20] Labeled glycans were dried in a vacuum centrifuge and redissolved in a known volume of water for further analysis.

Hydrophilic Interaction High Performance Liquid Chromatography (HILIC). Released glycans were then subjected to hydrophilic interaction high performance liquid chromatography (HILIC) on a 250 × 4.6 mm i.d. 5 μm particle packed TSKgel Amide 80 column (Anachem, Luton, U.K.) at 30 °C with 50 mM formic acid adjusted to pH 4.4 with ammonia solution as solvent A and acetonitrile as solvent B. The 60 min runs were on a 2795 Alliance separations module (Waters, Milford, MA). HPLCs were equipped with a Waters temperature control module and a Waters 2475 fluorescence detector set with excitation and emission wavelengths of 330 and 420 nm, respectively. The system was calibrated using an external standard of hydrolyzed and 2-AB-labeled glucose oligomers from which the retention times for the individual glycans were converted to glucose units (GU).[21] Glycans were analyzed on the basis of their elution positions and measured in glucose units then compared to reference values in the "GlycoBase" database (available at: http://glycobase.nibrt.ie) for structure assignment.[22]

Weak Anion Exchange (WAX)-HPLC. Glycans were separated according to the number of sialic acids by weak anion exchange HPLC. The analysis was performed using a Prozyme GlycoSep C 75 mm × 7.5 mm column (Prozyme, Leandro, CA)[21] at 30 °C with 20% (v/v) acetonitrile in water as solvent A and 0.1 M acetic acid adjusted to pH 7.0 with ammonia solution in 20% (v/v) acetonitrile as solvent B. Compounds were retained on the column according to their charge density, the higher charged compounds being retained the longest. A fetuin N-glycan standard was used for calibration.

Sialidase Digestion. Aliquots of the 2-AB-labeled glycan pool were dried down in 200-μL microcentrifuge tubes. To these, the following was added: 1 μL of 500 mM sodium acetate incubation buffer (pH 5.5), 1 μL (0.005 units) of ABS, *Arthrobacter ureafaciens* sialidase (releases α2−3, 6, 8 sialic acid, Prozyme) and H_2O to make up to 10 μL. This was incubated overnight (16−18 h) at 37 °C and then passed through a Micropure-EZ enzyme remover (Millipore, Billerica, MA) before applying to the HPLC.

Statistical Analysis. An analysis of the variable distributions was first conducted. Normality of distributions was checked using the Kolmogorov−Smirnov test, differences in age correlations with Spearman's rank test and gender differences with the Mann−Whitney test. SPSS version 13 was used in the analysis (SPSS, Inc., Chicago, IL). Pedigree information was used to establish relationships between respondents, as well as to confirm and check information used in the heritability analysis. Heritability analysis was conducted using polygenic models in Sequential Oligogenic Linkage Analysis Routines (SOLAR).[23] Age, sex, and age × sex interaction terms were used

Table 1. Glycan Structures Present in Different HPLC Peaks[a]

peak	structure	peak	structure	peak	structure
GP1	A2	GP9	A2F1G2S(3)1	GP16	A4G4S(6,6,6,6)4
GP2	A2B		A2F1G2S(6)1		A4G4S(3,6,6,6)4
	A1G1		M8D2, D3		A4BG4S4
	FA2		A2G2S(3,3)2		FA4G4S4
GP3	M5		A2G2S(3,6)2		A4F1G4S4
	FA2B		A2G2S(6,6)2		A4G4LacS4
	A2[6]G1		M8D1,D3		A4F2G4S4
	A2[6]BG1	GP10	A2BG2S(3,3)2		FA4F1G4S4
GP4	A2[3]G1		A2BG2S(3,6)2	DG1	A2
	A2[3]BG1		A2BG2S(6,6)2	DG2	A2B
	M4A1G1		A3BG3S(3)1		A1G1
	FA2[6]G1		A3BG3S(6)1		FA2
	FA2[6]BG1		FA2G2S(3,3)2	DG3	M5
	A1[6]G1S(3)1		FA2G2S(3,6)2		FA2B
	A1[6]G1S(6)1		FA2G2S(6,6)2		A2[6]G1
	FA2[3]G1	GP11	FA2BG2S(3,3)2		A2[6]BG1
	FA2[3]BG1		FA2BG2S(3,6)2	DG4	M4A1G1
	M6D1, D2		FA2BG2S(6,6)2		A2[3]G1
	A1[3]G1S(3)1		M9		A2[3]BG1
	A1[3]G1S(6)1	GP12	A2F1G2S2		FA2[6]BG1
GP5	M6D3		A3G3S(3,3)2		FA2[3]G1
	A2[6]G1S(3)1		A3G3S(3,6)2		FA2[3]BG1
	A2[6]G1S(6)1		A3G3S(6,6)2	DG5	M6D1, D2
	A2G2		A3BG3S(3,3)2		M6D3
	A2[3]G1S(3)1		A3BG3S(3,6)2		A2G2
	A2[3]G1S(6)1		A3BG3S(6,6)2		A2BG2
	A2BG2	GP13	A3G3F1S2	DG6	FA2G2
GP6	FA2[6]G1S(3)1		FA3G3S(3,3)2		M5A1G1
	FA2[6]G1S(6)1		FA3G3S(3,6)2		FA2BG2
	FA2[6]BG1S(3)1		FA3G3S(6,6)2	DG7	M7D3
	FA2[6]BG1S(6)1		FA3BG3S(3,3)2		A2F1G2
	M4A1G1S1		FA3BG3S(3,6)2		M7D1
	FA2G2		FA3BG3S(6,6)2	DG8	A3G3
	FA2[3]G1S(3)1		A3G3S(3,3,6)3		A2F2G2
	FA2[3]G1S(6)1		A3G3S(3,6,6)3		FA3G3
	A2BG1S1		A3G3S(6,6,6)3		M8D2, D3
	FA2[3]BG1S(3)1	GP14	A3F1G3S(3,3,6)3		M8D1,D3
	FA2[3]BG1S(6)1		FA3F1G3S(6,6,6)3	DG9	FA3BG3
GP7	FA2BG2		A4G4S(6,6)2		A3F1G3
	M7D3		A3F1G3S(3,6,6)3	DG10	M9
	A2G2S(3)1		A3F1G3S(6,6,6)3		FA3F1G3
	A2G2S(6)1		A4G4S(6,6,6)3	DG11	A4G4
	M7D1		A4F1G4S2		A4BG4
GP8	A2BG2S(3)1		A4G4S3		A3F2G3
	A2BG2S(6)1	GP15	A4G4S4		FA4G4
	M5A1G1S1		A4F1G4S3	DG12	A4F1G4
	FA2G2S(3)1			DG13	A4G4Lac
	FA2G2S(6)1				A4F2G4
	A3G3				FA4F1G4
	FA2BG2S(3)1				
	FA2BG2S(6)1				

[a] Structure abbreviations: all N-glycans have two core GlcNAcs; F at the start of the abbreviation indicates a core fucose α1-6 linked to the inner GlcNAc; M*x*, number (*x*) of mannose on core GlcNAcs; D1 indicates that the α1-2 mannose is on the Manα1-6Manα1-6 arm, D2 on the Manα1-3Manα1-6 arm, D3 on the Manα1-3 arm of M6 and on the Manα1-2Manα1-3 arm of M7 and M8; A*x*, number of antenna (GlcNAc) on trimannosyl core; A2, biantennary with both GlcNAcs as β1-2 linked; A3, triantennary with a GlcNAc linked β1-2 to both mannose and the third GlcNAc linked β1-4 to the α1-3 linked mannose; A4, GlcNAcs linked as A3 with additional GlcNAc β1-6 linked to α1-6 mannose; B, bisecting GlcNAc linked β1-4 to β1-3 mannose; G*x*, number (*x*) of β1-4 linked galactose on antenna; [3]G1 and [6]G1 indicates that the galactose is on the antenna of the α1-3 or α1-6 mannose; F(*x*), number (*x*) of fucose linked α1-3 to antenna GlcNAc; Lac(*x*), number (*x*) of lactosamine (Galβ1-4GlcNAc) extensions; S*x*, number (*x*) of sialic acids linked to galactose; the numbers 3 or 6 or in parentheses after S indicate whether the sialic acid is in an α2-3 or α2-6 linkage. If there is no linkage number, the exact link is unknown.

as predictor variables. Because of the non-normal data distribution found with some variables, rank transformations with *z*-score normalization procedures were used to obtain normal data distributions. The level of statistical significance was set at $P < 0.05$. Multiple linear regression was performed with a set of 16 predictor variables. Predictors were selected from a total of over 100 variables that were available for analysis, based on results from a data mining classification tree (CHAID).

Results

Recently published improvements in analytical procedures[20] created the possibility to reliably quantify glycans in a relatively

Table 2. Precision of the Analytical Procedure[a]

peak no.	mean	SD	CV
GP1	0.12	0.01	7.39
GP2	7.24	0.24	3.38
GP3	2.45	0.06	2.60
GP4	9.05	0.25	2.75
GP5	1.69	0.07	3.98
GP6	5.14	0.09	1.82
GP7	9.37	0.95	10.14
GP8	10.36	0.13	1.26
GP9	31.25	0.43	1.37
GP10	7.37	0.19	2.63
GP11	2.77	0.09	3.33
GP12	1.66	0.05	3.14
GP13	5.26	0.15	2.85
GP14	5.32	0.16	2.97
GP15	0.49	0.03	6.64
GP16	0.47	0.06	12.25
overall			**4.28**

[a] Plasma sample from the same individual was analyzed (released, labeled, separated by HPLC and integrated) 10 times to evaluate precision of the method. Mean values, standard deviation and coefficient of variation are presented for glycan structures separated into 16 peaks integrated from a hydrophilic interaction chromatogram. SD, standard deviation; CV, % coefficient of variation.

Table 3. Descriptive Statistics of the Plasma Glycome in the Sample of Adult Respondents from the Croatian Island of Vis[a]

	N	mean	SD	CV	median	IR	min	max
GP1	974	0.18	0.09	50.0	0.16	0.11	0.03	0.83
GP2	974	4.14	1.57	34.6	3.93	1.96	0.45	17.90
GP3	974	2.13	0.45	19.4	2.10	0.58	0.72	5.97
GP4	974	5.94	1.16	18.2	5.89	1.54	0.62	11.21
GP5	974	2.29	0.41	17.4	2.24	0.51	1.32	5.38
GP6	974	4.11	0.87	20.4	4.03	1.10	0.87	7.94
GP7	974	10.59	2.40	23.0	10.04	2.20	6.16	30.30
GP8	974	10.00	1.53	14.3	9.83	1.99	5.75	17.47
GP9	974	36.91	2.98	7.5	36.81	3.77	24.29	48.53
GP10	974	7.55	1.74	18.5	7.35	2.17	3.50	18.75
GP11	974	2.35	0.66	20.1	2.22	0.74	0.94	5.70
GP12	974	1.48	0.28	18.5	1.46	0.35	0.70	4.18
GP13	974	4.89	1.39	28.1	4.86	1.81	0.59	9.66
GP14	974	6.33	1.44	22.3	6.23	1.87	2.51	13.05
GP15	974	0.45	0.17	37.0	0.44	0.22	0.06	1.51
GP16	974	0.66	0.23	35.2	0.64	0.27	0.11	2.15
DG1	995	0.19	0.09	47.4	0.17	0.11	0.04	0.82
DG2	995	4.19	1.53	33.5	3.99	1.93	0.56	18.00
DG3	995	2.72	0.51	18.7	2.68	0.66	1.04	6.68
DG4	995	6.99	1.26	16.8	6.98	1.68	0.99	12.37
DG5	995	49.18	3.56	6.4	49.14	4.16	33.73	64.47
DG6	995	16.02	2.91	15.8	15.71	3.67	6.24	34.41
DG7	995	1.56	0.42	27.1	1.50	0.51	0.69	3.79
DG8	995	10.90	2.07	19.0	10.92	2.71	4.80	17.26
DG9	995	3.68	1.34	35.5	3.60	1.84	0.17	8.68
DG10	995	1.08	0.18	15.2	1.07	0.22	0.63	1.85
DG11	995	2.19	0.55	22.6	2.13	0.68	0.79	4.95
DG12	995	0.72	0.31	39.2	0.68	0.37	0.04	2.46
DG13	995	0.59	0.31	40.0	0.50	0.28	0.18	2.80
Monosialo	1006	24.58	3.29	21.0	24.13	4.13	13.51	44.44
Disialo	1006	58.43	2.35	14.2	58.62	2.89	44.61	66.00
Trisialo	1006	14.87	2.06	20.7	14.84	2.66	8.36	22.77
Tetrasialo	1006	2.11	0.69	27.9	2.10	0.97	0.37	5.09

[a] *N* - number of individuals, SD - standard deviation; CV - % coefficient of variation; IR - interquartile range; Min - minimal observed value; Max - maximal observed value

large number of samples. The aim was to evaluate variability and heritability of the human N-glycome. N-glycans were analyzed in plasma samples from 1008 adult individuals from the Croatian island of Vis. N-linked glycans were released from proteins using peptide N-glycosidase F, labeled with 2-aminobenzamide (2-AB) and analyzed by hydrophilic interaction high performance liquid chromatography (HILIC). Following release of α2−3, α2−6, and α2−8 linked sialic acid by *A. ureafaciens* sialidase, glycans were subjected to another HILIC analysis. Further analysis was performed by weak anion exchange (WAX) HPLC that separated glycans according to the number of attached sialic acids. In this way, we separated the plasma N-glycome into 33 different HPLC peaks. Individual glycan structures that are present in each individual peak are presented in Table 1.

Table 4. Age and Gender Related Glycans Levels in the Sample of Adult Respondents from the Croatian Island of Vis

		gender related glycans levels				
	age related change correlation r	median (men)	IR[b] (men)	median (women)	IR (women)	*P*
GP1	0.30[a]	0.16	0.10	0.15	0.10	0.001
GP2	0.53[a]	4.05	1.76	3.84	2.14	0.062
GP3	0.35[a]	2.04	0.57	2.14	0.60	0.006
GP4	−0.07[a]	5.98	1.43	5.83	1.57	0.053
GP5	−0.17[a]	2.15	0.46	2.29	0.52	<0.001
GP6	−0.48[a]	4.07	0.90	4.00	1.24	0.598
GP7	0.13[a]	10.06	2.00	10.03	2.38	0.799
GP8	−0.42[a]	9.76	1.65	9.92	2.24	0.082
GP9	−0.03	37.12	3.94	36.61	3.77	0.033
GP10	0.07[a]	7.50	2.24	7.25	2.18	0.013
GP11	0.04	2.20	0.79	2.22	0.70	0.243
GP12	−0.05	1.41	0.38	1.51	0.33	<0.001
GP13	−0.15[a]	4.48	1.78	5.07	1.67	<0.001
GP14	0.22[a]	6.55	1.91	6.03	1.84	<0.001
GP15	−0.13[a]	0.38	0.21	0.47	0.20	<0.001
GP16	−0.06	0.60	0.26	0.67	0.28	<0.001
DG1	0.29[a]	0.18	0.11	0.16	0.10	0.001
DG2	0.52[a]	4.03	1.75	3.90	2.08	0.092
DG3	0.34[a]	2.59	0.60	2.73	0.67	<0.001
DG4	0.00	7.05	1.49	6.95	1.75	0.038
DG5	−0.03	49.24	4.05	49.01	4.26	0.572
DG6	−0.28[a]	15.58	3.42	15.79	3.81	0.967
DG7	−0.01	1.64	0.49	1.40	0.44	<0.001
DG8	−0.09[a]	10.19	2.49	11.34	2.52	<0.001
DG9	0.13[a]	4.15	1.77	3.16	1.60	<0.001
DG10	0.15[a]	1.09	0.20	1.06	0.23	0.002
DG11	−0.02	1.98	0.58	2.27	0.69	<0.001
DG12	0.11[a]	0.76	0.39	0.62	0.34	<0.001
DG13	0.09[a]	0.51	0.34	0.49	0.25	0.010
Monosialo	−0.01	23.97	4.22	24.30	4.10	0.049
Disialo	−0.02	58.77	2.91	58.41	2.76	0.002
Trisialo	0.04	14.74	2.71	14.88	2.61	0.376
Tetrasialo	0.06	2.12	1.00	2.07	0.94	0.670

[a] Significant at the level of $P < 0.05$. [b] IR, interquartile range.

To determine the level of variation that can be expected due to limitations in the experimental methods used, the whole analytical procedure, including glycan release, labeling and chromatography was repeated 10 times for the same sample. Mean values, standard deviations (SD) and the precision, expressed as the coefficient of variation (CV) of a series of these measurements, are given in Table 2. Considering the number of steps in the analytical procedure and the existence of some partly resolved chromatographic peaks, the determined precision was considered to be very good. The average CV was only slightly above 4%. The CV of most peaks was actually even lower, while only GP7 and GP16 had CVs of 10.1%, and 12.3% respectively.

Table 5. Heritability (h) of the Plasma Glycome and Significance Levels for Age, Gender and Age × Gender as Covariates of the Polygenic Heritability Models

			covariates significance		
	h^2	P	age	gender	age × gender
GP1	0.464	<0.001	<0.001	<0.001	0.011
GP2	0.256	0.014	<0.001	0.004	0.002
GP3	0.235	0.021	<0.001	0.010	0.444
GP4	0.241	0.017	0.180	0.082	0.802
GP5	0.463	<0.001	<0.001	0.311	0.005
GP6	0.283	0.005	<0.001	0.817	<0.001
GP7	0.117	0.129	0.012	0.873	0.857
GP8	0.317	0.002	<0.001	0.003	<0.001
GP9	0.324	0.006	0.004	0.014	0.114
GP10	0.342	0.006	0.006	0.011	0.153
GP11	0.346	0.003	0.678	0.160	0.167
GP12	0.519	<0.001	0.007	<0.001	0.007
GP13	0.570	<0.001	<0.001	<0.001	<0.001
GP14	0.551	<0.001	<0.001	<0.001	0.730
GP15	0.381	<0.001	<0.001	<0.001	<0.001
GP16	0.240	0.029	0.001	<0.001	0.003
DG1	0.527	<0.001	<0.001	<0.001	0.036
DG2	0.273	0.008	<0.001	0.008	0.008
DG3	0.260	0.012	<0.001	<0.001	0.839
DG4	0.182	0.053	0.930	0.040	0.695
DG5	<0.010	0.500	0.060	0.486	0.173
DG6	0.187	0.050	0.003	0.673	<0.001
DG7	0.581	<0.001	0.072	<0.001	0.004
DG8	0.531	<0.001	0.002	<0.001	0.008
DG9	0.564	<0.001	<0.001	<0.001	0.032
DG10	0.455	<0.001	0.037	<0.001	0.111
DG11	0.426	<0.001	0.089	<0.001	0.027
DG12	0.316	<0.001	<0.001	<0.001	0.051
DG13	0.104	0.169	0.002	0.004	0.072
Monosialo	0.484	<0.001	0.100	0.050	0.010
Disialo	0.516	<0.001	0.013	<0.001	0.026
Trisialo	0.388	<0.001	0.952	0.410	0.157
Tetrasialo	0.367	<0.001	0.921	0.213	0.913

HILIC analysis separated the plasma glycome into 16 groups (GP1−GP16). The second HILIC step, performed after sialidase digestion, gave 13 groups of desialylated glycans (DG1−DG13). WAX-HPLC analysis divided the glycome into monosialylated, disialylated, trisialylated and tetrasialylated glycans. Individual results are presented as the relative percentage area of the total glycome. Since three separate chromatographic analyses were performed, the percentages add to 100% for each chromatography (HILIC, HILIC of desialylated glycans and WAX-HPLC). Basic descriptive statistics for these results are presented in Table 3, showing means, medians and the range of variation for each of the glycan groups analyzed. In this table, results for the whole data set (including relatives) are presented. To check whether the presence of relatives distorted sample representation, the same statistical analysis was performed on a data set with all relatives excluded (only singletons were included in that analysis). Since no unexpected statistically significant differences were observed, subsequent analysis of the effects of age and gender was performed on the complete data set.

The effects of age and gender on the plasma glycome are presented in Table 4. In 21 out of 33 analyzed glycan groups, statistically significant correlations with age were observed, while the remaining 12 glycan groups did not change with age. Glycans in GP1, GP2, GP3, DG1, DG2 and DG3 displayed the strongest increase with age, while glycans in GP6, GP8 and DG6 showed the largest negative correlation. A number of statistically significant gender differences were also observed (Table 4).

Variations observed in a human phenotype are generally a combination of genetic differences and environmental factors. Heritability is one of the most basic and often one of the first analyses ever to be made in a genetic study, since it represents the proportion of the trait variance that can be attributed to genetic factors, and it is often used as a screening tool to determine whether a trait may be suitable for gene mapping.[24] In this study, we performed the first estimation of heritability for different glycans. The results are presented in Table 5. A broad range of variation in heritability was observed, from insignificant or very low (e.g., DG5) to rather high (GP13, GP14, DG7, DG8, DG9). Notably, age, gender, and age−gender interaction effects were found to be statistically significant in majority of the glycan groups (Table 5).

Multiple linear regression analysis was performed to identify environmental determinants that affect plasma glycans. A group of 16 most important variables was selected from over 100 different variables available for analysis by using the data mining classification tree (CHAID). The results are presented in Table 6.

Discussion

With the use of a recently developed method for high-throughput analysis of glycans,[20] we performed the first study of variability and heritability in the human plasma glycome. Because of experimental limitations, we did not quantify individual glycan structures, but instead measured glycans partitioned into 33 groups containing several mostly similar glycan structures (Table 1). Despite the fact that different glycans within the same group could have changed in opposite directions, thus, diminishing the cumulative change, the variability of glycans in this population-based study was found to be rather high (Table 3). This apparently large variability of glycan structures should not be ignored in the development of diagnostic tools based on measuring alterations in glycan levels, since the magnitude of reported changes associated with a disease[25−29] is sometimes smaller than the observed natural variability revealed in this study. However, it should be noted that so far we only compared ratios of plasma glycans grouped into 33 chromatographic peaks and the possible presence or absence of some minor structures in specific individuals was not examined.

The median ratio of maximal and minimal observed values was 6.17 (with the highest ratios reaching levels of 20-fold or even greater). However, for glycans where 10-fold or higher ratios were observed, the high or the low end of the distribution curve was usually populated with a very small number of individuals or, in some cases, only a single individual. For example, glycan A4G4S4 which eluted as a major structure in GP15 represented on average 0.5% of the total plasma glycome, but in one individual, it was 1.5% and in only three more individuals between 1% and 1.2% of the total plasma glycome. The individual that was on the lowest extreme of GP1, GP2, GP3, GP4, GP5 and GP6, and on the highest extreme of GP9 and GP14 distribution curves represents an interesting case. Although the cause for the large disturbance of glycan synthesis in this individual is probably genetic, this study revealed a wide range of variation in the heritability of different glycans. While many glycan structures (GP1, GP5, GP12, GP13, GP14, DG1, DG7, DG8, DG9, DG10, DG11, mono- and disialylated glycans)

Table 6. Environmental Determinants Affecting the Plasma Glycome[a]

	age	gender	socioeconomic status	healthy food pattern	unhealthy food pattern	carbohydrates consumption	fruit and vegetables consumption	alcohol	smoking	skinfolds	insulin	fibrinogen	CRP	LDL cholesterol	HDL cholesterol	glucose
GP1	**0.30**	**−0.08**	−0.06	0.02	0.04	**−0.10**	0.02	0.06	0.05	−0.04	**0.10**	−0.06	−0.06	**0.08**	0.04	0.03
GP2	**0.46**	**−0.12**	0.00	−0.01	−0.05	0.04	0.04	−0.03	**−0.16**	**0.07**	0.04	−0.02	−0.01	−0.03	0.01	−0.01
GP3	**0.39**	**0.10**	−0.03	−0.01	−0.02	0.07	0.03	0.00	**0.07**	−0.05	**0.07**	**−0.09**	−0.06	**0.07**	−0.02	0.04
GP4	−0.03	**−0.09**	0.00	**−0.08**	−0.01	**0.08**	**0.10**	−0.03	**−0.15**	0.00	**0.07**	**−0.18**	−0.05	−0.05	0.01	−0.06
GP5	**−0.11**	**0.15**	−0.06	−0.02	−0.02	0.03	0.00	−0.02	0.03	**−0.14**	0.01	−0.03	**−0.10**	0.07	**0.07**	−0.03
GP6	**−0.39**	0.03	0.00	**−0.08**	0.01	**0.08**	0.06	0.00	**−0.09**	**−0.11**	0.05	**−0.21**	**−0.09**	−0.05	0.04	−0.01
GP7	0.03	−0.03	0.01	0.01	**−0.09**	−0.06	−0.05	−0.03	−0.01	**−0.08**	0.04	**0.22**	**−0.10**	**0.10**	**0.14**	−0.06
GP8	**−0.27**	**0.16**	−0.04	−0.02	0.03	**0.08**	−0.01	0.01	0.02	**−0.19**	−0.04	**−0.27**	−0.02	0.03	0.03	−0.01
GP9	−0.06	**−0.09**	0.05	0.04	−0.01	−0.04	0.00	−0.01	**0.09**	**0.10**	−0.06	**0.14**	0.00	**−0.08**	−0.04	0.03
GP10	**0.10**	−0.06	0.02	0.05	0.02	−0.05	−0.01	0.01	0.04	−0.04	0.07	−0.02	−0.03	−0.04	0.00	0.03
GP11	0.04	0.02	−0.03	−0.06	0.07	0.04	−0.02	0.05	−0.06	**0.11**	−0.06	**−0.12**	−0.05	**0.13**	−0.07	0.00
GP12	**−0.09**	**0.20**	**−0.08**	0.03	0.06	−0.05	−0.02	0.05	0.02	−0.03	−0.06	**−0.15**	**0.10**	**0.13**	−0.07	0.07
GP13	**−0.19**	**0.22**	−0.05	−0.02	0.03	−0.03	0.01	0.02	**−0.10**	0.04	−0.04	**−0.19**	**0.18**	**0.08**	**−0.11**	−0.01
GP14	**0.20**	**−0.13**	0.05	0.06	**0.08**	−0.06	−0.03	**0.07**	**0.19**	0.05	−0.03	**0.26**	**0.11**	0.01	−0.02	0.04
GP15	**−0.16**	**0.23**	−0.04	0.00	0.02	−0.02	0.02	−0.02	**−0.09**	−0.04	−0.05	**−0.11**	**0.09**	**0.13**	0.00	−0.02
GP16	**−0.15**	0.06	−0.04	0.03	0.02	−0.01	0.00	−0.04	−0.05	0.02	−0.05	0.09	**0.13**	0.05	0.03	0.05
DG1	**0.29**	−0.05	−0.02	0.06	0.01	−0.05	−0.01	**0.08**	0.04	**−0.08**	**0.10**	−0.02	−0.06	0.07	0.02	0.01
DG2	**0.45**	**−0.11**	0.01	−0.01	**−0.07**	0.03	0.04	−0.03	**−0.15**	**0.09**	0.03	−0.02	−0.01	−0.03	0.00	−0.01
DG3	**0.34**	**0.12**	−0.04	−0.01	−0.07	0.05	0.02	0.00	0.05	**−0.14**	0.05	−0.02	**−0.10**	**0.10**	**0.07**	0.01
DG4	0.02	**−0.10**	0.01	−0.06	−0.03	0.06	**0.09**	−0.03	**−0.16**	0.04	**0.07**	**−0.18**	−0.04	−0.05	0.01	−0.04
DG5	**−0.09**	−0.05	0.02	0.02	−0.04	−0.05	−0.04	−0.07	0.07	−0.01	−0.07	**0.19**	−0.05	−0.01	0.05	−0.01
DG6	**−0.18**	0.05	0.01	−0.02	0.04	0.01	0.02	0.03	−0.02	−0.01	0.04	**−0.22**	−0.05	−0.05	−0.02	0.02
DG7	0.03	**−0.27**	0.02	0.03	−0.01	0.07	−0.04	0.01	**0.14**	**−0.12**	0.00	**0.18**	**−0.10**	0.04	**0.10**	0.00
DG8	**−0.10**	**0.30**	−0.05	0.00	0.05	−0.05	0.00	0.03	−0.05	0.05	−0.04	**−0.17**	**0.17**	0.06	**−0.14**	0.01
DG9	**0.14**	**−0.27**	0.04	0.05	0.04	0.02	−0.04	0.06	**0.18**	**−0.06**	−0.02	**0.27**	0.03	0.03	**0.07**	0.00
DG10	**0.09**	**−0.16**	0.03	0.05	0.00	0.01	0.01	0.04	**0.09**	**0.15**	0.02	**0.10**	−0.03	**0.23**	−0.08	−0.02
DG11	−0.07	**0.25**	−0.05	0.02	0.02	−0.01	−0.01	0.03	−0.07	0.01	−0.05	−0.04	**0.09**	**0.09**	−0.03	0.02
DG12	**0.11**	**−0.23**	0.04	0.05	0.04	0.04	−0.02	0.04	**0.12**	**−0.07**	−0.02	**0.28**	−0.01	0.04	**0.11**	0.02
DG13	**0.11**	−0.05	0.02	0.03	0.01	0.05	−0.03	0.06	0.01	**−0.10**	−0.01	0.04	0.01	0.05	0.03	−0.01
Monosialo	0.01	0.06	0.01	−0.03	−0.05	0.00	−0.04	−0.03	−0.03	**−0.11**	0.05	0.01	**−0.10**	0.04	**0.11**	−0.05
Disialo	−0.03	**−0.15**	0.01	0.03	0.02	−0.03	0.05	−0.05	0.03	**0.13**	−0.03	−0.04	**−0.09**	**−0.11**	**−0.08**	0.06
Trisialo	0.01	0.07	−0.01	0.01	0.07	0.00	0.02	**0.10**	0.03	0.02	−0.05	0.02	**0.21**	0.05	**−0.10**	0.00
Tetrasialo	0.05	−0.01	−0.04	0.02	0.00	**0.09**	−0.01	0.02	−0.06	−0.02	−0.02	0.03	0.07	0.01	0.05	0.05

[a] Multiple linear regression analysis was performed. Statistically significant regression coefficients are shown in bold. Skinfolds were summed across five measurements (triceps, biceps, abdominal, subscapular and suprailiac). Smoking was scored as the pack-years, while alcohol was scored as the number of units that a person reported. Food consumption was scored from a food questionnaire, as the equally weighted sum of the several variables. Healthy food consumption was based on the olive oil or plant oil or avoidance of any fat use in the food preparation and fish and fish derivatives consumption. Unhealthy pattern was based on the animal fat use in food preparation, and consumption of red meat types. Carbohydrates were based on cake, chocolate, candy and sugar intake, fruit and vegetables as the sum of the fruit, leafy vegetables, root vegetables, flower vegetables and other vegetable types, while the protein intake was based on the pork, beef, lamb and various meat derivatives.

were found to be strongly influenced by genetic background (Table 5), other structures like GP7, DG4, DG5, DG6 and DG13 appeared to be under greater environmental control. The implications of these findings are multiple. First, it should be noted that heritability represents the proportion of the trait variance that can be attributed to genetic factors.[24] Because of this fact, heritability has often been one of the first analyses conducted in genetic epidemiology studies. Previous studies have well established the pattern of heritability for a wide variety of human traits, showing that the heritability estimates are in general higher for those traits that are not associated with fitness (e.g., height, tooth size), while fitness related traits have lower heritability (e.g., fertility).[30]

Heritability is also important as it can provide information about the underlying genetic structure of the trait, by reporting on additive and dominant variance components.[31,32] Although heritability provides important information on the genetic structure of the trait, the interpretation of heritability estimates is complicated by a number of factors.[24] Differences in methodological approaches and sampling schemes, variation in trait values over time, and population-specific differences can all influence heritability values,[24] resulting in a wide range of heritability estimates in different studies. The latter is largely due to the fact that since heritability is (in the simplest form) calculated as the ratio of genetic over genetic and environmental variance, estimates depend on the environment from which the sample has been collected. Care should therefore be taken in comparing heritability estimates from different populations.

A significant association between glycan structures and age was observed for some but not all glycans (Table 4). Glycans GP1, GP2, GP3 and DG2 displayed the strongest positive correlation with age, while glycans GP6 and GP8 showed the largest negative correlation. Since numerous glycans are present in these peaks, it is not possible to attribute age-dependent changes to specific glycans. However, a common characteristic of most of the glycans in peaks GP6 and GP8 is that they are core-fucosylated, while the majority of glycans in peaks GP1 and GP3 are not. Recently, it has been reported that core-fucosylation decreases with aging.[33] This finding is confirmed by our study, but it should be noted that we did not observe an age-dependent decrease in all core-fucosylated glycans. For example, all glycans in GP10 are core fucosylated and this group did not decrease with age. Some previous lectin studies also indicated that there is an age-dependent decrease of fucosylation of individual proteins in both humans[34] and animals.[35] One study of the effects of aging on the monosaccharide composition of the cell surface in the unexposed normal human epidermis also reported a decrease in fucose.[36] Taken together, these data suggest that at least some types of protein fucosylation are strongly age-dependent.

Similarly, gender had a strong modifying effect on some of these glycans, while others did not seem to be affected. A mixed model analysis showed that in some glycans (Table 5) gender−age interaction was also statistically significantly associated with these glycan levels, suggesting that the regulation of glycan levels in humans could be very complex and may include effects which are difficult to measure precisely and to use in the analysis. This argues for the use of large sample size studies which control for a number of possible confounding effects and the need for replication studies in different populations to confirm findings.

Associations with environmental variables were also very complex (Table 6). Socioeconomic status and dietary habits (including alcohol consumption) apparently have limited influence to a small number of glycan structures. On the other hand, smoking was found to have statistically significant effects on 14 groups of glycans, indicating that smoking habit is the most important environmental parameter that should be measured and controlled for in future studies investigating the relationship between plasma glycan levels and diseases, or in gene-association studies.

Skinfolds, as a measure of body fat, HDL cholesterol and LDL cholesterol were found to have statistically significant effects on numerous glycan groups. On the other hand, effects of insulin were generally lower and statistically significant for only five glycan groups, and blood glucose levels were not found to affect glycans at all. Multiple statistically significant correlations were observed between CRP and different glycans, probably reflecting complex changes in glycans that occur in inflammatory processes.[37] Numerous associations were also observed for fibrinogen, but since this protein is glycosylated and present in plasma in very variable concentration, the observed associations might also reflect changes in plasma glycome composition caused by changes in fibrinogen concentration.

Conclusions

By analyzing glycans in the plasma of 1008 individuals with defined pedigrees, we present the first comprehensive information about the variability and heritability of glycans in a human population. The variability in plasma glycan levels was found to be larger than expected, with the median ratio of maximal and minimal observed values of 6.17. Gender and age were also found to significantly affect plasma glycan distributions, although their effect was relatively small, generally in the range of up to 10%. The heritability of glycans was found to vary widely across individual glycan species, suggesting that the relative importance of genetic and environmental control varies across glycan species. These findings have important implications for the development of future diagnostic tests based on changes in levels of measured plasma glycans. A number of environmental variables (such as smoking) were found to be associated with plasma glycome components in a very complex manner, and these variables should perhaps be measured and included in the analysis of future studies investigating the relationship between plasma glycan levels and disease or disease traits and in gene-association studies.

Abbreviations: 2-AB, 2-aminobenzamide; ABS, sialidase from *Arthrobacter ureafaciens*; CV, coefficient of variation, GU, glucose unit; HILIC, Hydrophilic interaction high-performance liquid chromatography; HPLC, high-performance liquid chromatography; PNGase F, N-glycosidase F, SD, standard deviation, WAX, weak anion exchange. Structure abbreviations: all N-glycans have two core GlcNAcs; F at the start of the abbreviation indicates a core fucose α1-6 linked to the inner GlcNAc; M*x*, number (*x*) of mannose on core GlcNAcs; D1 indicates that the α1-2 mannose is on the Manα1-6Manα1-6 arm, D2 on the Manα1-3Manα1-6 arm, D3 on the Manα1-3 arm of M6 and on the Manα1-2Manα1-3 arm of M7 and M8; A*x*, number of antenna (GlcNAc) on trimannosyl core; A2, biantennary with both GlcNAcs as β1-2 linked; A3, triantennary with a GlcNAc linked β1-2 to both mannose and the third GlcNAc linked β1-4 to the α1-3 linked mannose; A4, GlcNAcs linked as A3 with additional GlcNAc β1-6 linked to α1-6 mannose; B, bisecting GlcNAc linked β1-4 to β1-3 mannose;

G*x*, number (*x*) of β1-4 linked galactose on antenna; [3]G1 and [6]G1 indicates that the galactose is on the antenna of the α1-3 or α1-6 mannose; F(*x*), number (*x*) of fucose linked α1-3 to antenna GlcNAc; Lac(*x*), number (*x*) of lactosamine (Galβ1-4GlcNAc) extensions; S*x*, number (*x*) of sialic acids linked to galactose; the numbers 3 or 6 or in parentheses after S indicate whether the sialic acid is in an α2-3 or α2-6 linkage. If there is no linkage number, the exact link is unknown.

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References

(1) Lauc, G. Sweet secret of multicellular life. *Biochim. Biophys. Acta* **2006**, *1760* (4), 525–6.
(2) Apweiler, R.; Hermjakob, H.; Sharon, N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta* **1999**, *1473* (1), 4–8.
(3) Schachter, H. The joys of HexNAc. The synthesis and function of N- and O-glycan branches. *Glycoconjugate J.* **2000**, *17* (7−9), 465–83.
(4) Nairn, A. V.; York, W. S.; Harris, K.; Hall, E. M.; Pierce, J. M.; Moremen, K. W. Regulation of glycan structures in animal tissues: transcript profiling of glycan-related genes. *J. Biol. Chem.* **2008**, *283* (25), 17298–313.
(5) Marek, K. W.; Vijay, I. K.; Marth, J. D. A recessive deletion in the GlcNAc-1-phosphotransferase gene results in peri-implantation embryonic lethality. *Glycobiology* **1999**, *9* (11), 1263–71.
(6) Freeze, H. H. Genetic defects in the human glycome. *Nat. Rev. Genet.* **2006**, *7* (7), 537–51.
(7) Vogt, G.; Chapgier, A.; Yang, K.; Chuzhanova, N.; Feinberg, J.; Fieschi, C.; Boisson-Dupuis, S.; Alcais, A.; Filipe-Santos, O.; Bustamante, J.; de Beaucoudrey, L.; Al-Mohsen, I.; Al-Hajjar, S.; Al-Ghonaium, A.; Adimi, P.; Mirsaeidi, M.; Khalilzadeh, S.; Rosenzweig, S.; de la Calle Martin, O.; Bauer, T. R.; Puck, J. M.; Ochs, H. D.; Furthner, D.; Engelhorn, C.; Belohradsky, B.; Mansouri, D.; Holland, S. M.; Schreiber, R. D.; Abel, L.; Cooper, D. N.; Soudais, C.; Casanova, J. L. Gains of glycosylation comprise an unexpectedly large group of pathogenic mutations. *Nat. Genet.* **2005**, *37* (7), 692–700.
(8) Vogt, G.; Vogt, B.; Chuzhanova, N.; Julenius, K.; Cooper, D. N.; Casanova, J. L. Gain-of-glycosylation mutations. *Curr. Opin. Genet. Dev.* **2007**, *17* (3), 245–51.
(9) Varki, A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **1993**, *3* (2), 97–130.
(10) Kobata, A. A journey to the world of glycobiology. *Glycoconjugate J.* **2000**, *17* (7−9), 443–64.
(11) Hizukuri, Y.; Yamanishi, Y.; Hashimoto, K.; Kanehisa, M. Extraction of species-specific glycan substructures. *Genome Inf.* **2004**, *15* (1), 69–81.
(12) Royle, L.; Matthews, E.; Corfield, A.; Berry, M.; Rudd, P. M.; Dwek, R. A.; Carrington, S. D. Glycan structures of ocular surface mucins in man, rabbit and dog display species differences. *Glycoconjugate J.* **2008**, *25* (8), 763–73.
(13) DeMarco, M. L.; Woods, R. J. Structural glycobiology: a game of snakes and ladders. *Glycobiology* **2008**, *18* (6), 426–40.
(14) Korner, C.; Lehle, L.; von Figura, K. Carbohydrate-deficient glycoprotein syndrome type 1: correction of the glycosylation defect by deprivation of glucose or supplementation of mannose. *Glycoconjugate J.* **1998**, *15* (5), 499–505.
(15) Ota, H.; Nakayama, J.; Momose, M.; Hayama, M.; Akamatsu, T.; Katsuyama, T.; Graham, D. Y.; Genta, R. M. Helicobacter pylori infection produces reversible glycosylation changes to gastric mucins. *Virchows Arch* **1998**, *433* (5), 419–26.
(16) Tangvoranuntakul, P.; Gagneux, P.; Diaz, S.; Bardor, M.; Varki, N.; Varki, A.; Muchmore, E. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (21), 12045–50.
(17) Rudan, I.; Campbell, H.; Rudan, P. Genetic epidemiological studies of eastern Adriatic Island isolates, Croatia: objective and strategies. *Coll. Antropol.* **1999**, *23* (2), 531–46.
(18) Rudan, I.; Biloglav, Z.; Vorko-Jovic, A.; Kujundzic-Tiljak, M.; Stevanovic, R.; Ropac, D.; Puntaric, D.; Cucevic, B.; Salzer, B.; Campbell, H. Effects of inbreeding, endogamy, genetic admixture, and outbreeding on human health: a (1001 Dalmatians) study. *Croat. Med. J.* **2006**, *47* (4), 601–10.
(19) Campbell, H.; Carothers, A. D.; Rudan, I.; Hayward, C.; Biloglav, Z.; Barac, L.; Pericic, M.; Janicijevic, B.; Smolej-Narancic, N.; Polasek, O.; Kolcic, I.; Weber, J. L.; Hastie, N. D.; Rudan, P.; Wright, A. F. Effects of genome-wide heterozygosity on a range of biomedically relevant human quantitative traits. *Hum. Mol. Genet.* **2007**, *16* (2), 233–41.
(20) Royle, L.; Campbell, M. P.; Radcliffe, C. M.; White, D. M.; Harvey, D. J.; Abrahams, J. L.; Kim, Y. G.; Henry, G. W.; Shadick, N. A.; Weinblatt, M. E.; Lee, D. M.; Rudd, P. M.; Dwek, R. A. HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal. Biochem.* **2008**, *376* (1), 1–12.
(21) Royle, L.; Radcliffe, C. M.; Dwek, R. A.; Rudd, P. M. Detailed structural analysis of N-glycans released from glycoproteins in SDS-PAGE gel bands using HPLC combined with exoglycosidase array digestions. *Methods Mol. Biol.* **2006**, *347*, 125–43.
(22) Campbell, M. P.; Royle, L.; Radcliffe, C. M.; Dwek, R. A.; Rudd, P. M. GlycoBase and autoGU: tools for HPLC-based glycan analysis. *Bioinformatics* **2008**, *24* (9), 1214–6.
(23) Almasy, L.; Blangero, J. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am. J. Hum. Genet.* **1998**, *62* (5), 1198–211.
(24) Visscher, P. M.; Hill, W. G.; Wray, N. R. Heritability in the genomics era—concepts and misconceptions. *Nat. Rev. Genet.* **2008**, *9* (4), 255–66.
(25) Flögel, M.; Lauc, G.; Gornik, I.; Maček, B. Fucosylation and galacosylation of IgG heavy chains differs between acute and remission phase of juvenile chronic arthritis. *Clin. Chem. Lab. Med.* **1998**, *36* (2), 99–102.
(26) Gornik, I.; Maravić, G.; Dumić, J.; Flögel, M.; Lauc, G. Fucosylation of IgG heavy chains is increased in rheumatoid arthritis. *Clin. Biochem.* **1999**, *32* (8), 605–8.
(27) Gornik, O.; Royle, L.; Harvey, D. J.; Radcliffe, C. M.; Saldova, R.; Dwek, R. A.; Rudd, P.; Lauc, G. Changes of serum glycans during sepsis and acute pancreatitis. *Glycobiology* **2007**, *17* (12), 1321–32.
(28) Gornik, O.; Gornik, I.; Gasparovic, V.; Lauc, G. Change in transferrin sialylation is a potential prognostic marker for severity of acute pancreatitis. *Clin. Biochem.* **2008**, *41* (7−8), 504–10.
(29) Arnold, J. N.; Saldova, R.; Hamid, U. M.; Rudd, P. M. Evaluation of the serum N-linked glycome for the diagnosis of cancer and chronic inflammation. *Proteomics* **2008**, *8* (16), 3284–93.
(30) Hartl, D. L.; Clark, A. G. *Principles of Population Genetics*, Fourth ed.; Sinauer Associates Inc.: Sunderland, MA, 2007.
(31) Abney, M.; McPeek, M. S.; Ober, C. Estimation of variance components of quantitative traits in inbred populations. *Am. J. Hum. Genet.* **2000**, *66* (2), 629–50.
(32) Ober, C.; Abney, M.; McPeek, M. S. The genetic dissection of complex traits in a founder population. *Am. J. Hum. Genet.* **2001**, *69* (5), 1068–79.
(33) Vanhooren, V.; Desmyter, L.; Liu, X. E.; Cardelli, M.; Franceschi, C.; Federico, A.; Libert, C.; Laroy, W.; Dewaele, S.; Contreras, R.; Chen, C. N-glycomic changes in serum proteins during human aging. *Rejuvenation Res.* **2007**, *10* (4), 521–31a.
(34) Przybylo, M.; Stepien, E.; Pfitzner, R.; Litynska, A.; Sadowski, J. Age effect on human aortic valvular glycoproteins. *Arch. Med. Res.* **2007**, *38* (5), 495–502.
(35) Przybylo, M.; Litynska, A. Changes in glycosylation of rat liver arylsulfatase B in relation to age. *Mech. Ageing. Dev.* **2000**, *113* (3), 193–203.
(36) Georgiou, S.; Pasmatzi, E.; Monastirli, A.; Sakkis, T.; Alachioti, S.; Tsambaos, D. Age-related alterations in the carbohydrate residue composition of the cell surface in the unexposed normal human epidermis. *Gerontology* **2005**, *51* (3), 155–60.
(37) Rudd, P. M.; Elliott, T.; Cresswell, P.; Wilson, I. A.; Dwek, R. A. Glycosylation and the immune system. *Science* **2001**, *291* (5512), 2370–6.

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