Effects of aging, body mass index, plasma lipid profiles, and smoking on human plasma *N*-glycans

Ana Knežević[2,*], Olga Gornik[2,*], Ozren Polašek[3],
Maja Pučić[4], Irma Redžić[2], Mislav Novokmet[4],
Pauline M Rudd[5], Alan F Wright[6], Harry Campbell[8],
Igor Rudan[7,8,9], and Gordan Lauc[1,2,4]

[2]University of Zagreb, Faculty of Pharmacy and Biochemistry, Ante Kovačića
1, 10000 Zagreb, Croatia, [3]Andrija Stampar School of Public Health,
University of Zagreb Medical School, Zagreb, Croatia, [4]Genos Ltd.,
Glycobiology Division, Planinska 1, 10000 Zagreb, Croatia, [5]National Institute
for Bioprocessing Research and Training, Dublin-Oxford Glycobiology Lab.,
Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland,
[6]MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine,
Western General Hospital, Crewe Road, Edinburgh, UK, [7]Department of
Public Health Sciences, The University of Edinburgh Medical School, Teviot
Place, Edinburgh, UK, [8]Croatian Centre for Global Health, University of Split
Medical School, Split, Croatia, and [9]Gen-info Ltd, Zagreb, Croatia

Protein glycosylation affects nearly all molecular interactions at the cell surface and in the intercellular space. Many of the physiological variations which are part of homeostatic mechanisms influence glycosylation. However, a comprehensive overview of changes in glycosylation caused by aging and common lifestyle parameters is still lacking. After analyzing *N*-glycans in the plasma of 1914 individuals from the Croatian islands of Vis and Korčula, we performed a comprehensive analysis of the dependence of different glycosylation features (position of fucose, level of galactosylation, sialylation and branching) on aging, smoking, body fat and plasma lipid status. A number of statistically significant associations were observed. Glycosylation changes with aging were especially evident in females, mostly in association with the transition from pre-menopausal to post-menopausal age. Levels of core-fucosylated, non-galactosylated, digalactosylated and disialylated biantennary glycans were shown to be mainly age dependent, but the level of branching and higher levels of galactosylation were found to correlate with lipid status. For the majority of glycans which we analyzed, all examined parameters explained up to 5% of the variance. The only notable exception were non-galactosylated glycans where 20% of the variance was explained mostly by age and blood pressure. In general, only a small fraction of the variability in glycan levels observed in a population was explained by age and other measured parameters, indicating that even in the absence of a genetic template, glycan levels are mostly determined by genetic background and/or specific pathophysiological processes.

Keywords: aging / body fat / human plasma glycome / *N*-glycans /
smoking

Introduction

Glycan chains attached to protein backbones are involved in nearly all molecular interactions at the cell surface and in the intercellular space. Changes in levels and composition of glycans were reported in numerous conditions (Packer et al. 2008). However, before we could assign the observed changes to the well-characterized physiologic and/or pathophysiological processes, a more extensive knowledge of baseline characteristics and natural variations in glycosylation around homeostasis is needed. Gender, race, age, reproductive cycle, developmental stage, environmental influences, dietary habits and lifestyle habits such as smoking and alcohol consumption, which are all well-known effect modifiers and confounders in epidemiology, could contribute to the variability of the human glycome. Until very recently, quantitative analysis of glycans in a large number of individuals was nearly an impossible task. Mass spectrometry and capillary electrophoresis can provide high sample throughput, but mass spectrometry is still not reliable enough for quantitative analysis of even much simpler peptide mixtures (Bell et al. 2009), and capillary electrophoresis lacks resolution for detailed analysis of complex biological samples (Callewaert et al. 2001).

High-performance liquid chromatography (HPLC) was a central tool for glycan analysis for decades, but it was only recently adapted for high-throughput glycan analysis (Royle et al. 2008). Using this method, we performed a first large-scale study of glycans in normal human population. By comparing plasma *N*-glycan levels in 1008 individuals, we revealed the high variability that exists in a human population and identified several environmental factors that were associated with changes in some glycans (Knežević et al. 2009). More recently, we demonstrated that the plasma glycan profile of a single individual is remarkably stable over a short period of time, and also for as long as 1 year (Gornik et al. 2009), indicating that glycan profiles may be under substantial genetic control.

One of the most prominent features of any individual is its age, but effects of aging on human plasma glycans are still mostly unknown (Klein 2008). Changes in glycan structures

[1]To whom correspondence should be addressed: Fax: +385 1 639 4400; e-mail:
glauc@pharma.hr
[*]These two authors contributed equally.

Table I. Age composition and the descriptives of the investigated glycans in men and women

	Both genders; median (IQR)	Men; median (IQR)	Women; median (IQR)	Gender differences (P)
Number of individuals	1914	742	1172	–
Age	57 (46–68)	58 (47–68)	56 (46–68)	0.145
Glycan feature				
Position of fucose				
Core fucosylated	23.02 (20.29–26.00)	23.09 (20.61–25.88)	23.01 (20.08–26.04)	0.275
Antennary fucosylated	2.96 (2.50–3.51)	3.17 (2.71–3.79)	2.82 (2.41–3.31)	<0.001
Level of sialylation				
Monosialylated	23.50 (21.78–25.92)	23.39 (21.75–25.85)	23.58 (21.81–26.01)	0.350
Disialylated	58.09 (56.42–59.50)	58.28 (56.72–59.85)	57.91 (56.28–59.34)	0.001
Trisialylated	15.00 (13.54–16.31)	14.93 (13.40–16.26)	15.03 (13.68–16.32)	0.159
Tetrasialylated	2.74 (2.02–3.89)	2.66 (1.93–3.69)	2.81 (2.06–3.98)	0.021
Degree of branching				
Biantennary	80.00 (78.22–81.74)	80.38 (78.58–82.21)	79.73 (78.03–81.45)	<0.001
Triantennary	16.28 (14.88–17.69)	16.02 (14.66–17.42)	16.39 (15.05–17.81)	<0.001
Tetraantennary	3.69 (3.16–4.28)	3.58 (3.05–4.18)	3.76 (3.24–4.33)	<0.001
Level of sialylation of biantennary glycans				
Monosialylated biantennary	30.19 (28.46–32.25)	30.01 (28.30–32.03)	30.27 (28.58–32.35)	0.011
Disialylated biantennary	71.08 (68.31–73.51)	71.32 (68.85–73.57)	70.86 (68.07–73.45)	0.012
Level of galactosylation				
Non-galactosylated	3.88 (3.12–4.75)	4.00 (3.33–4.79)	3.74 (2.98–4.73)	<0.001
Monogalactosylated	9.28 (8.33–10.23)	9.32 (8.43–10.29)	9.24 (8.27–10.21)	0.064
Digalactosylated	66.44 (64.71–68.26)	66.63 (64.89–68.43)	66.35 (64.52–68.16)	0.035
Trigalactosylated	13.29 (11.95–14.87)	13.06 (11.72–14.64)	13.42 (12.11–15.00)	0.003
Tetragalactosylated	1.35 (1.04–1.71)	1.23 (0.93–1.62)	1.43 (1.12–1.78)	<0.001
Biantennary non-galactosylated (A2)	0.16 (0.12–0.21)	0.17 (0.13–0.22)	0.16 (0.12–0.20)	<0.001

Glycans levels are presented as percentages of individual structural groups that add to 100% for each chromatographic analysis. For detailed description, see Materials and methods section; IQR, interquartile range.

Table II. Correlation coefficients of glycans and age in men and women

Glycan feature	Both genders; r, P	Men; r, P	Women; r, P
Position of fucose			
Core fucosylated	−0.22, $P < 0.001$	−0.08, $P = 0.025$	−0.31, $P < 0.001$
Antennary fucosylated	−0.02, $P = 0.305$	0.13, $P < 0.001$	−0.13, $P < 0.001$
Level of sialylation			
Monosialylated	−0.01, $P = 0.785$	0.13, $P < 0.001$	−0.09, $P = 0.001$
Disialylated	−0.02, $P = 0.508$	−0.11, $P = 0.002$	0.04, $P = 0.127$
Trisialylated	0.03, $P = 0.204$	−0.07, $P = 0.055$	0.10, $P = 0.001$
Tetrasialylated	0.02, $P = 0.463$	0.05, $P = 0.160$	0.00, $P = 0.930$
Degree of branching			
Biantennary	−0.04, $P = 0.057$	0.03, $P = 0.486$	−0.10, $P = 0.001$
Triantennary	0.04, $P = 0.084$	−0.04, $P = 0.282$	0.10, $P < 0.001$
Tetraantennary	0.05, $P = 0.017$	0.04, $P = 0.305$	0.08, $P = 0.009$
Level of sialylation of biantennary glycans			
Monosialylated biantennary	−0.06, $P = 0.011$	0.05, $P = 0.175$	−0.12, $P < 0.001$
Disialylated biantennary	0.25, $P < 0.001$	0.11, $P = 0.004$	0.33, $P < 0.001$
Level of galactosylation			
Non-galactosylated	0.46, $P < 0.001$	0.36, $P < 0.001$	0.52, $P < 0.001$
Monogalactosylated	0.05, $P = 0.038$	0.09, $P = 0.012$	0.02, $P = 0.520$
Digalactosylated	−0.29, $P < 0.001$	−0.18, $P < 0.001$	−0.36, $P < 0.001$
Trigalactosylated	0.06, $P = 0.010$	−0.04, $P = 0.329$	0.13, $P < 0.001$
Tetragalactosylated	−0.11, $P < 0.001$	−0.09, $P = 0.018$	−0.12, $P < 0.001$
Biantennary non-galactosylated (A2)	0.30, $P < 0.001$	0.18, $P < 0.001$	0.37, $P < 0.001$

with age were mainly analyzed for glycans on immunoglobulin G. The largest study was performed on 403 individuals, and the main observation was that non-galactosylated glycoforms and glycoforms containing bisecting GlcNAc increase with age (Yamada et al. 1997). Similar results were recently reported for the total human serum N-glycome in 100 individuals, but since the analysis was performed using capillary electrophoresis, inadequate resolution of the method limited this study to 10 most abundant glycan peaks (Vanhooren et al. 2007).

Smoking and dietary habits can influence life quality and life span. The World Health Organization claims that cardiovascular diseases are the world's largest killers, taking 17.1 million lives a year, and the most important behavioral risk factors of heart disease and stroke are unhealthy diet, physical inactivity and tobacco use. Behavioral risk factors are responsible for about 80% of coronary heart disease and cerebrovascular disease. Although there is no evidence that cardiovascular diseases and stress are directly associated with

Table III. Statistical significances (*P*) of differences between glycan levels after the sample breakdown in the gender and age groups

	All ages *P*	20–29 vs 30–39	30–39 vs 40–49	40–49 vs 50–59	50–59 vs 60–69	60–69 vs 70+
Men						
Position of fucose						
Core fucosylated	0.304	0.575	0.569	0.654	0.071	0.981
Antennary fucosylated	0.005	0.851	0.608	0.177	0.378	0.010
Level of sialylation						
Monosialylated	0.012	0.529	0.945	0.561	0.658	0.031
Disialylated	0.001	0.003	0.797	0.729	0.214	0.921
Trisialylated	0.019	0.067	0.873	0.534	0.663	0.013
Tetrasialylated	0.712	0.397	0.406	0.897	0.468	0.622
Degree of branching						
Biantennary	0.107	0.071	0.930	0.792	0.948	0.071
Triantennary	0.014	0.144	0.757	0.482	0.936	0.009
Tetraantennary	0.435	0.074	0.683	0.577	0.583	0.533
Level of sialylation of biantennary glycans						
Monosialylated biantennary	0.230	0.108	0.774	0.286	0.389	0.479
Disialylated biantennary	0.054	0.958	0.302	0.191	0.990	0.627
Level of galactosylation						
Non-galactosylated	<0.001	0.054	0.044	0.238	0.020	<0.001
Monogalactosylated	0.019	0.360	0.867	0.884	0.702	0.002
Digalactosylated	<0.001	0.008	0.201	0.859	0.292	0.194
Trigalactosylated	0.090	0.330	0.839	0.601	0.718	0.013
Tetragalactosylated	0.061	0.937	0.434	0.290	0.881	0.168
Biantennary non-galactosylated (A2)	<0.001	0.023	0.247	0.434	0.360	0.373
Women						
Position of fucose						
Core fucosylated	<0.001	0.115	0.922	<0.001	0.036	0.730
Antennary fucosylated	<0.001	0.023	0.804	0.615	0.694	0.039
Level of sialylation						
Monosialylated	0.001	0.391	0.880	<0.001	0.488	0.567
Disialylated	0.176	0.373	0.218	0.273	0.395	0.578
Trisialylated	<0.001	0.032	0.177	0.135	0.582	0.966
Tetrasialylated	0.010	0.544	0.117	0.001	0.151	0.152
Degree of branching						
Biantennary	<0.001	0.006	0.363	0.001	0.150	0.370
Triantennary	<0.001	0.007	0.636	<0.001	0.086	0.543
Tetraantennary	<0.001	0.066	0.369	0.024	0.776	0.144
Level of sialylation of biantennary glycans						
Monosialylated biantennary	0.001	0.306	0.594	0.011	0.349	0.842
Disialylated biantennary	<0.001	0.825	0.130	<0.001	0.010	0.894
Level of galactosylation						
Non-galactosylated	<0.001	0.573	0.221	<0.001	<0.001	<0.001
Monogalactosylated	0.230	0.321	0.874	0.476	0.853	0.073
Digalactosylated	<0.001	0.074	0.069	<0.001	0.141	0.063
Trigalactosylated	<0.001	0.015	0.500	<0.001	0.218	0.457
Tetragalactosylated	<0.001	0.006	0.552	0.010	0.108	0.025
Biantennary non-galactosylated (A2)	<0.001	0.041	<0.001	<0.001	0.019	0.929

changes in N-glycosylation, there is some evidence that smoking and weight affects N-glycosylation (Thompson et al. 1991) and that polymorphisms in some genes involved in glycosylation are associated with body mass (Aulchenko et al. 2009).

Here, we present the first comprehensive study of association between *N*-glycans in human plasma and some major variations usually present in a population: individuals' age, body mass characteristics, lipid biochemical status and smoking habits.

Results

By performing high-throughput HPLC analysis of 2-AB-labeled glycans, we analyzed the main *N*-glycan features in the plasma of 1914 individuals (742 males and 1172 females) between 18 and 98 years of age. Glycans were released from plasma proteins with PNGase F, labeled with 2-AB and analyzed by hydrophilic interaction (HILIC) and weak anion exchange (WAX) HPLC as described in the Materials and methods section. By performing three HPLC runs for each sample, we separated the total human *N*-glycome into 33 chromatographic peaks (16 groups of glycans before desialylation; 13 groups of desialylated glycans; 4 groups of differentially charged glycans). Structures of major glycans in each individual peak were reported previously (Knežević et al. 2009) and are shown in Supplementary Table 1. Individual glycan features (percentage of biantennary non-galactosylated, biantennary monogalactosylated, core fucosylated, etc.) were calculated by adding and subtracting the main glycan structures as described in the Materials and methods section.

Glycan levels are sex and age dependent

When we divided the population into gender groups, although the groups did not differ in their age composition, many of the

Table IV. Correlation coefficients between glycans and body weight parameters

Glycan feature	BMI	WH	Z	BF%	Systolic blood pressure	Diastolic blood pressure	Cholesterol	HDL	LDL	Triglycerides
Position of flucose										
Core fucosylated	−0.11[a]	−0.03	0.00	−0.06	−0.11[a]	−0.02	−0.18[a]	−0.14[a]	−0.15[a]	−0.02
Antennary fucosylated	−0.07	0.01	−0.14[a]	−0.13[a]	−0.03	−0.05	−0.04	0.03	−0.02	−0.10[a]
Degree of branching										
Biantennary	−0.07[a]	0.04	−0.05	−0.04	−0.07	−0.04	−0.25[a]	−0.18[a]	−0.20[a]	−0.10[a]
Triantennary	0.10[a]	−0.01	0.03	0.06	0.08[a]	0.04	0.25[a]	0.14[a]	0.19[a]	0.15[a]
Tetraantennary	−0.01	−0.08[a]	0.08	−0.01	0.06	0.01	0.18[a]	0.21[a]	0.17[a]	−0.05
Level of sialylation of biantennary glycans										
Monosialylated biantennary	−0.24[a]	−0.14[a]	0.17[a]	−0.07	−0.11[a]	−0.10[a]	0.00	0.10[a]	0.01	−0.12[a]
Disialylated biantennary	0.27[a]	0.20[a]	−0.16[a]	0.09[a]	0.21[a]	0.13[a]	0.10[a]	−0.05	0.08[a]	0.16[a]
Level of galactosylation										
Non−galactosylated	0.18[a]	0.28[a]	−0.12[a]	0.10[a]	0.32[a]	0.20[a]	0.02	−0.16[a]	0.04	0.09[a]
Monogalactosylated	−0.08[a]	0.01	0.00	−0.02	0.02	0.04	−0.13[a]	−0.12[a]	−0.07[a]	−0.09[a]
Digalactosylated	−0.13[a]	−0.11[a]	0.01	−0.09[a]	−0.24[a]	−0.15[a]	−0.17[a]	−0.04	−0.15[a]	−0.10[a]
Trigalactosylated	0.14[a]	0.02	0.00	0.06	0.10[a]	0.06	0.24[a]	0.14[a]	0.18[a]	0.16[a]
Tetragalactosylated	0.00	−0.17[a]	0.17[a]	−0.02	0.02	0.01	0.26[a]	0.37[a]	0.23[a]	−0.10[a]
Biantennary nongalactosylated (A2)	0.09[a]	0.15[a]	−0.16[a]	0.02	0.18[a]	0.11[a]	0.09[a]	−0.05	0.08[a]	0.09[a]

[a]$P \leq 0.001$; statistically significant correlation coefficients.

investigated glycans showed significant differences between the male and female groups, indicating that the levels of some glycans are sex dependent. These differences were generally rather small but nevertheless were statistically significant for many glycan groups (Table I).

In the next analysis step, all investigated glycans were correlated with age in order to identify glycan features for which the age composition of the population must always be taken in consideration in glycan studies. The results showed a number of significant correlations, usually larger in females than in males (Table II). The most considerable correlations were observed for biantennary non-galactosylated glycan, core-fucosylated glycans, disialylated forms of biantennary glycans, as well as non-galactosylated and digalactosylated glycans. Age-dependent increase in non-galactosylated glycans and corresponding decreases in digalactosylated glycans were observed in both males and females. The decreased level of digalactosylated glycans was accompanied with an increase in their sialylation. Interestingly, aging had opposing effects on fucosylation in males and females. Statistically significant decreases in both core and antennary fucose were observed in females, while an increase in antennary fucose was observed in males.

Since gender-specific differences in glycan changes could be the consequence of hormonal differences, the population was further divided into smaller age groups. Decade-based glycan analysis confirmed the existence of much stronger age-related patterns of glycan changes in females (Table III). While for males we detected only three significant associations when all the decades were compared together and only non-galactosylated glycans showed statistically significant difference between pairs of subsequent decades (in later age, over 60), in females we found significant differences between pairs of decade groups for almost all main groups of glycan character-

istics (Table III). The vast majority of statistically significant differences were restricted to differences between the age groups of 40–49 and 50–59, indicating that the majority of age-related changes in glycans were a consequence of transition from pre-menopausal to post-menopausal stages.

Glycan levels are related to body and plasma lipid status

Four body fat parameters (body mass index (BMI); waist to hip ratio (WH); bioelectrical resistive impedance (Z); and percentage of body fat (BF%)) were calculated as described in the Materials and methods section. Correlation coefficients between glycans and body fat parameters as well as systolic and diastolic blood pressures were calculated (Table IV). The pattern of statistically significant correlations ($P \leq 0.001$) was not the same for all four body fat parameters, but increased body fat and blood pressure were generally associated with an increase in the non-galactosylated and a decrease in digalactosylated glycans as well as an increase in sialylation of biantennary structures.

Levels of total blood cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol and triglycerides can be an indication of unhealthy dietary habits and lifestyle since even in genetically induced dyslipidemias levels of blood lipids can be controlled by diet (Stacpoole et al. 1981). Significant correlations ($P \leq 0.001$) in the direction of increased branching and sialylation and decreased core fucosylation were observed for all four lipid parameters. All three cholesterols were positively correlated with triantennary and tetraantennary structures, and this association was further confirmed by the same pattern of correlations between tri- and tetragalactosylated structures (Table IV).

Hyperlipidemic persons can often have normal body weight but, due to genetic predispositions or dietary habits, have high

Table V. Glycan levels in subpopulations of hyperlipidemic (*N* = 448) and normolipidemic (*N* = 1538) subjects presented trough median and 25th and 75th percentiles. In both groups, there are 25% males and 75% females.

Glycan feature	Hyperlipidemic; median (IQR)	Normolipidemic; median (IQR)	*P*
Position of fucose			
Core fucosylated	22.74 (19.95–25.52)	23.08 (20.23–25.93)	0.009
Antennary fucosylated	2.87 (2.41–3.32)	2.98 (2.47–3.49)	0.001
Degree of branching			
Biantennary	79.26 (77.54–80.98)	80.17 (78.39–81.95)	<0.001
Triantennary	16.78 (15.42–18.14)	16.08 (14.69–17.47)	<0.001
Tetraantennary	3.73 (3.18–4.28)	3.67 (3.10–4.25)	0.319
Level of sialylation of biantennary glycans			
Monosialylated biantennary	29.65 (27.92–31.37)	30.37 (28.45–32.29)	<0.001
Disialylated biantennary	72.19 (69.91–74.46)	70.73 (68.08–73.38)	<0.001
Level of galactosylation			
Non-galactosylated	3.94 (3.12–4.75)	3.87 (3.05–4.69)	0.096
Monogalactosylated	9.03 (8.04–10.02)	9.35 (8.41–10.29)	<0.001
Digalactosylated	65.94 (64.24–67.64)	66.61 (64.81–68.41)	<0.001
Trigalactosylated	13.97 (12.47–15.46)	13.10 (11.67–14.54)	<0.001
Tetragalactosylated	1.37 (1.03–1.70)	1.35 (1.02–1.68)	0.741
Biantennary non-galactosylated (A2)	0.17 (0.12–0.22)	0.16 (0.12–0.21)	0.001

Statistical significance (*P*) for differentiation of normolipidemic from hyperlipidemic group based on glycan levels is given.

blood cholesterol and/or triglycerides. As the primary aim of this study was to investigate lifestyle, and not obesity, we compared the hyperlipidemic group with the normolipidemic group. Statistically significant differences were observed for many glycan features (Table V).

Multivariate analysis

The pattern of correlations between body mass parameters and glycans was generally similar to the pattern of correlations between glycans and age, except for the monogalactosylated structures for which correlations were observed only for body fat parameters and not for aging. Since both body mass parameters and lipid status correlate with age, the possibility remained that some of the observed associations were a reflection of aging. To further investigate the significance of the impact of the analyzed personal characteristics on glycan levels, we performed a multiple regression analysis of age, body fat characteristics, lipid status and mean arterial pressure. Statistical significant associations (*P* values) are shown in Table VI and revealed which of the individual correlation coefficients shown in Table IV were the most statistically significant for changes in glycan levels. Levels of non-galactosylated glycans, digalactosylated glycans and core fucosylation were found to be influenced mostly by age, while the higher branching of glycans and their galactosylation levels were associated with plasma lipid status, with little or no age-dependent changes. Sialylation of biantennary structures was related to both age and body mass index.

Smoking is associated with increased branching and galactosylation of glycans

Smoking is one of the most important risk factors for premature mortality globally. The standard parameter for smoking exposure measurement is the "Pack Year" (number of cigarettes per day multiplied by years of smoking/20 cigarettes), but this parameter does not allow for separate testing of long- and short-term effects of smoking on glycans; thus, we used the number of cigarettes smoked per day and the number of years of smoking as two separate parameters. These parameters were also examined in people who stopped smoking at least 5 years ago. Correlation coefficients between different smoking parameters and glycan features are presented in Table VII. The strongest positive correlation of cigarettes smoked per day was observed with tetraantennary (*P* < 0.001) and tetragalactosylated (*P* < 0.001) glycans, while moderate correlations were also observed for core-fucosylated (*P* = 0.015) and biantennary glycans (*P* = 0.005).

To investigate differences in glycan levels between smokers, ex-smokers and non-smokers, the population was divided into three groups. Since we showed that age is an important factor that affects glycan levels, we attempted to control for the influence of age by selecting three subpopulations which comprised the same number of individuals of matching age. Comparison of glycan levels between these groups identified statistically significant differences in the levels of antennary fucosylated and non-galactosylated glycans (Table VIII).

Discussion

Using a recently developed method for high-throughput analysis of glycans, we performed the first comprehensive study of changes in total human plasma *N*-glycans with age and some factors that principally reflect lifestyle habits. Although aging is one of the most studied biological processes, little is known about the genetic and molecular mechanisms which influence this process in humans. Aging itself can be defined as a process which is genetically pre-determined, but this genetic influence must not be considered as programmed but rather as a physiological process that results from constant interaction between the genome and the environment (Ostojic et al. 2009). Considering the localization, sequence and function of protein glycosylation (Varki et al. 1999), it is logical to expect that glycosylation of plasma proteins would change during life in humans and that these changes will comprise many glycan structures.

Our results show that changes in levels of glycan features with age are sex dependent and generally more evident in women than in men. It is interesting that some glycosylation features have reciprocal age dependence in these two sex

Table VI. The association of various predictor variables and glycan features; linear regression analysis with percent of variance explained for each glycan feature with six predictors

| | Position of fucose | | Degree of branching | | | Level of sialylation of biantennary glycans | | | Level of galactosylation | | | | |
	Core fucosylated	Antennary fucosylated	Biantennary	Triantennary	Tetraantennary	Monosialylated Biantennary	Disialylated Biantennary	Nongalactosylated	Monogalactosylated	Digalactosylated	Trigalactosylated	Tetragalactosylated	Biantennary nongalactosylated (A2)
Age	<0.001	0.909	0.514	0.972	0.054	0.733	<0.001	<0.001	0.022	<0.001	0.662	0.006	<0.001
BMI	0.205	0.890	0.599	0.245	0.231	<0.001	<0.001	0.099	0.061	0.742	0.028	0.965	0.529
WH	0.562	0.823	0.938	0.898	0.939	0.363	0.366	0.695	0.488	0.644	0.683	0.611	0.151
HDL	0.002	0.142	<0.001	<0.001	<0.001	0.157	0.794	0.001	0.006	0.115	<0.001	<0.001	0.274
LDL	0.003	0.834	<0.001	<0.001	<0.001	0.292	0.345	0.006	0.003	0.002	<0.001	<0.001	0.918
Mean arterial pressure	0.117	0.155	0.753	0.443	0.381	0.009	0.043	<0.001	0.123	0.003	0.146	0.704	0.646
Variance explained (%)	0.065	0.003	0.053	0.049	0.035	0.018	0.062	0.197	0.019	0.088	0.052	0.086	0.048

Table VII. Correlation coefficients between glycans and smoking habit parameters for smokers and ex-smokers. Smokers are characterized as persons who have smoked for longer than 5 years and ex-smokers as persons who quit smoking at least 5 years ago

| | Smokers | | Ex–smokers | | |
Glycan feature	Cigarettes per day (DC)	Years of smoking (SY)	Cigarettes per day (ExDC)	Years of smoking (ExSY)	Years since ex–smoker stopped smoking (ExStop)
Position of fucose					
Core fucosylated	–0.19	–0.13	–0.21	–0.13	–0.08
Antennary fucosylated	0.09	0.17	0.21	0.20	–0.07
Degree of branching					
Biantennary	–0.22	0.07	–0.12	–0.01	–0.07
Triantennary	0.17	–0.08	0.12	0.02	0.04
Tetraantennary	0.29[a]	–0.04	0.07	–0.04	0.17
Level of sialylation of biantennary glycans					
Monosialylated biantennary	0.03	–0.04	–0.13	–0.10	0.07
Disialylated biantennary	0.09	0.13	0.20	0.13	0.02
Level of galactosylation					
Nongalactosylated	–0.14	0.15	–0.21	0.25[a]	0.04
Monogalactosylated	–0.16	0.00	–0.28[a]	–0.04	0.07
Digalactosylated	–0.07	0.00	0.05	–0.16	–0.18
Trigalactosylated	0.17	–0.06	0.15	0.03	0.04
Tetragalactosylated	0.34[a]	–0.11	0.16	–0.19	0.18
Biantennary nongalactosylated (A2)	0.01	0.20	–0.01	0.15	0.09

[a]$P \leq 0.001$; statistically significant correlation coefficients.

groups. While in women antennary fucosylation was decreasing with age, in men it increased at the equivalent rate. The level of monosialylated glycans in men rose with age on the account of di- and trisialylated glycans, while in women sialylation was almost not changing during life. A particularly important observation was the increase in sialylation of digalactosylated glycans that was found to accompany a decrease in the total level of digalactosylated glycans in both aging and increased body fat and blood pressure. This indicated that normal glycoprotein production exceeds the maximal capacity

of the sialylation machinery and that full sialylation can be achieved only when the production levels are decreased. This has important implications for the production of recombinant glycoproteins in human cell lines.

Both fucose and sialic acid are sugars involved in many pathological processes (Sillanaukee et al. 1999; Becker and Lowe 2003; Yasukawa et al. 2005), including inflammation, host immune response and tumor metastasis. Opposite changes in these glycan traits may be connected to immunological status, which changes with aging depending on sex, since sex hor-

Table VIII. Levels of *N*-glycan subclasses by subpopulation of smokers (*N* = 175), non-smokers (*N* = 175) and ex-smokers (*N* = 175) presented trough median and 25th and 75th percentiles. In both groups, males are present as 47.4% and females as 52.6%

Glycan feature	Smokers; median (IQR)	Non-smokers; median (IQR)	Ex-smokers; median (IQR)	Smokers vs Non-smokers vs ex-smokers (*P*)	Smokers vs non-smokers (*P*)	Smokers vs ex-smokers (*P*)	Non-smokers vs ex-smokers (*P*)
Position of fucose							
Core fucosylated	22.48 (19.63–25.32)	23.33 (20.53–26.14)	22.34 (19.72–24.97)	0.065	0.036	0.877	0.052
Antennary fucosylated	3.32 (2.71–3.92)	2.81 (2.30–3.33)	2.97 (2.51–3.43)	<0.001	<0.001	0.002	0.047
Degree of branching							
Biantennary	79.20 (77.20–81.19)	79.98 (78.28–81.69)	79.26 (77.13–81.39)	0.018	0.008	0.674	0.028
Triantennary	16.83 (15.49–18.16)	16.29 (14.92–17.67)	16.80 (15.19–18.41)	0.042	0.017	0.683	0.056
Tetraantennary	4.00 (3.36–4.63)	3.66 (3.11–4.22)	3.88 (3.29–4.47)	0.020	0.010	0.688	0.026
Level of sialylation of biantennary glycans							
Monosialylated biantennary	29.77 (27.99–31.54)	30.23 (28.39–32.07)	29.85 (27.93–31.78)	0.110	0.060	0.867	0.081
Disialylated biantennary	72.04 (69.84–74.24)	70.96 (68.57–73.35)	71.75 (69.04–74.47)	0.017	0.005	0.508	0.047
Level of galactosylation							
Non-galactosylated	3.51 (2.76–4.25)	3.95 (3.22–4.69)	3.84 (3.12–4.56)	<0.001	<0.001	0.012	0.047
Monogalactosylated	8.91 (8.03–9.78)	9.42 (8.49–10.36)	8.98 (8.01–9.96)	0.008	0.003	0.510	0.024
Digalactosylated	66.29 (64.59–67.98)	66.57 (64.65–68.49)	66.20 (64.69–67.72)	0.701	0.995	0.438	0.496
Trigalactosylated	13.96 (12.39–15.53)	13.39 (12.06–14.72)	13.99 (12.29–15.70)	0.046	0.028	0.937	0.037
Tetragalactosylated	1.44 (1.03–1.84)	1.45 (1.09–1.81)	1.54 (1.25–1.84)	0.112	0.593	0.046	0.130
Biantennary non-galactosylated (A2)	0.17 (0.12–0.23)	0.16 (0.12–0.21)	0.15 (0.10–0.20)	0.230	0.242	0.091	0.671

Statistical significance (*P*) for differentiation of all three or separate pairs of groups based on glycan levels are given.

mones were shown to exhibit opposite inflammatory effects. In contrast to antennary fucose, a discernable negative correlation for core-fucosylated glycans was observed only in the female group, suggesting different roles for these two forms of fucosylation in the biology of aging.

The most prominent difference in glycan levels in women are between age groups of 40–49 and 50–59, which implies the influence of hormonal status, since in this life period women usually enter the menopause and their hormonal status changes. The influence of hormones on glycan structures has been reported previously, and it is known that the level of estrogen correlates negatively with the sialyl Lewis X (tri-sialylated antennary fucosylated) glycan structure (Brinkman-Vander Linden et al. 1996). We did not find significant changes in total antennary fucosylation between these two critical age groups. Moreover, a modest decline with age was observed, suggesting that estrogen influence could be limited to sialyl Lewis X structure and not antennary fucosylation in general. It is also known that galactosylation of IgG increases during pregnancy, supporting the theory of hormonal influence on glycosylation changes. In men, statistically significant changes involve only a few glycan features and occur in later age groups. The only observed exception was galactosylation, for which the best linear associations with aging were detected for non-galactosylated glycan structures in both sex groups. These glycan structures are typical of IgG glycans and thus mainly reflect increased agalactosylation of IgG with aging. The increase in non-galactosylated structures was accompanied by a decrease in digalactosylated structures, while the level of monogalactosylated structures stayed the same, confirming the initial observation of glycan changes on IgG from over 20 years ago (Parekh, Roitt, Isenberg, Dwek and Rademacher 1988).

Although it is known that the process of "hormonal" aging in men exists, only a limited number of men experience the problems of this gradual loss of testosterone. However, menopause, a complete cessation of reproductive ability caused by the shutting down of the female reproductive system, occurs at once and affects the whole female population. It can be objected that the evident changes in glycosylation in women are a consequence of hormonal changes rather than the aging process itself and that changes in men better reflect the real glycan behavior caused by genetically pre-determined aspects of aging. However, women usually live longer than men, and maybe the linkage between hormonal status and aging should not be considered separately. Improvements in healthcare have also extended the human lifespan, and, although the process of aging is partially under genetic control it is also further influenced by the environment, and therefore the biological age of the individual does not necessarily correspond to chronological age (Wahlin et al. 2006).

Estimation of body fat composition can be performed in several ways, and each method has its limitations (Lukaski et al. 1985; Deurenberg et al. 1991; Turconi et al. 2006; Henneberg and Ulijaszek 2010). To avoid errors of individual estimations, we calculated all four scores to achieve the most reliable results. When we correlated these body fat parameters with the main structural features of glycans, levels of non-galactosylated and disialylated biantennary glycans were found to positively correlate with all four body fat parameters, but since these glycans were also strongly correlated with age after including all these characteristics in a multivariate analysis, the association of body fat parameters with these glycans diminished.

Human body fat levels are often connected with lipid status; therefore, we also looked for associations between glycans and cholesterol and lipoproteins. All lipoproteins transport cholesterol, and for many of them N-glycosylation is essential for function (Vukmirica et al. 2002; Vinals et al. 2003; Qu et al. 2006; Skropeta et al. 2007), but there are few data describing structural features of these N-linked glycans (Harazono et al. 2005). Correlation of glycan features with these biochemical parameters revealed that tetragalactosylated structures posi-

tively correlate with all of them, except triglycerides, for which correlation was in the opposite direction. It is unlikely that correlations between LDL and HDL and these glycans are the consequence of changes in the glycosylation of lipoproteins since most glycans on apoproteins are mono- or disialylated biantennary structures. Apo B100, which is part of LDL, is highly glycosylated, and its disialylated structures were shown to be decreased in hypercholesterolemia in rabbits (Millar 2001). It is known that obesity results in low-grade chronic inflammation (Dupuis et al. 2010), and there is evidence that C-reactive protein is elevated with weight gain (Saito et al. 2003), and increased tri- and tetra-sialylated structures are usually connected to inflammation (Gornik and Lauc 2008). Also, although in the absence of acute or chronic inflammation, HDL acts as an anti-inflammatory agent, with the onset of a systemic inflammatory state such as atherosclerosis, HDL becomes pro-inflammatory, enhancing the inflammatory response (Parekh, Roitt, Isenberg, Dwek, Ansell, et al. 1988; Van Lenten et al. 1995).

Smoking is an important behavioral risk factor for premature mortality that usually reflects lifestyle and attitudes toward personal health. Our results indicated that the number of cigarettes smoked per day is associated with increased levels of tetraantennary ($P < 0.001$) and tetragalactosylated ($P < 0.001$) structures and decreased levels of biantennary structures ($P = 0.005$) (Table VII). These changes reflect increased N-glycan branching in smokers and might be associated with smoking-related inflammation since these changes are associated with inflammatory processes (Gornik and Lauc 2008). This could also partly be the consequence of a decrease in IgG concentration, since higher consumption of cigarettes was reported to result in decreased IgG levels (McMillan et al. 1997). Core fucosylation was also decreased in smokers ($P = 0.015$) (Table VII), confirming the observation of decreased FUT8 activity in mice exposed to cigarette smoke in laboratory conditions (Taniguchi 2009). These novel observations represent preliminary findings which merit further study and replication in other populations. They provide some initial data on associations between important behavioral health risks and changes in levels of N-glycans which may yield new insights into mechanisms through which these risks may be mediated.

Conclusions

For the majority of glycans which we analyzed, all parameters together explained only up to 5% of the variance. The only notable exceptions were non-galactosylated glycans where 20% of the variance was explained. Since for the majority of glycans only a small fraction of variability observed in a population was explained by age and lifestyle parameters, it appears that even in the absence of a genetic template, glycan levels are mostly determined by genetic background and/or specific pathophysiological processes. Nevertheless, changes in glycan levels associated with age and environmental influences should be taken into consideration when glycan changes in different pathological conditions are being studied. Age is a particularly significant factor that relates to the glycosylation profiles of females, especially around menopausal age, which is the life period during which many pathological conditions in women originate. Glycosylation changes are a sensitive indicator of changes in the external or internal environment of the cell. Our increasing understanding of the inter-relatedness of the many systems that underpin both glycosylation and disease processes will provide tools with which we probe the relationships between them.

Materials and methods

Plasma samples

Blood samples were collected from unselected examinees from two Croatian Adriatic islands, Vis and Korcula as part of a larger genetic epidemiology program (Rudan et al. 1999, 2006, 2009). The project aims were initially to describe the variability and genetic epidemiology of isolated island communities (Carothers et al. 2006; Polasek et al. 2006; Campbell et al. 2007; Vitart et al. 2008), while in the later stages the focus has changed towards gene mapping efforts, some of which included notable successes (Vitart et al. 2008; Johansson et al. 2009; Dupuis et al. 2010). All these successes support the suggestion that isolated communities present highly interesting research resources in human genetics (Vitart et al. 2006). Participants were recruited on the basis of the official voting registers and contacts with the local stakeholders and religious communities in both islands. Sampling on the Vis Island took place in 2003 and 2004, while in Korcula Island it took place in 2007. All examinees were given detailed study information prior to the enrolment, and they had to sign the informed consent to enter the study. The study was approved by the appropriate ethical committees in both Croatia and Scotland. A total of 1991 individuals were recruited; 77 of them lacked data for some of the analyzed parameters and were excluded from the study, which was performed on 1914 individuals.

Over 250 different phenotypic traits of each subject were measured, supplemented by the blood samples. Blood was taken in epruvetes containing anticoagulant. Each blood sample was immediately processed; plasmas were separated and stored at −70°C.

Glycan analysis

Glycan release and labeling was performed as reported previously (Knežević et al. 2009). Plasma proteins were immobilized in a block of sodium dodecyl sulfate-polyacrylamide gel, and N-glycans were released by digestion with recombinant N-glycosidase F. This was done in a 96-well microtiter plate to achieve the best throughput of sample preparation. After extraction, glycans were fluorescently labeled with 2-aminobenzamide.

Hydrophilic interaction high-performance liquid chromatography

Released glycans were 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 (Tosoh Bioscience, Stuttgart, Germany) at 30°C with 50 mM formic acid adjusted to pH 4.4 with ammonia solution as solvent A and acetonitrile as solvent B. Sixty-minute runs were performed with 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).

The chromatograms obtained were all separated in the same manner to 16 chromatographic areas and 13 for desialylated glycans, considering the peak resolutions and similarity of glycan structures present as described before. The amounts of glycans present in each area were expressed as percent of total integrated chromatogram (amount of total glycan structures/total serum *N*-glycome).

Weak anion exchange-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, USA) 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 96-well polymerase chain reaction plates. To these, the following were 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 AcroPrep™ 96 Filter Plates, 350 µL well, 10K (Pall Corporation, Port Washington, NY, USA) before applying to the HPLC.

Glycan structural features

Levels of glycans sharing the same structural features were approximated by adding the structures having the same characteristic, from either HILIC, WAX or after sialidase treatment-integrated glycan profiles (individual glycan structures present in each glycan group that were reported previously are shown in Supplementary Table 1). Glycans were quantified from WAX profiles according the level of sialylation (monosialylated, disialylated, trisialylated, tetrasialylated). Glycan features were defined as: core-fucosylated glycans (FUC-C) = DG6/(DG5 + DG6) * 100; antennary fucosylated glycans (FUC-A) = DG7/(DG5 + DG7) * 100; biantennary glycans (BA) = DG1 + DG2 + DG3 + DG4 + DG5 + DG6 + DG7; monosialylated biantennary glycans (BAMS) = (G7 + G8)/(DG5 + DG6 + DG7) * 100; disialylated biantennary glycans BADS = (G9 + G10 + G11)/(DG5 + DG6 + DG7) * 100; triantennary glycans (TRIA) = DG8 + DG9 + DG10; tetraantennary glycans (TA) = DG11 + DG12 + DG13; non-galactosylated glycans (G0) = DG1 + DG2; monogalactosylated glycans (G1) = DG3+DG4; digalactosylated glycans (G2) = DG5 + DG6 + DG7; trigalactosylated glycans (G3) = GP12 + GP13 + GP14; tetragalactosylated glycans (G4) = GP15 + GP16; biantennary non-galactosylated glycan (A2) = (GP1 + DG1)/2.

Observed parameters of dietary habits and smoking

Estimation of body fat composition can be done in several ways, but they do not uniformly represent dietary habits. In this study, we used four anthropometric body fat estimation parameters: BMI, WH, bioelectrical resistive impedance (Z) and percentage of body fat calculated by Durnin–Womersley equation from four skinfold measurements (BF%). All subjects were measured for height, weight, hip circumference, waist circumference and skinfold thickness using calipers at four sites: biceps, triceps, subscapular and suprailiac. Bioelectrical resistive impedance was measured through resistance (*R*) and reactivity (Xc) just for Vis samples using STA-BIA analyser (Akern, Italy).

From these measured values, desirable parameters were calculated as follows:

$$BMI = \text{weight (kg)/height}^2 \text{ (m}^2)$$
$$WH = \text{waist circumference (mm)/hip circumference (mm)}$$
$$Z = \sqrt{(R^2(\Omega) + Xc^2(\Omega))}$$
$$BF\% = 495/(d - 450), \text{ where density } (d) \; d = c - m * \log S,$$
$$S = \text{sum of all four skinfolds (mm)}$$

and *c* and *m* are age/sex-related constants (Durnin and Womersley 1974).

We recorded systolic and diastolic blood pressure as average value of two independent measurements.

Biochemical parameters (lipoproteins, total blood cholesterol and triglycerides) can be more precise in estimating dietary habits than anthropometric measurements.

Total blood cholesterol was measured by photometry with cholesterol oxidase, triglycerides by UV photometry with glycerolphosphate-oxidase, HDL-cholesterol by homogene enzyme method with modified polyethylene glycol and acyclohexane-sulfate, and LDL-cholesterol was calculated according to Friedewald if the triglycerides were lower than 4.6 mmol/L (lab referent range).

Familial combined hyperlipidemia is the most common genetic dyslipidemia and a major risk factor for early coronary heart disease but dyslipidemia with same physiological effect (high cholesterol and triglycerides) can be also the result of bad dietary habits. We defined a subgroup of hyperlipidemic subjects as: total cholesterol >5.2 mmol/L and triglycerides >1.69 mmol/L.

Effects of smoking were based on average day consumption of cigarettes (DC) and number of smoking years (SY) for smokers and average day consumption of cigarettes (ExDC), number of smoking years (ExSY) and number of years since ex-smoker stopped smoking (ExStop) for ex-smokers. A smoker was defined as a subject who smoked for at least 5 years, ex-smokers were defined as subjects who smoked in the past for at least 5 years and stopped smoking at least 5 years ago. Non-smokers were subjects who never smoked. To avoid sex and age corrections, we created a random subpopulation of 175 smokers, 175 non-smokers and 175 ex-smokers matching by average age and percentage of males and females (in each group there were 47.4% males and 52.6% females).

Statistical analysis

The descriptive part of the statistical analysis was aimed at showing the basic characteristics of the population. The population was then tested for normality using Smirnov–Kolmogorov test, and nonparametric statistical tests were further used. Mann–Whitney test was used to analyze differences in levels of glycans between groups (males/females,

normolipidemic/hyperlipidemic and smokers/ex-smokers/non-smokers), while the Spearman's rank correlation was calculated for association of glycans' structures and age as well as all observed parameters of smoking and dietary habits. The multiple linear regression was used to analyze the association of selected predictor variables and each glycan feature.

Significance level was set at $P \leq 0.001$.

Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Acknowledgements

Funding

Abbreviations

BF%, percentage of body fat; BMI, body mass index; HDL, high-density lipoprotein; HILIC, hydrophilic interaction; HPLC, High-performance liquid chromatography; LDL, low-density lipoprotein; WAX, weak anion exchange; WH, waist to hip ratio.

References

Aulchenko YS, Ripatti S, Lindqvist I, Boomsma D, Heid IM, Pramstaller PP, Penninx BW, Janssens AC, Wilson JF, Spector T, et al. 2009. Loci influencing lipid levels and coronary heart disease risk in 16 European population cohorts. *Nat Genet*. 41:47–55.

Becker DJ, Lowe JB. 2003. Fucose: biosynthesis and biological function in mammals. *Glycobiology*. 13:41R–53R.

Bell AW, Deutsch EW, Au CE, Kearney RE, Beavis R, Sechi S, Nilsson T, Bergeron JJ, Beardslee TA, Chappell T, et al. 2009. A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. *Nat Methods*. 6:423–430.

Brinkman-Vander Linden CM, Havenaar EC, Van Ommen CR, Van Kamp GJ, Gooren LJ, Van Dijk W. 1996. Oral estrogen treatment induces a decrease in expression of sialyl Lewis x on a1-acid glycoprotein in females and male-to-female transsexuals. *Glycobiology*. 6:407–412.

Callewaert N, Geysens S, Molemans F, Contreras R. 2001. Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard DNA-sequencing equipment. *Glycobiology*. 11:275–281.

Campbell H, Carothers AD, Rudan I, Hayward C, Biloglav Z, Barac L, Pericic M, Janicijevic B, Smolej-Narancic N, Polasek O, et al. 2007. Effects of genome-wide heterozygosity on a range of biomedically relevant human quantitative traits. *Hum Mol Genet*. 16:233–241.

Carothers AD, Rudan I, Kolcic I, Polasek O, Hayward C, Wright AF, Campbell H, Teague P, Hastie ND, Weber JL. 2006. Estimating human inbreeding coefficients: comparison of genealogical and marker heterozygosity approaches. *Ann Hum Genet*. 70:666–676.

Deurenberg P, Weststrate JA, Seidell JC. 1991. Body mass index as a measure of body fatness: age- and sex-specific prediction formulas. *Br J Nutr*. 65:105–114.

Dupuis J, Langenberg C, Prokopenko I, Saxena R, Soranzo N, Jackson AU, Wheeler E, Glazer NL, Bouatia-Naji N, Gloyn AL. 2010. New genetic loci implicated in fasting glucose homeostasis and their impact on type 2 diabetes risk. *Nat Genet*. 42:105–116.

Durnin JV, Womersley J. 1974. Body fat assessed from total body density and its estimation from skinfold thickness: measurements on 481 men and women aged from 16 to 72 years. *Br J Nutr*. 32:77–97.

Gornik O, Lauc G. 2008. Glycosylation of serum proteins in inflammatory diseases. *Dis Markers*. 25:267–278.

Gornik O, Wagner J, Pučić M, Knežević A, Redžić I, Lauc G. 2009. Stability of *N*-glycan profiles in human plasma. *Glycobiology*. 19:1547–1553.

Harazono A, Kawasaki N, Kawanishi T, Hayakawa T. 2005. Site-specific glycosylation analysis of human apolipoprotein B100 using LC/ESI MS/MS. *Glycobiology*. 15:447–462.

Henneberg M, Ulijaszek SJ. 2010. Body frame dimensions are related to obesity and fatness: lean trunk size, skinfolds, and body mass index. *Am J Hum Biol*. 22:83–91.

Johansson A, Marroni F, Hayward C, Franklin CS, Kirichenko AV, Jonasson I, Hicks AA, Vitart V, Isaacs A, Axenovich T, et al. 2009. Linkage and genome-wide association analysis of obesity-related phenotypes: association of weight with the MGAT1 gene. *Obesity*. Silver Spring.

Klein A. 2008. Human total serum *N*-glycome. *Adv Clin Chem*. 46:51–85.

Knežević A, Polašek O, Gornik O, Rudan I, Campbell H, Hayward C, Wright A, Kolčić I, O'Donoghue N, Bones J, et al. 2009. Variability, heritability and environmental determinants of human plasma *N*-glycome. *J Proteome Res*. 8:694–701.

Lukaski HC, Johnson PE, Bolonchuk WW, Lykken GI. 1985. Assessment of fat-free mass using bioelectrical impedance measurements of the human body. *Am J Clin Nutr*. 41:810–817.

McMillan SA, Douglas JP, Archbold GP, McCrum EE, Evans AE. 1997. Effect of low to moderate levels of smoking and alcohol consumption on serum immunoglobulin concentrations. *J Clin Pathol*. 50:819–822.

Millar JS. 2001. The sialylation of plasma lipoproteins. *Atherosclerosis*. 154:1–13.

Ostojic S, Pereza N, Kapovic M. 2009. A current genetic and epigenetic view on human aging mechanisms. *Coll Antropol*. 33:687–699.

Packer NH, von der Lieth CW, Aoki-Kinoshita KF, Lebrilla CB, Paulson JC, Raman R, Rudd P, Sasisekharan R, Taniguchi N, York WS. 2008. Frontiers in glycomics: bioinformatics and biomarkers in disease. An NIH white paper prepared from discussions by the focus groups at a workshop on the NIH campus, Bethesda MD (September 11–13, 2006). *Proteomics*. 8:8–20.

Parekh R, Roitt I, Isenberg D, Dwek R, Rademacher T. 1988. Age-related galactosylation of the N-linked oligosaccharides of human serum IgG. *J Exp Med*. 167:1731–1736.

Parekh RB, Roitt IM, Isenberg DA, Dwek RA, Ansell BM, Rademacher TW. 1988. Galactosylation of IgG associated oligosaccharides: reduction in patients with adult and juvenile onset rheumatoid arthritis and relation to disease activity. *Lancet*. 1:966–969.

Polasek O, Kolcic I, Smoljanovic A, Stojanovic D, Grgic M, Ebling B, Klaric M, Milas J, Puntaric D. 2006. Demonstrating reduced environmental and genetic diversity in human isolates by analysis of blood lipid levels. *Croat Med J*. 47:649–655.

Qu SJ, Fan HZ, Gillard BK, Pownall HJ. 2006. N-Glycosylation is required for secretion-competent human plasma phospholipid transfer protein. *Protein J*. 25:167–173.

Royle L, Campbell MP, Radcliffe CM, White DM, Harvey DJ, Abrahams JL, Kim YG, Henry GW, Shadick NA, Weinblatt ME, et al. 2008. HPLC-based analysis of serum *N*-glycans on a 96-well plate platform with dedicated database software. *Anal Biochem*. 376:1–12.

Rudan I, Biloglav Z, Vorko-Jovic A, Kujundzic-Tiljak M, Stevanovic R, Ropac D, Puntaric D, Cucevic B, Salzer B, Campbell H. 2006. Effects of inbreeding, endogamy, genetic admixture, and outbreeding on human health: a (1001 Dalmatians) study. *Croat Med J*. 47:601–610.

Rudan I, Rudan P. 1999. Genetic epidemiological studies of eastern Adriatic Island isolates, Croatia: objective and strategies. *Coll Antropol*. 23:531–546.

Rudan I, Marusic A, Jankovic S, Rotim K, Boban M, Lauc G, Grkovic I, Dogas Z, Zemunik T, Vatavuk Z, et al. 2009. "10001 Dalmatians:" Croatia launches its national biobank. *Croat Med J*. 50:4–6.

Saito I, Yonemasu K, Inami F. 2003. Association of body mass index, body fat, and weight gain with inflammation markers among rural residents in Japan. *Circ J*. 67:323–329.

Sillanaukee P, Ponnio M, Jaaskelainen IP. 1999. Occurrence of sialic acids in healthy humans and different disorders. *European journal of clinical investigation*. 29:413–425.

Skropeta D, Settasatian C, McMahon MR, Shearston K, Caiazza D, McGrath KC, Jin W, Rader DJ, Barter PJ, Rye KA. 2007. N-Glycosylation regulates endothelial lipase-mediated phospholipid hydrolysis in apoE- and apoA-I-containing high density lipoproteins. *J Lipid Res*. 48:2047–2057.

Stacpoole PW, Grundy SM, Swift LL, Greene HL, Slonim AE, Burr IM. 1981. Elevated cholesterol and bile acid synthesis in an adult patient with homozygous familial hypercholesterolemia. Reduction by a high glucose diet. *J Clin Invest*. 68:1166–1171.

Taniguchi N. 2009. Role of "Glycan cycle" in disease; lessons from functional glycomics. *Glycoconjugate J*. 26:825–825.

Thompson S, Matta KL, Turner GA. 1991. Changes in fucose metabolism associated with heavy drinking and smoking: a preliminary report. *Clin Chim Acta*. 201:59–64.

Turconi G, Guarcello M, Maccarini L, Bazzano R, Zaccardo A, Roggi C. 2006. BMI values and other anthropometric and functional measurements as predictors of obesity in a selected group of adolescents. *Eur J Nutr*. 45:136–143.

Van Lenten BJ, Hama SY, de Beer FC, Stafforini DM, McIntyre TM, Prescott SM, La Du BN, Fogelman AM, Navab M. 1995. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J Clin Invest*. 96:2758–2767.

Vanhooren V, Desmyter L, Liu XE, Cardelli M, Franceschi C, Federico A, Libert C, Laroy W, Dewaele S, Contreras R, et al. 2007. N-glycomic changes in serum proteins during human aging. *Rejuvenation Res*. 10:521–531a.

Varki A, et al. 1999. *Essentials of glycobiology*. New York: Cold Spring Harbor Laboratory Press.

Vinals M, Xu S, Vasile E, Krieger M. 2003. Identification of the N-linked glycosylation sites on the high density lipoprotein (HDL) receptor SR-BI and assessment of their effects on HDL binding and selective lipid uptake. *J Biol Chem*. 278:5325–5332.

Vitart V, Biloglav Z, Hayward C, Janicijevic B, Smolej-Narancic N, Barac L, Pericic M, Klaric IM, Skaric-Juric T, Barbalic M, et al. 2006. 3000 years of solitude: extreme differentiation in the island isolates of Dalmatia, Croatia. *Eur J Hum Genet*. 14:478–487.

Vitart V, Rudan I, Hayward C, Gray NK, Floyd J, Palmer CN, Knott SA, Kolcic I, Polasek O, Graessler J, et al. 2008. SLC2A9 is a newly identified urate transporter influencing serum urate concentration, urate excretion and gout. *Nat Genet*. 40:437–442.

Vukmirica J, Nishimaki-Mogami T, Tran K, Shan J, McLeod RS, Yuan J, Yao Z. 2002. The N-linked oligosaccharides at the amino terminus of human apoB are important for the assembly and secretion of VLDL. *J Lipid Res*. 43:1496–1507.

Wahlin A, MacDonald SW, deFrias CM, Nilsson LG, Dixon RA. 2006. How do health and biological age influence chronological age and sex differences in cognitive aging: moderating, mediating, or both? *Psychology and aging*. 21:318–332.

Yamada E, Tsukamoto Y, Sasaki R, Yagyu K, Takahashi N. 1997. Structural changes of immunoglobulin G oligosaccharides with age in healthy human serum. *Glycoconjugate J*. 14:401–405.

Yasukawa Z, Sato C, Kitajima K. 2005. Inflammation-dependent changes in alpha2, 3-, alpha2, 6-, and alpha2, 8-sialic acid glycotopes on serum glycoproteins in mice. *Glycobiology*. 15:827–837.