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Stability of *N*-glycan profiles in human plasma

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Glycan heterogeneity was shown to be associated with numerous diseases and glycan analysis has a great diagnostic potential. Recently, we reported high biological variability of human plasma *N*-glycome at the level of population. The observed variations were larger than changes reported to be associated with some diseases; thus, it was of great importance to examine the temporal constancy of human *N*-glycome before glycosylation changes could be routinely analyzed in diagnostic laboratories. Plasma samples were taken from 12 healthy individuals. The blood was drawn on seven occasions during 5 days. *N*-Linked glycans, released from plasma proteins, were separated using hydrophilic interaction high-performance liquid chromatography into 16 groups (GP1-GP16) and quantified. The results showed very small variation in all glycan groups, indicating very good temporal stability of *N*-glycome in a single individual. Coefficients of variation from 1.6% for GP8 to 11.4% for GP1 were observed. The average coefficient of variation was 5.6%. These variations were comparable to those observed when analytical procedure was tested for its precision. Good stability of plasma *N*-glycome in healthy individuals implies that glycosylation is under significant genetic control. Changes observed in glycan profiles are consequence of environmental influences and physiologic responses and therefore have a significant diagnostic potential.

Keywords: glycan analysis/human plasma/glycome stability/ *N*-glycans/protein glycosylation

Introduction

Recent advances in technical sciences, molecular biology, and pharmacy significantly improved medicine and healthcare. However, everyday clinical problem of early diagnosis and prognosis of many pathological conditions using simple human material, such as plasma, still remains a big issue (Dellinger et al. 2004; Besselink et al. 2007). New potential disease markers are continuously being identified, but both markers and methods for their determination should be rigorously tested and optimized before they could be applied for early diagnosis and/or treatment.

Almost all extracellular proteins in higher eukaryotic organisms are glycosylated (Apweiler et al. 1999) and their glycan parts can be present in various structural forms, resulting in different glycoforms of the same molecule. This structural variability reflects the origin of the molecule and reveals the physiological and biochemical conditions in the cell and tissue that produced it at the moment of the release. Glycan changes were shown to be associated with many diseases (De Graaf et al. 1993; Brinkman-van der Linden et al. 1996; Axford 1999, 2001; Gornik et al. 1999; Anderson et al. 2002; Higai et al. 2003; Block et al. 2005; Croce et al. 2005; Grzymislawski et al. 2006; Wuhrer 2007; Gornik and Lauc 2008; Miyoshi et al. 2008; Tanabe et al. 2008; Wuhrer et al. 2009). The reported changes vary from minor to considerable differences that can also be very specific for certain condition; thus, studies on serum protein glycosylation offer a good basis for diagnosis and prognosis of many diseases (Hashimoto et al. 2004; Gornik et al. 2008). It was shown that glycan structures change under many pathological conditions, both of acute and chronic nature (Canellada and Margni 2002; Higai et al. 2005). These changes occur very early in acute processes (Chavan et al. 2005; Piagnerelli et al. 2005; Gornik et al. 2007) and sometimes even follow progression of the disease (Hashimoto et al. 2004; Piagnerelli et al. 2005; Ceciliani and Pocacqua 2007).

Since branched structures of sugars make analysis of glycoconjugates very challenging, different approaches using various analytical methods have been applied in studying protein glycosylation patterns aiming to reveal as many potential disease markers in the most reliable, fast, and simple manner (Sagi et al. 2002; Morelle et al. 2006; Royle et al. 2006; Gornik and Lauc 2007; Gornik et al. 2007). Until recently, detailed quantitative analysis of glycans was generally performed on a very limited number of patients, but the latest advance in analytical procedures created the possibility of reliably quantifying glycans in a large number of samples giving the glycoanalysis more realistic diagnostic potential (Royle et al. 2008). Using the newly developed method, we recently performed the first study of glycan variability on the level of population. By analyzing *N*-linked glycans in plasma of 1008 individuals, we observed rather large biological variability in a studied population and significant age- and gender-specific differences (Knezevic et al. 2009). Since the observed variability was larger than some changes previously reported to be associated with a disease, it was of great importance to examine the temporal stability of *N*-glycome in human individuals to be able to realistically evaluate diagnostic potential of changes observed in different diseases.

Results

We tested the stability of the human plasma *N*-glycan profile within an individual by using a recently improved quantitative method for glycan analysis in which hydrophilic interaction high-performance liquid chromatography (HILIC) separates

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Table I. Glycan structures present in different HPLC peaks[a]

Peak	Structure	Peak	Structure	Peak	Structure
GP1	A2				
	A2B		FA2BG2		A2F1G2S2
			M7D3		A3G3S(3,3)2
GP2	A1G1	GP7	A2G2S(3)1		A3G3S(3,6)2
	FA2		A2G2S(6)1	GP12	A3G3S(6,6)2
			M7D1		A3BG3S(3,3)2
	M5				A3BG3S(3,6)2
GP3	FA2B				A3BG3S(6,6)2
	A2[6]G1		A2BG2S(3)1		
	A2[6]BG1		A2BG2S(6)1		
			M5A1G1S1		A3F1G3S2
	A2[3]G1	GP8	FA2G2S(3)1		FA3G3S(3,3)2
	A2[3]BG1		FA2G2S(6)1		FA3G3S(3,6)2
	M4A1G1		A3G3		FA3G3S(6,6)2
	FA2[6]G1		FA2BG2S(3)1	GP13	FA3BG3S(3,3)2
	FA2[6]BG1		FA2BG2S(6)1		FA3BG3S(3,6)2
GP4	A1[6]G1S(3)1				FA3BG3S(6,6)2
	A1[6]G1S(6)1		A2F1G2S(3)1		A3G3S(3,3,6)3
	FA2[3]G1		A2F1G2S(6)1		A3G3S(3,6,6)3
	FA2[3]BG1		M8D2, D3		A3G3S(6,6,6)3
	M6D1, D2	GP9	A2G2S(3,3)2		
	A1[3]G1S(3)1		A2G2S(3,6)2		
	A1[3]G1S(6)1		A2G2S(6,6)2		A3F1G3S(3,3,6)3
			M8D1,D3		FA3G3F1S(6,6,6)3
	M6D3				A4G4S(6,6)2
	A2[6]G1S(3)1			GP14	A3F1G3S(3,6,6)3
	A2[6]G1S(6)1		A2BG2S(3,3)2		A3F1G3S(6,6,6)3
GP5	A2G2		A2BG2S(3,6)2		A4G4S(6,6,6)3
	A2[3]G1S(3)1		A2BG2S(6,6)2		A4F1G4S2
	A2[3]G1S(6)1	GP10	A3BG3S(3)1		A4G4S3
	A2BG2		A3BG3S(6)1		
			FA2G2S(3,3)2	GP15	A4G4S4
			FA2G2S(3,6)2		A4F1G4S3
	FA2[6]G1S(3)1		FA2G2S(6,6)2		
	FA2[6]G1S(6)1				A4G4S(6,6,6,6)4
	FA2[6]BG1S(3)1		FA2BG2S(3,3)2		A4G4S(3,6,6,6)4
	FA2[6]BG1S(6)1	GP11	FA2BG2S(3,6)2		A4BG4S4
	M4A1G1S1		FA2BG2S(6,6)2		FA4G4S4
GP6	FA2G2		M9	GP16	A4F1G4S4
	FA2[3]G1S(3)1				A4G4LacS4
	FA2[3]G1S(6)1				A4F2G4S4
	A2BG1S1				FA4F1G4S4
	FA2[3]BG1S(3)1				
	FA2[3]BG1S(6)1				

[a]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.

labeled glycans into 16 groups (GP1-GP16). Groups were formed as in our previous study on glycome variability in a population (Knezevic et al. 2009) and the glycan structures present in each group correspond to those given in Table I. The region of each glycan group is highlighted on the chromatogram in Figure 1.

To be able to interpret our results, first we determined 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 tested for its precision. When the whole procedure was repeated 10 times on the same occasion (sample 1), the average coefficient of variation (CV) of 4.3% was obtained (Table II). The CV of most peaks was actually even lower, while only GP7 and GP16 had CVs of 10.1%, and 12.3%, respectively. The analytical procedure was also repeated 10 times by two different analysts (sample 2) and analyzed by HPLC on 10 different days to determine inter-precision. The average CV of 7.04 was obtained (Table II). Considering the number of steps in the analytical procedure and the existence of some partly unresolved chromatographic peaks, the determined precision was considered to be very good.

To determine temporal variability of the human plasma *N*-glycome, glycans in plasma samples from 12 individuals taken

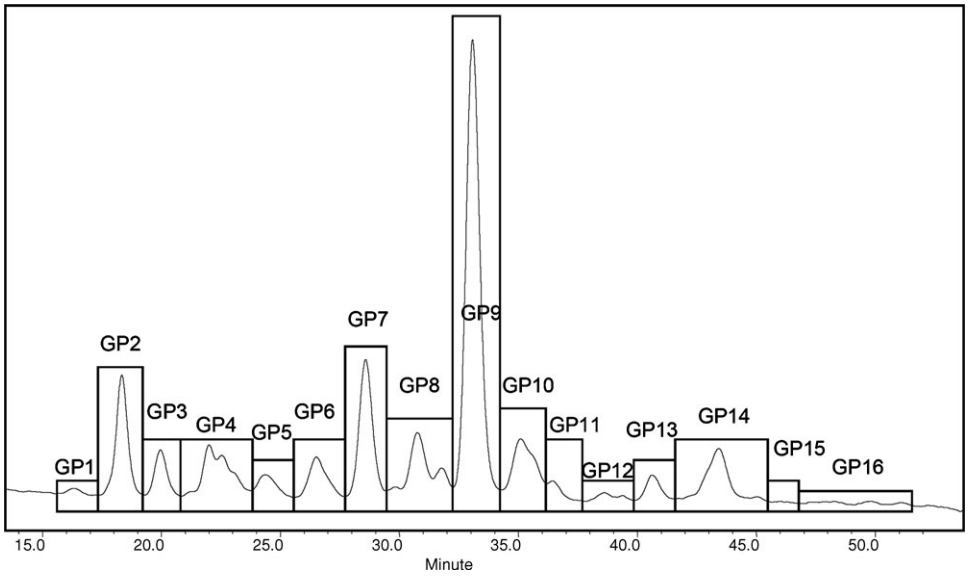

Fig. 1. *N*-Glycan profile from the human plasma sample divided into 16 groups of glycans. The glycan structures present in each group correspond to those given in Table I. Glycans were released from proteins, labeled and separated using hydrophilic interaction high-performance liquid chromatography.

Table II. Precision of the analytical procedure

	Precision (same day; sample 1)			Precision (different days; sample 2)		
Peak no.	Mean	SD	CV	Mean	SD	CV
GP1	0.12	0.01	7.39	0.11	0.01	12.73
GP2	7.24	0.24	3.38	3.03	0.42	14.02
GP3	2.45	0.06	2.60	2.37	0.13	5.39
GP4	9.05	0.25	2.75	5.49	0.70	12.78
GP5	1.69	0.07	3.98	2.82	0.19	6.82
GP6	5.14	0.09	1.82	4.25	0.37	8.61
GP7	9.37	0.95	10.14	9.65	0.76	7.86
GP8	10.36	0.13	1.26	9.40	0.35	3.68
GP9	31.25	0.43	1.37	39.25	1.11	2.83
GP10	7.37	0.19	2.63	5.82	0.33	5.71
GP11	2.77	0.09	3.33	1.84	0.10	5.42
GP12	1.66	0.05	3.14	2.35	0.18	7.50
GP13	5.26	0.15	2.85	4.73	0.15	3.09
GP14	5.32	0.16	2.97	7.19	0.20	2.71
GP15	0.49	0.03	6.64	0.59	0.06	9.50
GP16	0.47	0.06	12.25	1.12	0.04	3.95
Average			4.28			7.04

Plasma samples were analyzed (released, labeled, separated, and integrated) 10 times to evaluate precision of the method. The plasma sample from one individual (sample 1) was analyzed 10 times in a sequence, while sample 2 (from a different individual) was analyzed 10 times on different days and by different analysts. Mean values, standard deviation, and coefficient of variation are presented for glycan structures separated into 16 peaks (expressed as a % of total glycome) integrated from a hydrophilic interaction chromatogram. SD, standard deviation; CV, % coefficient of variation.

Table III. Variability in human plasma *N*-glycome

Peak no.	Variability in an individual CV (%)	Min CV (%)	Max CV (%)	Variability in a population CV (%)[a]
GP1	11.41	3.19	24.15	50.0
GP2	6.71	2.62	12.15	34.6
GP3	4.02	1.30	8.00	19.4
GP4	5.43	2.74	8.35	18.2
GP5	5.16	2.81	8.03	17.4
GP6	4.39	1.88	8.43	20.4
GP7	3.85	1.28	11.34	23.0
GP8	1.63	1.10	2.66	14.3
GP9	1.75	1.05	2.75	7.5
GP10	2.59	1.56	3.73	18.5
GP11	6.61	3.46	12.10	20.1
GP12	6.77	3.87	9.89	18.5
GP13	4.38	2.22	7.61	28.1
GP14	4.05	1.36	6.06	22.3
GP15	9.48	4.81	15.31	37.0
GP16	10.66	5.85	14.69	35.2
Average	5.56			24.0

Plasma samples from 12 individuals were taken on seven occasions during 5 days and analyzed to evaluate plasma *N*-glycan profile stability. Mean values and minimum and maximum of coefficients of variation (CV) of all 12 persons measurements are presented for glycan structures separated into 16 peaks integrated from a hydrophilic interaction chromatogram.
[a]The variability of glycan groups measured in population of 1008 individuals from the Croatian island Vis (Knezevic et al. 2009).

on seven occasions through 5 days (three times on day 1, than once per day) were compared. All chromatograms obtained from the same person through days coincided extremely well (Figure 2), indicating very good temporal stability of *N*-glycome in an individual. Statistically significant changes were not found for any of the glycan groups (lowest $P = 0.658$ for GP5; P close to 1 for all other groups). Results showed very small variation in all groups of glycans and are given in Table III as average coefficients of variations (CV) for each group of measurements. Average variations between 1.6% for GP8 and 11.4% for GP1, with mean average variation of 5.6%, were observed. These variations are comparable to those obtained when the same sample was analyzed on different occasions and confirm extremely good stability of human plasma *N*-glycan profiles. Contrary to glycome stability within an individual, in our recent study we observed very high level of glycome variability in a population

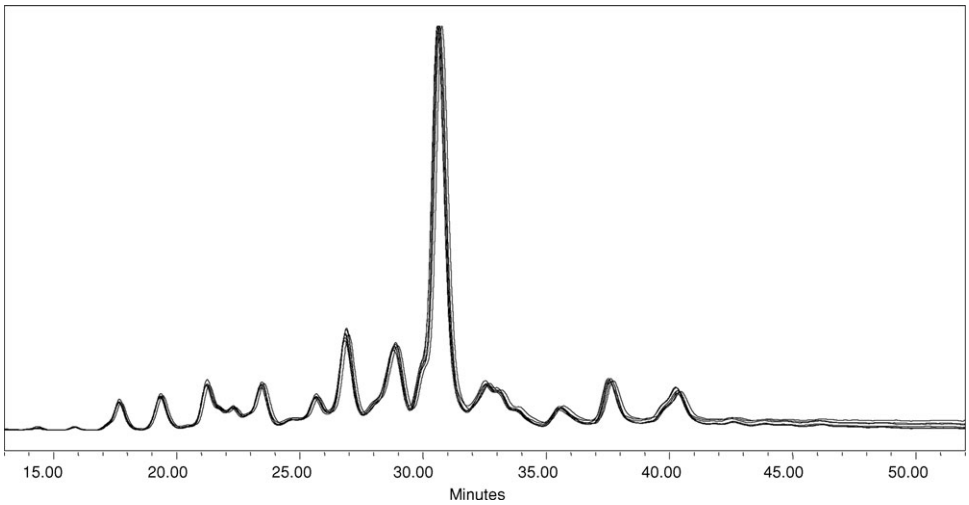

Fig. 2. Overlay of seven *N*-glycan chromatograms from plasma samples from the same individual on seven occasions during 5 days. Glycans were released from proteins, labeled and separated using hydrophilic interaction high-performance liquid chromatography.

of 1008 individuals with the CV of glycan structures from 7.5% up to even 50% (Knezevic et al. 2009).

Different from the 5 day period, during which no change in psychophysical condition of our examinees occurred, when plasma samples where taken from eight individuals (participants in a larger study) after a period of approximately 1 year somewhat greater differences in glycan profiles were observed (average CV = 8%, from 3% for GP5 to 20% for GP11), but only two glycan groups GP11 and GP16 were found to change with statistical significance ($P = 0.014$, $P = 0.016$, respectively). Although these results show good stability of plasma glycan profiles even after longer period of time, detailed examination of individual chromatograms (Figure 3) revealed considerable changes in some glycans in some individuals, for instance almost 40% increase in GP1 was observed in person 7.

Discussion

With the use of a recently developed method for high throughput analysis of glycans, we performed the study on intra-individual stability of human plasma *N*-glycome during time. Our results show good stability of human plasma *N*-glycan profiles in individuals with time and are of great importance for further implementation of glycan analysis in diagnostic laboratories. Many studies that reported changes of glycan structures under various pathological conditions have also shown their occurrence very early in acute pathological processes (Chavan et al. 2005; Gornik et al. 2007) as well as variation through days (Chavan et al. 2005; Gornik et al. 2007, 2008). Thus, the knowledge that glycan profiles are stable and that normal everyday activities do not have global effects on protein glycosylation gives greater significance to those findings.

Due to experimental limitations, we did not quantify glycan structures individually, but instead divided them into 16 groups containing most similar glycan structures. More detailed structural analysis would require several HPLC analysis and glycosidase digestions, what is currently not realistic for high throughput analysis needed for diagnostic use. This "reduced" analysis approach was already used by us in the study on glycan variability within a population (Knezevic et al. 2009) and was proven to be adequate for quantitative studies of *N*-glycome. In that study, we observed a rather high level of variability between 1008 individuals with the CV of certain glycan groups reaching 50%. In contrast to that now we have showed that during 5 days, glycan profiles practically do not change at all within a single individual. The observed variability in the profiles was very small with average CV of 5.6%, and all 16 glycan groups showed excellent stability through all seven measurements, with high statistical significance.

The observed variability in glycan profiles through seven measurements is comparable to the variability caused by limitations in the analytical procedure where the average CVs for intraprecision were only slightly above 4% and 7% for interprecision, which further supports our conclusions that glycan profiles are very stable. Although variability in glycan profiles is generally very small, it differs for different individuals and varies between glycan groups. For example, for glycan group 1, for which the highest average CV of 11.4% was observed, the average CV for one person was approximately 24%, while in an another was only slightly above 3%. This was the biggest deviation observed, while for other glycan groups the difference between maximum and minimum CV values were between 1.5% and 10.5%. These differences are also largely consequence of limitations in the analytical procedure since some very small chromatographic areas, such as GP1, make peak integration less accurate resulting in higher percent of variability.

We followed glycan profiles through relatively short period of time (5 days), to secure maximal stability in person's psychophysical condition since it is known that various environmental changes that affect homeostasis can also change protein glycosylation. This can also be seen from glycan profiles obtained from the same individuals after a longer period of time, where glycan profiles showed a somewhat greater variability than that within the 5 days. Although for most individuals glycan profiles did not change and *N*-glycome appears to be stable even in longer periods, for some individuals considerable changes in some glycan structures were observed (Figure 3). It is outside of

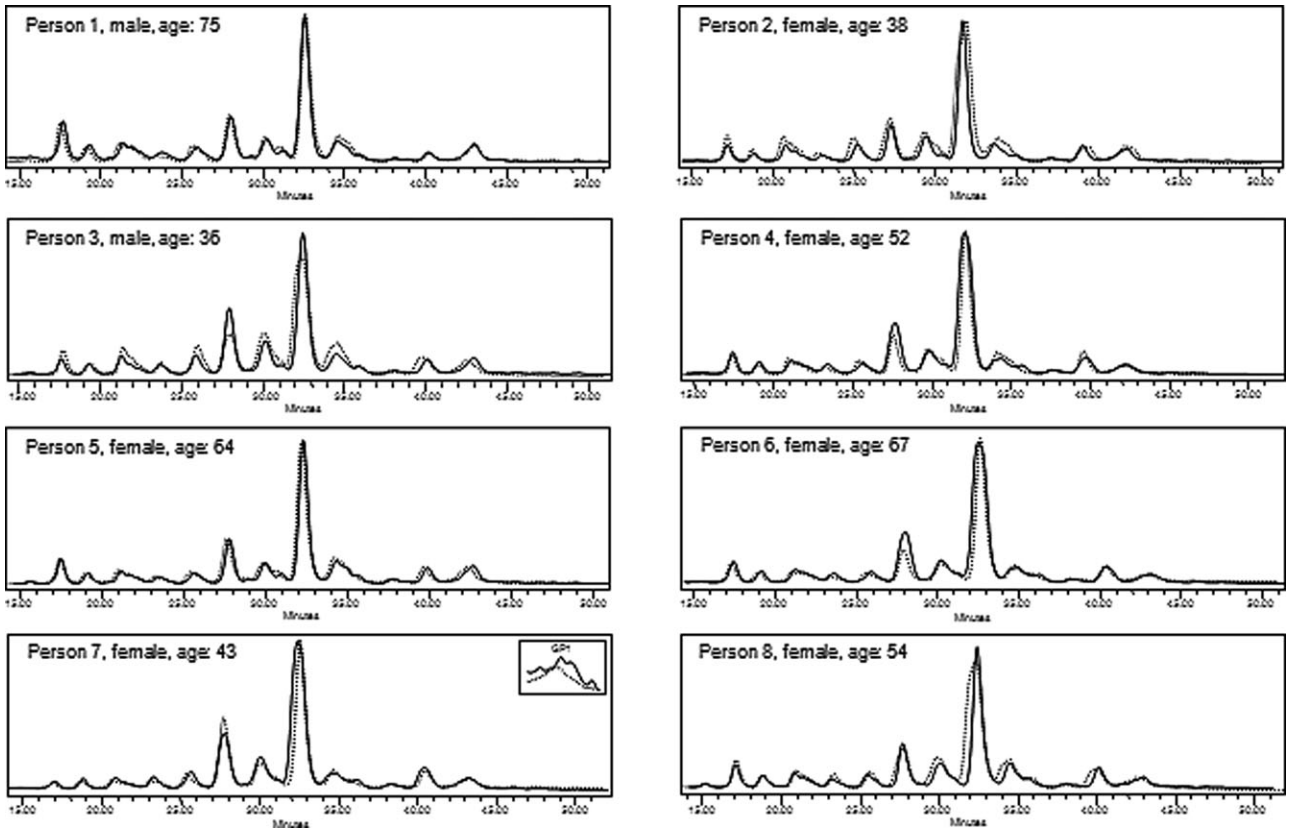

Fig. 3. *N*-Glycan HPLC profiles from eight individuals that were sampled twice with an interval of approximately 1 year. Glycans were released from proteins, labeled and separated using hydrophilic interaction high-performance liquid chromatography. In general, glycan patterns in most of the individuals did not change significantly after a year, but some glycans in some individuals did change considerably. The largest observed change was a nearly 40% increase in GP1 that was observed in person 7.

the scope of this study to evaluate (patho)physiological causes of these changes, but this observation is very important since it demonstrated that changes are limited to some specific glycan structures and indicate their potential diagnostic value.

Contrary to the great stability within an individual, glycan profiles have large variability at the population level. Therefore, we can conclude that, although glycan parts of glycoproteins are not directly encoded in genes, glycan profiles are under dominant genetic control. Hundreds of glycosyltransferases, transporters, transcription factors, and other proteins are involved in the synthesis of glycans, but on the global level the stability of this process is remarkable. Changes occurring in person's glycan profile are thus consequences of environmental influences and pathophysiological processes and therefore carry a great potential diagnostic value.

Material and methods

Plasma samples

Plasma samples were taken from 12 human volunteers, 6 males and 6 females. Their median age was 29.5 (26–38). The blood was drawn on seven occasions. First three portions were taken on the same (First) day at 8 am, 1 pm, and 6 pm, while next four blood portions were taken during next 4 days (daily) at 8 am. Plasmas were separated by centrifugation within 30 min after blood drawn and stored at −20°C. All participants were of good psychophysical condition without any chronic disease or acute pathological condition. They did not take any medications. From female participants, the blood was taken during second week of the menstrual cycle.

To determine stability of glycan profiles after a longer period, eight plasma samples (6 women, 2 men; age 53.5 (36–75)) from our previous study on glycan analysis (Knezevic et al. 2009) were used. These eight persons are habitants of island Vis and their plasma samples have been taken twice with an interval of approximately 1 year, and then stored at −80°C until analysis (between 2 and 3 years).

This study was performed in conformance to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the ethics committee of University of Osijek, School of Medicine. All participants have signed the informed consent for participation in the study.

Glycan release and labeling

The plasma proteins were immobilized in a block of SDS–polyacrylamide gel (Sigma-Aldrich, Saint Louis, MO) and *N*-glycans were released by digestion with recombinant *N*-glycosidase F (Prozyme, Leandro, CA). This was done as described previously (Royle et al. 2008) in a 96-well microtiter and protein precipitation filter plate (Whatman Inc., NJ) to achieve

the best throughput of sample preparation. After extraction, glycans were fluorescently labeled with 2-aminobenzamide (Prozyme).

Hydrophilic interaction high-performance liquid chromatography (HILIC)

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 (Anachem, Luton, UK) 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 a 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 (Prozyme) from which the retention times for the individual glycans were converted to glucose units (GU) (Royle et al. 2006).

The obtained chromatograms were separated into 16 chromatographic areas, regarding the peak resolutions and similarity of glycan structures as described previously (Knezevic et al. 2009). The amounts of glycans present in each area were expressed as percentage of total integrated chromatogram (amount of total glycan structures, total serum *N*-glycome).

Precision of the analytical procedure

The whole analytical procedure, including glycan release, labeling, and chromatography was repeated 10 times on the same occasion (sample 1) as well as 10 times (five times by each analyst) on different days (sample 2). The precision was expressed as the CV of series of these measurements. Mean values and standard deviations are also given for each group measurements.

Statistical analysis

Changes in levels of glycans through seven measurements were tested using the Kruskal–Wallis nonparametric statistical test. The level of statistical significance was set at $P < 0.05$. Results for the variability of measured glycan levels are expressed as average coefficient of variation (CV = Sd/mean × 100) since it best describes the variation in a group of measurements and allows the comparison with results for analytical precision. The average CV for each group of glycans was calculated as a mean value of CVs from all 12 persons (seven measurements for each group of glycans). Mean, minimal, and maximal values are given. This aberration from conservative statistical presentation was chosen to acquire best transparency of results.

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Conflict of interest statement

None declared.

Abbreviations

2 AB, 2 aminobenzamide; CV, coefficient of variation; GU, glucose units; HILIC, hydrophilic interaction high-performance liquid chromatography; SD, standard deviation; SDS, sodium dodecyl sulfate.

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