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

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Abstract

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 plasma of 1,914 individuals from 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, nongalactosylated, 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 nongalactosylated 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.

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).

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 1,008 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, but also for as long as one 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 ageing on human plasma glycans are still mostly unknown (Klein 2008). Changes in glycan structures with age were mainly analyzed for glycans on immunoglobulin G. The largest study was performed on 403 individuals and the main observation was that nongalactosylated 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 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 plasma of 1,914 individuals (742 males and 1172 females) between 18 an 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 nongalactosylated, 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 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 1).

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 2). The most considerable correlations were observed for biantennary nongalactosylated glycan, core-fucosylated glycans, disialylated forms of biantennary glycans, as well as nongalactosylated and digalactosylated glycans. Age-dependant increase in nongalactosylated 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 3). While for males we detected only three significant associations when all the decades were compared together, and only nongalactosylated 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 characteristics (Table 3). 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 4). 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 nongalactosylated and a decrease in digalactosylated glycans as well as an increase in sialylation of biantennary structures.

Levels of total blood cholesterol, HDL-cholesterol, 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 tetra galactosylated structures (Table 4).

Hyperlipidemic persons can often have normal body weight, but due to genetic predispositions or dietary habits have high 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 5).

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 6 and revealed which of the individual correlation coefficients shown in Table 4 were the most statistically significant for changes in glycan levels. Levels of nongalactosylated 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-dependant 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 five years ago. Correlations coefficients between different smoking parameters and glycan features are presented in Table 6. 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, exsmokers and nonsmokers, 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 nongalactosylated glycans (Table 8).

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 result from constant interaction between the genome and the environment (Ostojic, et al. 2009). Considering the localization, sequence and function of protein glycosylation (Varki and 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 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, 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 (Becker and Lowe 2003, Sillanaukee, et al. 1999, 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 hormones 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 (trisialylated antennary fucosylated) glycan structure (Brinkman-Van der 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 nongalactosylated 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 nongalactosylated 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, R., et al. 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 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 (Deurenberg, et al. 1991, Henneberg and Ulijaszek , Lukaski, et al. 1985, Turconi, et al. 2006). To avoid errors of individual estimations, we calculated all 4 scores to achieve the most reliable results. When we correlated these body fat parameters with the main structural features of glycans, levels of nongalactosylated 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 (Qu, et al. 2006, Skropeta, et al. 2007, Vinals, et al. 2003, Vukmirica, et al. 2002), but there is little 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 positively 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 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 CRP is elevated with weight gain (Saito, et al. 2003) and increased tri- and tetrasialylated 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, R. B., 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 3). 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 4), 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 exception were nongalactosylated 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 underpins 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 a part of a larger genetic epidemiology program (Rudan, et al. 2006, Rudan, et al. 1999, Rudan, et al. 2009). The project aims were initially to describe the variability and genetic epidemiology of isolated island communities (Campbell, et al. 2007, Carothers, et al. 2006, Polasek, et al. 2006, Vitart, et al. 2008), while in the later stages the focus has changed towards gene mapping efforts, some of which included notable successes (Dupuis, et al. 2010, Johansson, et al. 2009, Vitart, et al. 2008). 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. 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 individual.

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

Glycan analysis

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

Hydrophilic interaction high performance liquid chromatography (HILIC).

Released glycans were subjected to hydrophilic interaction high performance liquid chromatography (HILIC) on a 250 x 4.6 mm i.d. 5μm 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. 60 min 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, regarding the peak resolutions and similarity of glycan structures present as described before. The amounts of glycans present in each area were expressed as % of total integrated chromatogram (amount of total glycan structures/ total serum N-glycome).

*Weak anion exchange (WAX)-HPL*C.

Glycans were separated according to the number of sialic acids by weak anion exchange HPLC. The analysis was performed using a Prozyme GlycoSep C 75mm x 7.5mm 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 2AB-labeled glycan pool were dried down in 96 well PCR plates. 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 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 same characteristic, from either HILIC, WAX or after sialidase treatment integrated glycan profiles (individual glycan structures present in each glycan group 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; Nongalactosylated 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 nongalactosylated 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: Body mass index (BMI), waist to hip ratio (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 trough 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 = weight (kg) / height^2 (m^2)
WH = waist circumference (mm) / hip circumference (mm)
$Z = \sqrt{(R^2\,(\Omega) + Xc^2\,(\Omega)}$
BF% = 495/(d-450), where density (d) d = c – m * log S, S = 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 (CHOD PAP), triglycerides by UV photometry with glycerolphosphate-oxidase (GPO PAP), HDL

cholesterol by homogene enzyme method with modified PEG and acyclohexane-sulphate 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 result of bad dietary habits. We defined 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 past for at least 5 years and stopped smoking at least five 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 was 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-Kolmogrov 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 analyse the association of selected predictor variables and each glycan feature.
Significance level was set at $P \leq 0.001$.

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Table 1. 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				
Nongalactosylated	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 nongalactosylated (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 2. 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						
Nongalactosylated	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
Trigalacosylated	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 nongalactosylated (A2)	0.30	P<0.001	0.18	P<0.001	0.37	P<0.001

Table 3. 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						
Nongalactosylated	<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
Trigalacosylated	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 nongalactosylated (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						
Nongalactosylated	<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
Trigalacosylated	<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 nongalactosylated (A2)	<0.001	0.041	<0.001	<0.001	0.019	0.929

Table 4 Correlation coefficients between glycans and body weight parameters. Statistically significant correlation coefficients (P≤0.001) are presented as shaded gray.

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

Table 5 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 is 25% males and 75% females. Statistical significance (P) for differentiation normolipidemic from hyperlipidemic group based on glycan levels is given.

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			
Nongalactosylated	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 nongalactosylated (A2)	0.17 (0.12 - 0.22)	0.16 (0.12 - 0.21)	0.001

Table 6. 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 7 Correlation coefficients between glycans and smoking habits 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. Statistically significant correlation coefficients (P≤0.001) are presented as shaded gray.

Glycan feature	Smokers		ExSmokers		
	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	-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	0.04
Monogalactosylated	-0.16	0.00	-0.28	-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	-0.11	0.16	-0.19	0.18
Biantennary nongalactosylated (A2)	0.01	0.20	-0.01	0.15	0.09

Table 8 Levels of N-glycan sub-classes by subpopulation of smokers (N=175), nonsmokers (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 %. Statistical significance (P) for differentiation of all three or separate pairs of groups based on glycan levels are given.

Glycan feature	Smokers; median (IQR)	Nonsmokers; median (IQR)	Ex-smokers; median (IQR)	Smokers *vs* Nonsmok. vs. Ex-smokers (P)	Smokers *vs* Nonsmok. (P)	Smokers *vs* Ex-smokers (P)	Nonsmok. *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							
Nongalactosylated	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 nongalactosylated	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