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Plasma protein N-glycan profiles are associated with calendar age, familial longevity and health

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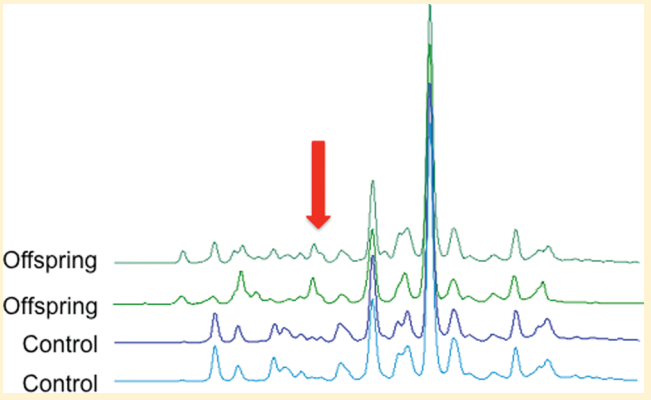
ABSTRACT: The development of medical interventions for the preservation of disease-free longevity would be facilitated by markers that predict healthy aging. Altered protein N-glycosylation patterns have been found with increasing age and several disease states. Here we investigate whether glycans derived from the total glycoprotein pool in plasma mark familial longevity and distinguish healthy from unhealthy aging. Total plasma N-glycan profiles of 2396 middle aged participants in the Leiden Longevity Study (LLS) were obtained by glycan release, labeling, and subsequent HPLC analysis with fluorescence detection. After normalization and batch correction, several regression strategies were applied to evaluate associations between glycan patterns, familial longevity, and healthy aging. Two N-glycan features (LC-7 and LC-8) were identified to be more abundant in plasma of the offspring of long-lived individuals as compared to controls. These results were not confounded by the altered lipid status or glucose homeostasis of the offspring. Furthermore, a decrease in levels of LC-8 was associated with the occurrence of myocardial infarction (p = 0.049, coefficient = −0.065), indicating that plasma glycosylation patterns do not only mark familial longevity but may also reflect healthy aging. In conclusion, we describe two glycan features, of which increased levels mark familial longevity and decreased levels of one of these features mark the presence of cardiovascular disease.



KEYWORDS: human plasma, N-glycosylation, longevity, aging

INTRODUCTION

Glycosylation is the enzymatic addition of oligosaccharides (also known as glycans) to proteins and lipids. In N-glycosylation, the glycans are attached to the asparagine residues in the protein. N-Glycans have important functions in many biological processes such as protein folding,[1] protein clearance,[2] cell adhesion,[2−4] receptor binding, and receptor activation.[5,6] Protein N-glycosylation may be very diverse and is a dynamic equilibrium: in a given physiological state, the glycan signature is highly reproducible;[7,8] however, when the physiological state changes, for example, due to aging or disease, the glycosylation machinery of affected cells in an organism may be altered, and the glycan pattern can change dramatically.[7] Therefore, protein N-glycosylation patterns may represent an important group of potential biomarkers of health and disease.[9]

Since the biological variation in plasma N-glycan is rather large,[10] larger sample sizes are required for biological interpretation. The analysis methods required for the evaluation in larger sample sets have only recently been developed.

Total plasma N-glycosylation patterns were found to be associated with calendar age in a study population of 100 Belgian individuals. It has previously been reported that elderly individuals above 50 years of age showed increased levels of non-galactosylated glycans, whereas the levels of galactosylated glycan structures decreased with increasing calendar age.[11] Even in an exceptionally high-age group, these associations between glycosylation and calendar age have been observed.[11] In a more recent study,[12] comprising a larger sample set, changes in levels of glycan features have also been observed with increasing age and were sex specific. In general, females showed more profound associations between glycan patterns and age than males, while

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some glycans showed opposite associations in males compared to females. Interestingly, glycosylation patterns of women changed most dramatically between the age groups 40−49 years and 50−59 years, suggesting an influence of the hormonal changes associated with entrance of the menopause. Irrespective of being influenced by calendar age, plasma N-glycosylation was shown to be associated with body fat parameters as well as lipid status (total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglyceride levels) in the same study.[12] Several glycan features mainly including tetra- and trisialylated compounds correlated positively with cholesterol and lipoproteins. Changes in glycosylation could also be observed in smoking individuals.[12]

Since it is unknown whether N-glycosylation patterns mark health or disease, the aim of our study is to investigate whether changes in N-glycosylation are associated with healthy aging and/or disease. Such markers might provide targets for specific interventions aimed at preservation of disease-free longevity. Therefore, we evaluated N-glycosylation patterns in the offspring of nonagenarian siblings (N = 1671) and controls (N = 744) from the Leiden Longevity Study (LLS).[13] This offspring shows 30% lower mortality rate and lower prevalence of myocardial infarction, hypertension, and type 2 diabetes.[14] Also, beneficial metabolic profiles were observed in the offspring as compared to controls such as lower glucose levels,[15] larger LDL particle sizes, and lower triglyceride levels.[16] To identify parameters that mark familial longevity, the offspring are compared to their partners. In such a comparison, the offspring are regarded to represent individuals with a higher susceptibility to become long-lived, while their partners, representing the general population, serve as controls. In the comparison of offspring of nonagenarian siblings and controls, we tested for association N-glycosylation patterns with familial longevity and healthy aging. Second, we assessed whether the longevity markers also associated with the presence or absence of disease.

EXPERIMENTAL SECTION

Participants

In the Leiden Longevity study, Caucasian families were recruited if at least two long-lived siblings were alive and fulfilled the age criterion of 89 years or older for males and 91 year or older for females, representing less than 0.5% of the Dutch population in 2001.[13] In total, 944 long-lived proband siblings were included, 1671 offspring with a mean age of 59 (st.dev 6.5), and 744 partners with a mean age of 58 (st.dev. 7.5). The partners serve as control individuals and will be mentioned as such from now. Information on medical history was requested from the participants' treating physicians. The study protocol was approved by the Leiden University Medical Centre ethical committee, and an informed consent was signed by all participants prior to participation in the study.

Phenotypic Parameters

All serum measurements were performed with fully automated equipment. For insulin, the Immulite 2500 from DPC (Los Angeles, CA) was applied. For glucose, total cholesterol, HDL-cholesterol (HDL-C), triglycerides and CRP the Hitachi Modular or the Cobas Integra 800, both from Roche, Almere, The Netherlands were applied. For free triiodothyronine, the Modular E170 was used from Roche, Almere, The Netherlands. CV's of these measurements were below 5%. LDL-cholesterol level (LDL-C) was calculated using the Friedewald formula (LDL-C = total cholesterol − HDL-C − (triglycerides/2.2); unit mmol/L) and set to missing if plasma triglyceride concentration exceeded 4.52 mmol/L. Body mass index was calculated from self-reported weight and height.

N-Glycan Preparation

N-glycans from the total protein pool in plasma from the offspring as well as the controls were released, labeled with 2-aminobenzoic acid and purified using Hydrophilic Interaction Liquid Chromatography (HILIC)-SPE as previously described.[17] Briefly, 20 μL of 2% SDS were added to 10 μL of plasma, randomly distributed in 28 96-well plates. In each plate four control samples were added to monitor sample processing. After protein denaturation and subsequent addition of NP-40, N-glycans were released overnight using PNGaseF. Without intermediate purification, the N-glycans were labeled with 2-aminobenzoic acid (2-AA) in the presence of $NaCNBH_3$ and acetic acid for 2 h at 65 °C. HILIC-SPE was performed using 40 mg cellulose in 96-well 0.45 μm GHP-filter plates (Pall). All wells of the filter plate were washed using water and subsequently equilibrated using acetonitrile (ACN)/water (80:20 v/v). The labeled N-glycan samples were then applied to the wells in 80% ACN, and the wells were washed using ACN/water (80:20 v/v). Purified 2-AA labeled N-glycans were eluted into 0.8 mL deep well collection plates (Abgene via Westburg, Leusden, The Netherlands) using 400 μL water.

HILIC-HPLC Analysis

Purified 2-AA labeled N-glycans were separated using hydrophilic interaction-high performance liquid chromatography (HILIC-HPLC) with trapping columns in dual mode as previously described.[17] In the Ultimate LC system (Dionex, Sunnyvale, CA), a Famos autosampler, a Switchos module with a loading pump and an Ultimate module with two pumps were connected. A nanovalve was used to connect the system to the detector. Chromeleon software (Dionex) was used to control the system. Two 2.0 mm × 10 mm TSK gel-Amide 80 trapping columns (Tosoh Biosciences, Stuttgart, Germany) and two 2.0 mm × 250 mm TSK gel-Amide 80 analytical columns (Tosoh Biosciences) were used for the separation of 2-AA labeled N-glycans, while a fluorescence detector (FP-2020 plus; Jasco, Easton, MD) was used for detection.

Briefly, 50 μL of aqueous eluate from the HILIC SPE were mixed with 150 μL ACN in a 96-deep-well plate. After a 20 μL injection, the 2-AA-labeled N-glycans were trapped on the trapping column and washed using ACN/50 mM ammonium formate (80:20, v/v; pH 4.4). Subsequently the 2-AA labeled N-glycans were separated on the analytical column using a linear gradient of ACN (solvent A) and 50 mM ammonium formate (pH 4.4; solvent B), resulting in a total analysis time of 106 min. The control samples were carefully examined by eye to eliminate large batch-effects. The HILIC HPLC method was, however, previously shown to be highly reproducible.[17]

Data Preprocessing

HILIC-HPLC chromatograms were exported from Chromeleon wp 6.50 as ASCII files and were loaded into Matlab (version 2007a) software (The Mathworks, Inc., Natick, MA). The data were normalized and subsequently prealigned using the peak of highest intensity. After data reduction by cropping of the data to the range of 30−80 min, alignment of the data was performed by correlation optimized warping (COW) according to the method described by Skov et al.,[18] which included reference sample generation, segment length and slack size optimization and alignment. Manual peak picking was performed and resulted in 26 areas under the curve.

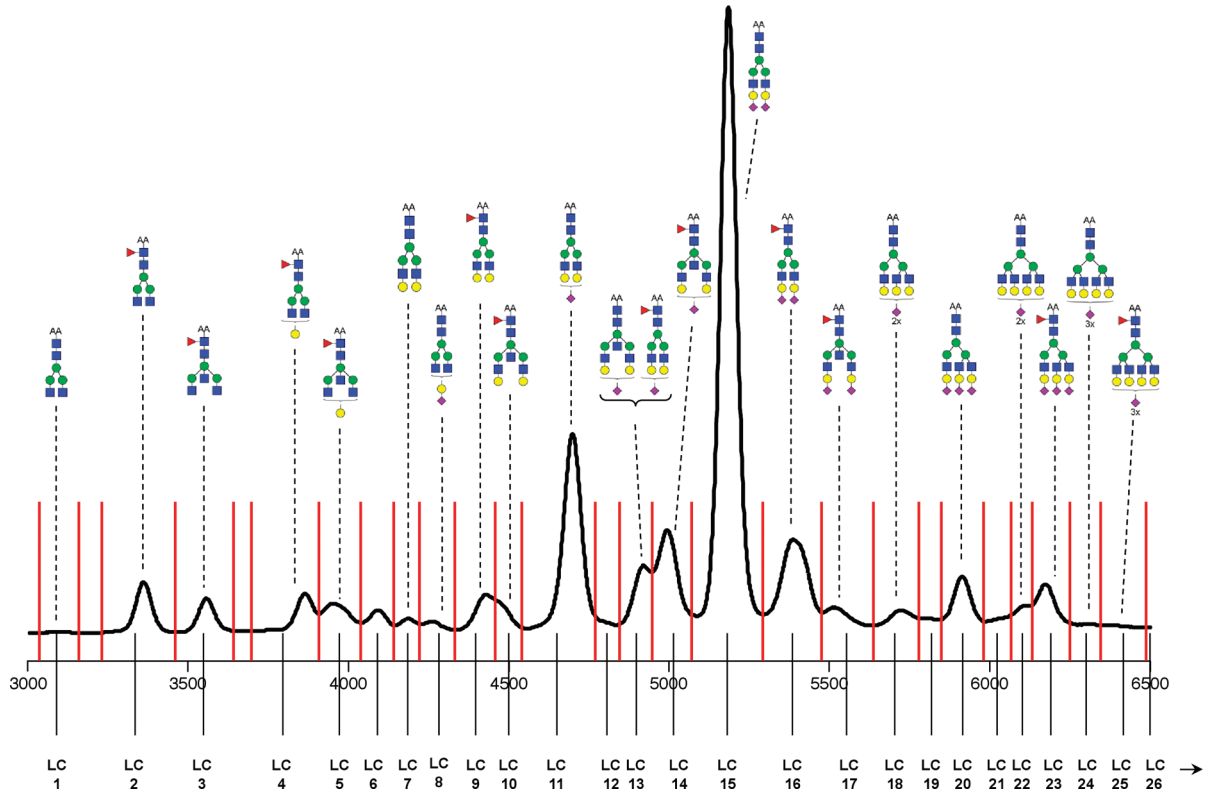

Figure 1. Typical HILIC-HPLC-FL chromatogram of plasma derived N-glycans labeled with 2-AA. Only high-abundant glycans are annotated. Glycan compositions are given in terms of N-acetylglucosamine (blue square), mannose (green circle), galactose (yellow circle), sialic acid (purple diamond), and fucose (red triangle).

Statistics

The samples of 2395 participants were divided over 28 individual plates to record plasma N-glycosylation patterns. To correct for batch effects, the 26 plasma N-glycosylation values were regressed on the categorical variable batch memberships. The standardized residuals of this model were used for further statistical analysis.

Since we have multiple offspring from the same family, the sandwich estimator was used to obtain valid standard errors. *P*-values <0.05 were regarded statistically significant. In all analyses, sibling relations among offspring were taken into account by the use of robust standard errors clustered on sibling pair. First, linear regression was performed with N-glycan features as dependent variable, age, sex and the interaction between age and sex as covariates. To determine potential biomarkers for longevity, logistic regression was applied with offspring/control allocation as dependent variable, age, sex and the age*sex interaction as covariates and glycosylation feature as independent variable, where offspring of nonagenarian siblings are coded as 1 and controls as 0.

To explore relationships between LC-7 and LC-8 and other phenotypic parameters, linear regression was applied with LC7 and LC8 as dependent variable, age, sex and the age*sex interaction as covatiates, and BMI, levels of cholesterol, HDL-cholesterol, LDL-cholesterol, triglyceride, glucose or insulin as independent univariate variable, while adjusting for offspring/control allocation. To evaluate whether the association between LC-7 and LC-8 was independent of these covariates, the covariates were included in the logistic regression model for classification of offspring/control allocation. Finally, linear regression was used to explore relationships between LC-7 and LC-8 and disease status—incidence of MI, CVA or diabetes—adjusting for age, sex and their interaction.

Analyses were performed using STATA 10 (StataCorp LP, College Station, TX) and R version 2.9.0 (R Development Core Team).

RESULTS

Analysis

Glycans were released from plasma proteins using PNGaseF, labeled with 2-AA, and subsequently analyzed by HILIC-HPLC-FL as previously reported.[17] A typical HILIC-HPLC-FL chromatogram of 2-AA labeled N-glycans from plasma is depicted in Figure 1, where only high-abundant glycans have been annotated. To allow statistical evaluation, chromatograms were normalized to the highest peak, aligned, and subsequently integrated according to the 26 intervals indicated. The human plasma glycome has been studied extensively (e.g.,[17,19]), and it is common knowledge that more than 100 glycan structures are present on human plasma glycoproteins. One should, therefore, consider that most glycan intervals will contain several—potentially dozens—glycan structures. The repeatability of the sample preparation and profiling procedure using HILIC-HPLC-FL was reported previously and was shown to remain below 9% for all glycan features.[17]

Glycan integrals could be obtained for 2395 individuals from the LLS (1662 Offspring and 733 controls) between 30 and 80 years of age. The average age was 59.3 years. A description of the studied samples is depicted in depicted in Table 1.

Plasma N-Glycosylation Changes with Calendar Age and Is Sex-Specific

Several groups previously published on the sex-dependency of glycosylation patterns of plasma glycoproteins (both total plasma as well as specifically IgG) and their changes with chronological

Table 1. Descriptives of Offspring of Nonagenarian Sibling Pairs and Controls from the Leiden Longevity Study

	total (2395 individuals)		offspring (1662 individuals)		control (733 individuals)		
	mean	Std/(95% C.I.)	mean	Std/(95% C.I.)	mean	Std/(95% C.I.)	P^a
Age	59.3	6.8	59.4	6.6	58.9	7.3	0.079
% of male individuals	45%	50%	47%	50%	42%	49%	0.028
Body Mass Index	25.42	3.6	25.33	3.6	25.61	3.61	0.117
Totaal Cholesterol (mmol/L)	5.59	1.19	5.57	1.2	5.62	1.16	0.425
HDL cholesterol (mmol/L)	1.44	0.45	1.45	0.45	1.42	0.47	0.151
LDL cholesterol (mmol/L)	3.33	0.98	3.33	0.99	3.34	0.95	0.829
Triglyceride (mmol/L)	1.56	(0.68−3.65)	1.52	(0.66−3.63)	1.64	(0.72−4.14)	0.002
Glucose (mmol/L)	5.87	1.54	5.79	1.37	6.04	1.86	0.000
Insulin (mU/L)	16.52	(4.00−64.00)	16.01	(4.00−61.00)	17.71	(4.00−69.60)	0.008
CRP (mg/L)	1.42	(0.31−9.16)	1.41	(0.31−9.26)	1.46	(0.32−8.55)	0.506

[a] *P*-values were generated by comparison of the offspring with the controls using univariate linear regression.

Table 2. Associations of Total Plasma N-Glycosylation with Age, Sex, and the Age*Sex Interaction[a]

	change with increasing calendar age	*P*	sex-related difference[b]	*P*
LC-1	+	**0.000**	−	0.879
LC-2	+	**0.000**	+	0.022
LC-3	+	**0.000**	−	0.018
LC-4	−	0.127	+	**0.000**
LC-5	−	0.459	+	0.007
LC-6	−	0.442	−	0.039
LC-7	−	0.137	−	0.004
LC-8	+	0.010	−	**0.000**
LC-9	−	**0.000**	+	0.019
LC-10	−	**0.000**	+	0.107
LC-11	−	0.741	−	**0.000**
LC-12	+	0.917	−	0.735
LC-13	−	**0.000**	+	0.005
LC-14	−	**0.000**	−	**0.002**
LC-15				
LC-16	+	0.890	+	**0.000**
LC-17	−	0.045	−	**0.000**
LC-18	−	0.147	−	**0.000**
LC-19	+	0.145	+	**0.000**
LC-20	-	**0.001**	−	**0.000**
LC-21	+	0.113	−	**0.000**
LC-22	+	**0.001**	−	**0.000**
LC-23	+	**0.000**	+	**0.000**
LC-24	+	**0.000**	+	0.463
LC-25	+	0.098	−	**0.000**
LC-26	+	0.013	−	0.124

[a] As normalization was performed using feature LC-15, no values are obtained for LC-15. Significant ($P < 0.002$, after Bonferroni correction) results are highlighted in bold. [b] Female = 0, male = 1.

age.[12,20−24] We assessed the effects of chronological age and sex in the current data set (Table 2).

Clearly, 11 glycan features are strongly associated with calendar age. Consistent with previous findings,[12] nongalactosylated glycans in features LC-1 to LC-3 increase with age, while core fucosylated non- and monosialylated bigalactosylated glycans in features LC-9, LC-10, LC-13 and LC-14 decrease with age. Interestingly, the triantennary trisialylated glycan in feature LC-20 decreased with calendar age, while its fucosylated counterpart in LC-23 was positively correlated with calendar age. Levels of several tetraantennary glycans (in glycan features LC-22 and LC-24) increased with calendar age.

Thirteen glycan features showed different levels in males compared to females (see Table 2): the levels of monosialylated glycan features LC-8, LC-11 and LC-14 decreased with age in males. Others, like LC-4, LC-16, LC-19 and LC-23 increased with age in males. Since the cohort has a wide age-range, we tested the interaction between age and sex in the plasma N-glycosylation features. Four glycan features (LC-7, LC-9, LC-10 and LC-13) showed significant positive interaction between age and sex, indicating that the decline of the levels of these glycan features with age is more profound in females than in males, as previously reported. Age, sex and the age and sex interaction were included in all further analyses.

Plasma N-Glycosylation Features Are Associated with Longevity

The observation that N-glycosylation patterns change with age is not new and does not explain to which extent it marks physiological aging or subclinical and clinically diagnosed disease. A first indication of the value of the glycan features as biomarkers comes from a comparison of the more healthy offspring of long-lived siblings and controls. Thus, to identify N-glycan features that associate with familial longevity, for each glycan feature a logistic regression model was fitted with longevity status as outcome. The results are depicted in Table 3, with significant ($p < 0.002$, after correction for multiple testing) results highlighted in bold.

The levels of two glycan features, both with nonfucosylated glycans as their major glycan (LC-7 and LC-8), are significantly different between the controls and the offspring. The odds ratios for LC-7 and LC-8 depicted in Table 3 are above 1, indicating that elevated levels of LC-7 and LC-8 could be observed in the offspring, relative to controls. This is confirmed in Figure 2, where averages of the standardized values, adjusted for age, sex and the age*sex interaction are plotted. Interestingly, these N-glycan features are not significantly associated to calendar age but they do have the tendency to change with age in a sex-specific manner (Table 2). In Figure 3, HILIC-HPLC-FL chromatograms of the control-offspring couple with the largest difference in LC-7 and LC-8 are depicted to visualize the effect.

Table 3. Associations between Glycosylation Features and Longevity[a]

			95% C.I.	
glycan feature	odds ratio	*P*	lower bound	upper bound
LC-1	1.096	0.041	1.004	1.196
LC-2	1.038	0.457	0.941	1.144
LC-3	1.046	0.378	0.947	1.154
LC-4	1.018	0.72	0.925	1.12
LC-5	1.045	0.37	0.949	1.151
LC-6	1.017	0.744	0.921	1.122
LC-7	**1.209**	**0.001**	1.078	1.357
LC-8	**1.174**	**0.001**	1.068	1.291
LC-9	1.035	0.503	0.936	1.144
LC-10	1.019	0.718	0.922	1.125
LC-11	1.085	0.087	0.988	1.191
LC-12	1.018	0.766	0.907	1.142
LC-13	0.996	0.93	0.902	1.099
LC-14	1.06	0.34	0.941	1.193
LC-15	–	–	–	–
LC-16	0.935	0.182	0.847	1.032
LC-17	1.038	0.461	0.94	1.145
LC-18	1.013	0.798	0.92	1.114
LC-19	1.079	0.111	0.982	1.186
LC-20	0.989	0.827	0.893	1.094
LC-21	1.014	0.773	0.922	1.115
LC-22	0.939	0.208	0.85	1.036
LC-23	1.027	0.596	0.931	1.133
LC-24	0.989	0.827	0.899	1.088
LC-25	1.046	0.336	0.955	1.146
LC-26	1.051	0.271	0.962	1.148

[a] For all glycan features, odds ratios are reported together with their 95% confidence interval and their respective *p*-values. Significant results are highlighted in bold ($p < 0.002$, after Bonferroni correction). Odds ratios >1 indicate that the offspring has higher levels of a glycan feature than the controls, while odds ratios <1 indicate that the offspring has a lower levels of a glycan features than the controls.

Association of Glycan Features with Body Mass Index and Plasma Lipid Parameters as well as Glucose Homeostasis Parameters

Since lipid parameters have been associated with glycan features[12] and with familial longevity,[16] we tested whether the association of glycan features LC-7 and LC-8 with longevity is confounded by the association with lipid parameters. Furthermore, since one of the major marks of healthy aging in the LLS is the preserved glucose homeostasis and insulin sensitivity,[15] illustrated by the fact that plasma levels of glucose and insulin are significantly lower in the offspring compared to the controls in the current study (see Table 1), we also tested for association of LC-7 and LC-8 with glucose and insulin levels. The association of BMI as well as plasma concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, glucose and insulin with the glycosylation features was evaluated (Table 4). Interestingly, all effect sizes are larger for LC-8 than for LC-7. We did not find significant associations of the two glycan features with cholesterol, LDL-cholesterol and glucose levels. However, high levels of LC-8 associate with high HDL-cholesterol levels and low triglyceride levels and both glycan features are negatively correlated with BMI. These are all strong predicting phenotypes of cardiovascular health. The association of LC-8 levels follows the well-known negative correlation of HDL-cholesterol levels and the positive correlation of BMI and triglyceride levels with the risk of cardiovascular disease. High levels of LC-8 further associate with higher insulin level.

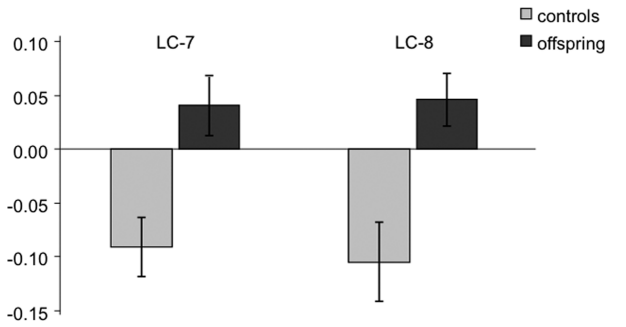

Figure 2. Glycan features LC-7 and LC-8 are associated with familial longevity. Mean values for controls (light gray) and offspring (dark gray) are depicted with their SEM.

To evaluate whether the association of LC-7 and LC-8 with longevity was actually based on association with the metabolic parameters, we then performed logistic regression with offspring/control allocation as dependent variable and LC-7 or LC-8 in the model together with one of the lipid or glucose parameters. Upon inclusion of these parameters, the association between longevity and glycosylation features remained significant: for LC-7 the P values were 0.002, 0.006, 0.008, and 0.008 upon inclusion of BMI, HDL concentration, triglyceride- and insulin levels, respectively. For LC-8 these P values were 0.001, 0.005, 0.014 and 0.006, respectively. Therefore, the observed associations of LC-7 and LC-8 with familial longevity may be regarded independent of the metabolic parameters in Table 4.

Plasma N-Glycosylation Features and their Association to Healthy Aging

Then we tested in the studied population whether LC-7 and LC-8 are indicative parameters of the health status. Given the results in Table 4 we would expect the features to mark cardiovascular health, which is marked by plasma C-reactive protein (CRP) levels. A high level of CRP, indicating a chronic inflammatory response, is a marker for decreased (cardiovascular) health.[25] Because N-glycosylation has previously been reported to be correlated to CRP levels,[26] the associations between glycosylation and CRP levels in total plasma were evaluated for LC-7 and LC-8. Both glycans were negatively associated with CRP levels ($p < 0.000$). This indicates that the glycosylation features are not only altered by familial longevity and metabolic health, but may also mark an individual's inflammatory status.

This leads us directly to the question whether LC-7 and LC-8 could be markers for metabolic health and cardiovascular disease. Even though the number of diagnosed individuals in the LLS study cohort is limited, we tested for association of the glycan features and metabolic disease: myocardial infarction (MI), cerebrovascular accident (CVA) and diabetes in the complete cohort (Table 5). No significant associations could be observed for CVA and diabetes. For myocardial infaction a boarderline significant negative association was observed, indicating that high levels of LC-8 are related to decreased incidence of MI.

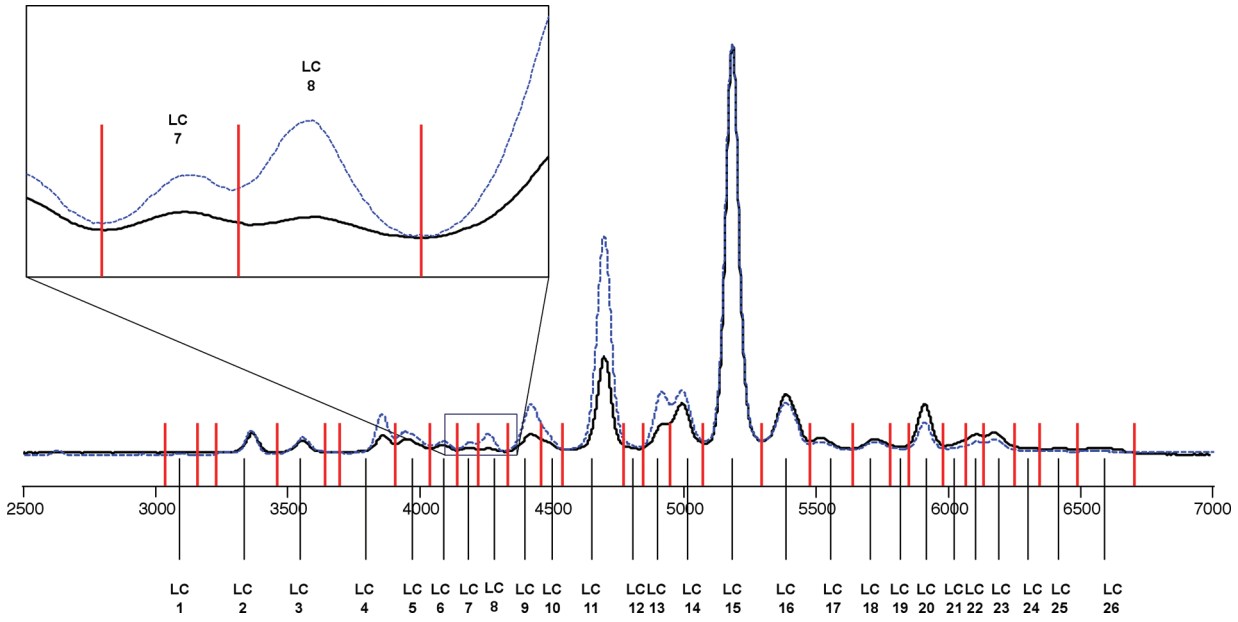

Figure 3. HILIC-HPLC-FL chromatograms of a control-offspring couple with the largest difference in LC-7 and LC-8. Control is depicted with a continuous line, while the offspring is depicted with a dotted line. The inset displays the LC-7 and LC-8 region.

Table 4. Direction of Association of LC-7 and LC-8 with Lipid and Glucose Parameters[a]

	LC-7		LC-8	
Parameter	direction of coef.	*P*	direction of coef.	*P*
BMI	−	**0.000**	−	**0.000**
Cholesterol	+	0.645	−	0.062
HDL-cholesterol	+	0.106	+	**0.000**
LDL-cholesterol	+	0.586	−	0.084
Triglyceride	−	0.050	−	**0.000**
Glucose	−	0.151	−	0.004
Insulin	−	0.004	+	**0.000**

[a] Significant results are highlighted in bold ($p < 0.002$, after Bonferroni correction).

Table 5. Association between Glycan Fractions and Health Parameters[a]

		LC-7		LC-8	
	% of participants diagnosed	coef.	*P*	coef.	*P*
MI	2.8	−0.028	0.406	**-0.065**	**0.049**
CVA	3.0	0.019	0.679	0.013	0.753
diabetes	5.2	−0.004	0.828	0.005	0.824

[a] Significant results are highlighted in bold ($p < 0.05$).

As the numbers of individuals to which MI has occurred is low in the study population (2.8%), this finding has to be replicated in other studies.

DISCUSSION

In this study, we confirmed that glycan features change with calender age and that levels of glycans may differ between males and females (e.g., see refs 12, 20, 23, and 24). The most considerable correlations with calendar age were observed for the biantennary nongalactosylated glycan, core-fucosylated glycans, disialylated forms of biantennary glycans, as well as nongalactosylated and digalactosylated glycans. Because, we aimed to identify total plasma protein N-glycosylation based markers that reflect familial longevity and healthy aging, we compared the middle-aged offspring of nonagenarian siblings, representing healthy agers and controls. Two N-glycan features LC-7 and LC-8 were identified that are more abundant in plasma of the offspring. These glycans did not show a significant association with calendar age in the total population, but it was observed that the levels of LC-7 tend to increase with calendar age in males.

Since longevity has previously been associated with glucose homeostasis, insulin sensitivity and lipid parameters, we investigated whether LC-7 and LC-8 actually mark the effect of one of these parameters on longevity. While LC-7 could only be associated with BMI, LC-8 was associated with BMI, levels of HDL-cholesterol, triglycerides and insulin. The association of LC-7 and LC-8 with familial longevity was, however, found to be independent of these metabolic phenotypes. Blood glucose levels are biochemically not directly linked to glycan levels and this is corroborated by the fact that features of glycosylation associate with familial longevity independent of glucose homeostasis.

Serum CRP levels have been described as a marker for inflammation and the risk for cardiovascular disease[25] and therefore reflect decreased health. As the two glycosylation features LC-7 and LC-8 are both negatively correlated with CRP levels, it may be concluded that these glycan-features also reflect an individual's inflammatory health status. Although the prevalence of disease is still rather low in the middle aged offspring and controls, we observed association of higher LC-8 levels with lower MI prevalence, which is a characteristic of the long-lived LLS families.[14] This finding, however, needs replication in other studies. Upon follow-up of the individuals of the LLS, the association may be investigated further as more individuals will be diagnosed with metabolic and cardiovascular diseases.

Interestingly, glycan features LC-7 and LC-8 show very similar behavior in their association with familial longevity and these

features elute subsequently during analysis. This raises the question whether these features partially consist of the same components. However, there are also differences between the two features, for example, the association between glycosylation and insulin level is not observed for LC-7, but is highly significant for LC-8, indicating that the components of LC-7 and LC-8 are not completely the same. Currently, the glycan annotation of the features is performed using fractionation of one standard plasma sample from a healthy individual,[17] and only the most abundant glycans in the fractions are annotated. LC-7 is annotated as a nonfucosylated diantennary nonsialylated glycan, while LC-8 is annotated as a nonfucosylated diantennary glycan with one truncated antenna and one sialylated antenna. Based on previous work and the GlycoBase database,[27] which was recently developed for the annotation of 2-AB-labeled glycans in HILIC-LC analysis, it is clear that also less abundant glycans elute in glycan fractions LC-7 and LC-8. Therefore, there is a need to further identify the exact composition of the longevity associating glycan features LC-7 and LC-8. To do so, it would be important to study the composition of LC-7 and LC-8 in selected samples of the LLS, derived from both offspring and controls as the current annotation is based on one control sample only. The most suitable technique for such analyses would be LC−MS, as the availability of mass spectrometric data largely facilitates the confirmation of the annotation.

As the current method comprises release of N-glycans from all plasma glycoproteins, the N-glycosylation pattern reflects N-glycan on this total glycoprotein pool. Altered glycosylation patterns may, therefore, be due to changes in plasma glycoprotein concentrations, but may also be caused by altered glycosylation of one or more glycoproteins. It could thus be beneficial to generate in parallel quantitative protein profiles, to allow identification of altered protein expression. Alternatively, the glycosylation pattern of a given specific glycoprotein may be monitored, similarly to our previous study on IgG glycosylation.[21]

Given that the plasma profiles generated in this study reflect the glycosylation of a pool of glycoproteins, and assuming that the changes in glycosylation are caused by altered glycosylation and not by protein expression, it would be interesting to investigate whether the changes in the levels of these glycan(s) are caused by the attachment to a single glycoprotein, a group or even several groups of glycoproteins. Such future studies would need the use of extensive lectin-, antibody- or chromatographic-purification steps.

The regulation of glycosylation is a very complex cellular process, and the biological pathways involved in longevity and healthy aging have only started to become unraveled. Currently, there is no clear regulator that would link familial longevity with the regulation of the plasma N-glycan profile. To allow further in-depth analysis, the glycosylation in long-lived mice with various genetic backgrounds, such as the GHRKO and the FIRKO mice described in[28] and,[29] respectively, could be studied. As mice can be bred under standardized conditions, and additional parameters could be analyzed more easily than in humans, such a model would facilitate the search for alterations associatied with longevity that could regulate protein N-glycosylation.

In conclusion, we found two glycan features, of which increased levels in human plasma mark familial longevity and healthy aging. These features also associate to metabolic parameters (s.a. BMI and insulin levels) and one of them with the risk of MI. Yet the association with familial longevity does not depend on the metabolic factors. The two N-glycan features are not correlated with glucose level, a previously found marker for human longevity,[15] and may therefore be considered a novel group of markers. Further studies are needed to identify regulatory pathways that cause such altered glycosylation.

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