

# Human Plasma Glycome in Attention-Deficit Hyperactivity Disorder and Autism Spectrum Disorders\*<sup>§</sup>

Nela Pivac‡, <sup>d</sup> Ana Knežević§, <sup>d</sup> Olga Gornik§, Maja Pučić¶, Wilmar Igl||, Hilde Peeters\*\*, An Crepel\*\*, Jean Steyaert\*\*, Mislav Novokmet¶, Irma Redžić§, Matea Nikolac‡, Vesna Novković Hercigonja‡‡, Katarina Dodig Ćurković§§, Mario Ćurković¶¶, Gordana Nedić‡, Dorotea Muck-Seler‡, Fran Brovečki|||, Igor Rudan,<sup>ab</sup> and Gordan Lauc§¶<sup>c</sup>

Over a half of all proteins are glycosylated, and their proper glycosylation is essential for normal function. Unfortunately, because of structural complexity of nonlinear branched glycans and the absence of genetic template for their synthesis, the knowledge about glycans is lagging significantly behind the knowledge about proteins or DNA. Using a recently developed quantitative high throughput glycan analysis method we quantified components of the plasma *N*-glycome in 99 children with attention-deficit hyperactivity disorder (ADHD), 81 child and 5 adults with autism spectrum disorder, and a total of 340 matching healthy controls. No changes in plasma glycome were found to associate with autism spectrum disorder, but several highly significant associations were observed with ADHD. Further structural analysis of plasma glycans revealed that ADHD is associated with increased antennary fucosylation of biantennary glycans and decreased levels of some complex glycans with three or four antennas. The design of this study prevented any functional conclusions about the observed associations, but specific differences in glycosylation appears to be strongly associated with ADHD and warrants further studies in this direction. *Molecular & Cellular Proteomics* 10: 10.1074/mcp.M110.004200, 1–7, 2011.

From the ‡Division of Molecular Medicine, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia, §University of Zagreb, Faculty of Pharmacy and Biochemistry, Ante Kovačića 1, 10000 Zagreb, Croatia, ¶Genos Ltd., Glycobiology Division, Planinska 1, 10000 Zagreb, Croatia, ||Department of Genetics and Pathology, Rudbeck Laboratory; Uppsala University; Uppsala, Sweden, \*\*Center of Human Genetics, University Hospitals Leuven, Leuven, Belgium, ‡‡Polyclinic Kocijan/Hercigonja, Lipovecka 17, 10000 Zagreb, Croatia, §§Clinical Hospital Osijek, Department of Child and Adolescent Psychiatry, J. Huttlera 4, 31000 Osijek, Croatia, ¶¶Primary Care Unit Osijek, Park Kralja Petra Krešimira 4, 31000 Osijek, Croatia, |||University of Zagreb Medical School, 10000 Zagreb, Croatia, <sup>a</sup>Croatian Centre for Global Health; University of Split Medical School; 21000 Split, Croatia, <sup>b</sup>Centre for Population Health Sciences, The University of Edinburgh Medical School, Teviot Place, Edinburgh, UK

Received August 10, 2010, and in revised form, October 12, 2010  
Published, MCP Papers in Press, October 25, 2010, DOI 10.1074/mcp.M110.004200

Attention-deficit hyperactivity disorder (ADHD)<sup>1</sup> and autism spectrum disorders (ASDs) are both highly heritable multifactorial and clinically heterogeneous childhood-onset neurodevelopmental disorders (1, 2). The hallmarks of ADHD are severe inattention, hyperactivity, and impulsivity whereas the characteristic symptoms of ASD include impaired communication and social interaction skills as well as repetitive and restricted behavior and interests (3). Several pieces of evidence point toward some shared heritability and thus genetic factors contributing to the occurrence of both ADHD and ASD (4). Children with ADHD and ASD frequently share symptoms of impaired communication skills, impaired social interactions (4), and develop difficulties in familial, educational, and academic functioning (5, 6). The abnormal functioning of the dopaminergic and serotonergic systems (5, 7–9), among other neurotransmitters, were repeatedly found in ADHD and ASD, and therefore the candidate genes common for both disorders are the dopaminergic transporter gene, dopaminergic receptor type 3 and type 4 genes, serotonin transporter gene, and genes for catechol-*o*-methyl-transferase and monoamine oxidase type A (4), although genome-wide association studies did not confirm with certainty all these candidates (10). A shared genetic basis is further suggested by the higher frequency of ADHD and ASD traits in children with a defined genetic diagnosis such as 22q11 deletion syndrome, XYY syndrome, and microdeletions or duplications in the chromosome 15q13.2-q13.3 region (11, 12). However, the molecular agents related to various biological processes and interactions between different risk factors responsible for development of ADHD and ASD remain elusive (6).

Protein *N*-glycosylation is an ancient metabolic pathway invented before the diversification of Archea, Bacteria, and Eukarya, which still exists in all three domains of life (13, 14). At least 2000 different glycan determinants have been added

<sup>1</sup> The abbreviations used are: ADHD, attention-deficit hyperactivity disorder; ASD, autism spectrum disorder; HILIC, high performance liquid chromatography; HPLC, high pressure liquid chromatography.

to the backbone of mammalian polypeptides (15) and the complexity of the glycoproteome is estimated to be several orders of magnitude greater than for the proteome itself (16). *N*-glycosylation is essential for multicellular life and its complete absence is embryonically lethal (17). Disregulation of glycosylation is associated with a wide range of diseases, including cancer, diabetes, and cardiovascular, congenital, immunological, and infectious disorders (18–20), but because of experimental difficulties in analyzing complex glycan structures, the knowledge about glycan structure and function is still lagging behind the knowledge about other macromolecules (21). However, recent technological advances have allowed reliable, high-throughput quantification of *N*-glycans (22–24), which now permit large scale studies aimed at understanding of the role of protein glycosylation in complex diseases.

Variations in glycosylation are of great physiological significance because changes in glycans significantly change the structure and function of polypeptide parts of glycoproteins (25). Proper glycosylation of membrane receptors is particularly important as it modulates adaptive properties of the cell and affects communication between cells (26). Altered glycosylation and ensuing differences in function of various receptors could be a predisposing factor in different psychiatric disorders, and indeed some of them were reported to be associated with altered protein glycosylation (27–31). Here we report the first analysis of plasma *N*-glycans in patients with ADHD and ASD.

### EXPERIMENTAL PROCEDURES

**Sample Population and Study Design**—The study included 99 medication-free children with ADHD ( $9.00 \pm 2.78$  years old), recruited sequentially from the Polyclinic Kocijan/Hercigonja, Zagreb, diagnosed using psychiatric interview (APA, 1994), according to the DSM-IV criteria, separate interviews with the children and their parents, psychological interview and psychological tests, physical examination, and the Conners' Rating Scale for Parents-short version (32). Demographical data were uniform for all children: they were Caucasians of urban origin, attended kindergarten or primary school, and were coming from the undisturbed families, with average intellectual abilities. Exclusion criteria were comorbid depression, schizophrenia, oppositional defiant disorder, conduct disorder, borderline personality disorder and somatic disorders, all assessed by psychiatric interview based on DSM-IV criteria (APA 1994), or pharmacotherapy with carbamazepine, clomipramine, lamotrigine or selective serotonin reuptake inhibitors.

Control group included 175 healthy children ( $10.94 \pm 4.01$ ), attending their regular physical exams, served as controls. Somatic or other psychiatric comorbidities were excluded according to DSM-IV criteria and the Child Symptoms Inventory-4 (APA 1994). All parents signed written informed consent.

Sixty families with one or more children with ASD were included. These families were selected from the Autism Family Study by the Leuven Autism Research consortium (LAuRes). All patients have a clinical diagnosis of ASD according to DSM-IV criteria. All diagnoses were made by a child psychiatrist and a multidisciplinary team in the Expert Center for Autism in Leuven. All patients, siblings, and parents have been examined by a clinical geneticist and patients with known underlying genetic conditions were excluded. The criterion for inclusion in this

study was normal intelligence of all family members. All family members have a normal karyotype and FMR1 mutations and premutations were excluded. In total the sample includes 251 individuals (81 children and 5 adults with ASD and 165 individuals without ASD).

Blood samples (4 ml) were taken during routine laboratory tests from a forearm vein into a plastic syringe with 1 ml of acid citrate dextrose anticoagulant at 08.00 a.m. following overnight fasting.

Local ethics committees approved all protocols. The study was carried out in accordance with the Helsinki declaration, and the samples were coded in sequential way without recording any personal information about the patients.

**Glycan Release and Labeling**—Glycan release and labeling was performed as reported previously (33). 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. Following extraction, glycans were fluorescently labeled with 2-aminobenzamide.

**Hydrophilic Interaction High Performance Liquid Chromatography (HILIC)**—Released glycans were subjected to hydrophilic interaction high performance liquid chromatography (HILIC) on a  $250 \times 4.6$  mm i.d. 5- $\mu$ m particle packed TSK gel Amide 80 column (Tosoh Bioscience, Stuttgart, Germany) at 30 °C with 50 mM formic acid adjusted to pH 4.4 with ammonia solution as solvent A and acetonitrile as solvent B. Sixty-minute runs were performed with fluorescence detector set with excitation and emission wavelengths of 330 and 420 nm, respectively. The system was calibrated using an external standard of hydrolyzed and 2-AB-labeled glucose oligomers from which the retention times for the individual glycans were converted to glucose units.

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

**Weak Anion Exchange High Performance Liquid Chromatography (WAX-HPLC)**—Glycans were separated according to the number of sialic acids by weak anion exchange HPLC. The analysis was performed using a Prozyme GlycoSep C 75 mm  $\times$  7.5 mm column (Prozyme, Leandro, CA) 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 samples were dried down in 96-well PCR plates. To these, the following was added: 1  $\mu$ l of 250 mM sodium phosphate incubation buffer (pH 6.0), 0.5  $\mu$ l (0.0025 U) of ABS, *Arthrobacter ureafaciens* sialidase (releases  $\alpha$ 2–3,6,8 sialic acid, Prozyme) and H<sub>2</sub>O to make up to 5  $\mu$ l. This was incubated overnight (16–18 h) at 37 °C and then passed through AcroPrep™ 96 Filter Plates, 350  $\mu$ l well, 10K (Pall Corporation, Port Washington, NY) before applying to the HPLC.

**Fucosidase Digestion**—To examine differences in levels of structures present in peak DG7 between ADHD samples and controls, aliquots of the 2AB-labeled glycans were mixed together to make an ADHD glycan pool and a control glycan pool. These pooled samples were dried down and concentrated pool samples were applied on HILIC column. Peaks DG7 were collected from both glycan pools and dried down in PCR tubes before desired digestion. To these, the following was added: 1  $\mu$ l of 250 mM sodium acetate incubation buffer (pH 5.0), 1  $\mu$ l (0.003 mU) of AMF, *Almond meal* fucosidase (releases

TABLE I

Descriptive parameters of plasma glycome in children with ADHD and controls. Glycan structures present in each glycan peaks (GP1–16, DG1–13) are shown in Supplementary Table I, and the way other glycans traits were calculated is described in the Materials and Methods section. Associations between ADHD and *N*-glycans were analyzed using a standard linear fixed effects model (*i.e.* linear regression) with *N*-glycans as the criterion and ADHD as the predictor. Additionally, sex and age were used as covariates. Associations with statistical significance below the pre-set level of  $p < 0.001$  are shown in bold.

	ADHD				Controls				Linear regression with ADHD as a predictor	
	N	Median	Percentiles		N	Median	Percentiles		P	$\beta$
			25th	75th			25th	75th		
Age	99	9.00	7.00	11.00	173	11.00	6.00	15.00		
Glycan feature										
GP1	96	0.07	0.05	0.09	175	0.06	0.04	0.08	4.04E-01	0.12
GP2	96	3.39	2.71	4.02	175	3.17	2.57	3.79	7.15E-01	-0.05
GP3	96	1.66	1.52	1.86	175	1.65	1.47	1.84	9.69E-01	0.01
GP4	96	5.65	4.84	6.53	175	5.70	4.84	6.22	9.92E-01	0.00
GP5	96	2.21	2.01	2.41	175	2.12	1.92	2.39	7.91E-03	0.37
GP6	96	4.47	3.86	5.05	175	4.28	3.83	4.80	3.80E-02	0.30
GP7	96	8.97	8.48	9.57	175	8.82	8.34	9.60	1.76E-01	0.19
GP8	96	10.32	9.72	10.84	175	10.29	9.57	10.98	3.98E-01	-0.12
GP9	96	39.80	37.74	41.85	175	39.28	37.83	41.58	3.33E-01	0.13
GP10	96	6.06	5.54	6.69	175	6.48	5.90	7.01	3.18E-01	-0.14
GP11	96	1.45	1.33	1.56	175	1.29	1.08	1.41	<b>1.22E-13</b>	0.99
GP12	96	1.70	1.48	2.01	175	1.77	1.53	2.17	<b>2.84E-06</b>	-0.53
GP13	96	6.02	5.06	6.95	175	5.98	5.03	6.72	6.05E-01	-0.07
GP14	96	5.27	4.53	6.24	175	6.04	5.21	6.87	2.44E-03	-0.42
GP15	96	0.84	0.69	1.03	175	0.76	0.59	0.95	7.94E-01	0.03
GP16	96	1.49	1.29	1.75	175	1.51	1.25	1.89	2.36E-03	-0.38
DG1	96	0.08	0.07	0.11	175	0.09	0.07	0.11	3.08E-01	-0.14
DG2	96	3.67	2.94	4.20	175	3.36	2.76	3.96	8.58E-01	0.02
DG3	96	2.32	2.11	2.58	175	2.29	2.07	2.53	4.62E-01	0.10
DG4	96	6.73	5.84	7.71	175	6.72	5.86	7.37	9.25E-01	0.01
DG5	96	50.54	48.60	52.95	175	50.68	48.22	53.20	1.66E-01	0.19
DG6	96	14.54	12.70	16.03	175	14.73	13.28	16.52	2.41E-01	-0.17
DG7	96	1.54	1.31	1.87	175	1.39	1.16	1.65	<b>1.02E-04</b>	0.53
DG8	96	11.65	10.25	13.08	175	11.69	10.53	13.08	2.80E-01	-0.15
DG9	96	2.80	2.32	3.64	175	3.33	2.58	4.13	1.29E-02	-0.35
DG10	96	0.98	0.89	1.06	175	0.93	0.85	1.02	1.63E-02	0.34
DG11	96	2.83	2.49	3.43	175	2.72	2.32	3.30	7.92E-01	-0.03
DG12	96	0.80	0.61	1.01	175	0.83	0.65	1.03	1.90E-01	-0.19
DG13	96	0.72	0.54	0.80	175	0.63	0.50	0.75	2.40E-01	0.16
Monosialo	88	21.68	20.40	22.76	175	21.59	20.06	23.41	7.31E-01	0.05
Disialo	88	60.77	59.22	61.92	175	59.54	58.27	61.26	3.45E-02	0.27
Trisialo	88	15.07	13.42	16.71	175	15.48	14.07	17.00	6.95E-02	-0.25
Tetrasialo	88	2.72	2.34	3.13	175	2.79	2.34	3.51	1.24E-03	-0.40
FUC-C	96	22.38	19.14	24.59	175	22.41	20.15	24.98	2.01E-01	-0.18
FUC-A	96	2.98	2.49	3.48	175	2.69	2.30	3.18	<b>9.56E-04</b>	0.46
BAMS	96	28.75	27.62	30.09	175	28.80	27.40	30.27	7.95E-01	-0.04
BADS	96	70.63	68.65	72.71	175	70.46	68.65	72.48	5.18E-01	0.09
BA	96	80.00	78.19	81.71	175	79.60	77.91	81.37	4.07E-02	0.27
TRIA	96	15.46	14.25	16.91	175	16.12	14.93	17.21	1.52E-02	-0.33
TA	96	4.37	3.99	5.05	175	4.23	3.59	5.01	5.93E-01	-0.07
G0	96	3.75	3.00	4.28	175	3.43	2.86	4.07	8.89E-01	0.02
G1	96	9.09	8.20	10.01	175	9.00	8.19	9.73	8.59E-01	0.03
G2	96	67.11	65.22	68.60	175	67.16	65.20	68.74	8.71E-02	0.22
G3	96	13.17	11.58	14.70	175	13.86	12.54	15.31	1.21E-03	-0.42
G4	96	2.38	1.97	2.73	175	2.25	1.91	2.83	4.18E-02	-0.25
A2	96	0.08	0.06	0.11	175	0.07	0.06	0.10	8.73E-01	0.02

$\alpha$ 1–3,4 fucose, Prozyme) and H<sub>2</sub>O to make up to 5  $\mu$ l. This was incubated overnight (16–18 h) at 37 °C and then passed through AcroPrep™ 96 Filter Plates, 350  $\mu$ l well, 10K (Pall Corporation, Port Washington, NY) before applying to the HPLC.

**Glycan Structural Features**—Levels of glycans sharing the same structural features were approximated by adding the structures having the same characteristic, from either HILIC, weak anion exchange, or following sialidase treatment integrated glycan profiles (individual glycan structures present in each glycan group that were reported

previously are shown in Supplemental Table 1). Glycans were quantified from weak anion exchange profiles according to the level of sialylation (monosialylated, disialylated, trisialylated, and 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 = DG1+DG2+DG3+DG4+DG5+DG6+DG7; Monosialylated biantennary glycans = (GP7+GP8)/(DG5+DG6+DG7)\*100; Disialylated biantennary glycans BADS = (GP9+GP10+GP11)/(DG5+DG6+DG7)\*100;

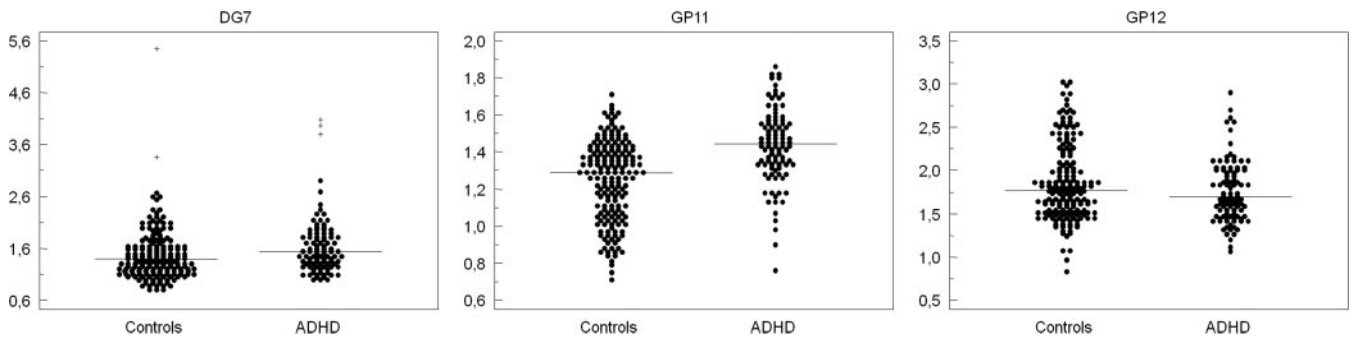


FIG. 1. Levels of glycan groups DG7, GP11 and GP12 in ADHD and controls. Plasma glycome of ADHD patients and controls was separated into 13 desialylated chromatographic peaks (DG series) and 16 sialylated chromatographic peaks (GP series) and expressed as percentages of the total plasma glycome. The majority of peaks did not differ between groups, but statistically significant differences were observed for DG7, GP11 and GP12. Each individual is represented as a black dot and median level is marked with a horizontal line.

Triantennary glycans = DG8+DG9+DG10; Tetraantennary glycans = 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.

**Statistical Analysis**—Associations between ADHD and N-glycans were analyzed using a standard linear fixed effects model (i.e. linear regression) with N-glycans as the criterion and ADHD as the predictor. Additionally, sex and age were used as covariates. The analysis was performed using the *lm()* and *anova()* functions of the statistical analysis system R (Version 2.7.2) (34).

Associations between ASD and N-glycans were calculated using a linear mixed effects model with N-glycans as the criterion and ASD as the predictor (fixed effect). Additionally, sex and age were used as covariates (both fixed effects). The pedigree structure (i.e. father, mother, child) was modeled as a random effect. We used a linear mixed effects model because a standard linear fixed effects model is not adequate for this data structure and will give progressive results, i.e. to low *p* values. The sample is clustered in families, and therefore each individual of two related individuals contains less “information” than a sample consisting of two unrelated individuals. The analysis was performed using the statistical analysis program SOLAR (<http://solar.sfbgenetics.org/>) (35).

Multiple tests on associations between mental disorders and plasma N-glycans were performed. To account for false positive results because of  $\alpha$  inflation, we performed a Bonferroni adjustment per disorder. Therefore, we used an  $\alpha$  error threshold of  $0.05/46 = 0.001$ . Because we are aware of the fact that this method is conservative if applied to correlated traits such as plasma N-glycans, as the method is based on the assumption of independent traits, we have taken this into account in the discussion.

RESULTS

Using a recently developed method for high throughput quantitative analysis of glycans we analyzed the plasma glycome in 99 medication-free children with ADHD ( $9.00 \pm 2.78$  years old) and 175 healthy control children ( $10.94 \pm 4.01$  years old). Three HPLC analyses were performed for each sample resulting in 33 directly quantified groups of glycans. The exact composition of the each glycan group is shown in Supplemental Table 1. Further 13 glycosylation features were derived from the measured values as described in the Materials and Methods section, resulting in

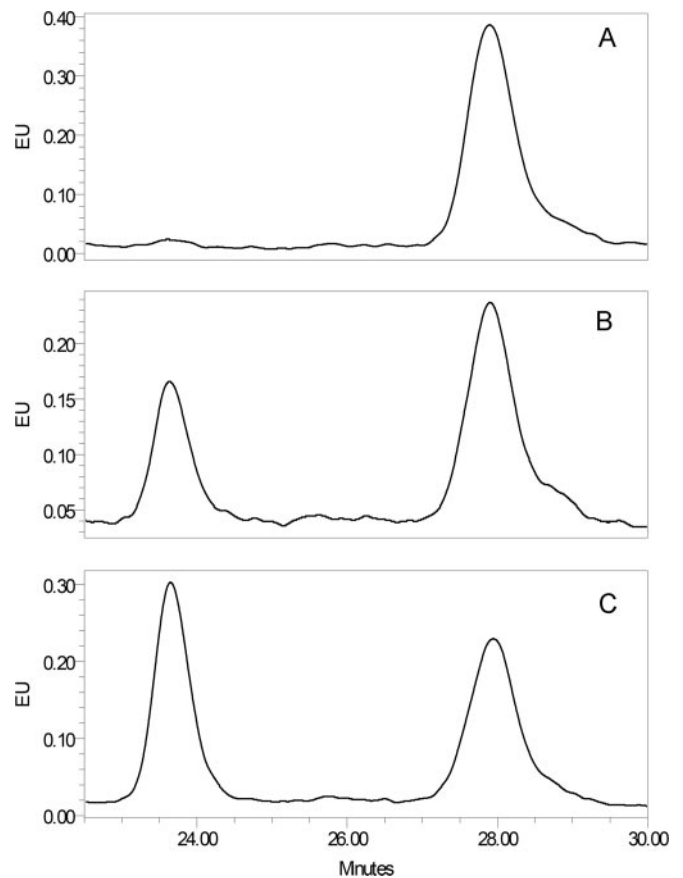


FIG. 2. Structural analysis of glycans associated with ADHD. A, Glycan peak DG7 isolated by HPLC from the control glycan pool. This peak consists of two structures: A2FG2 and M7, which both elute at 26 min. B, Analysis of DG7 from control glycan pool following digestion with  $\alpha(1-3,4)$  fucosidase that converts A2FG2 to A2G2. The new peak eluted at 23.75 min, contrary to M7 structure, which remained on the original of DG7 peak. C, Analysis of DG7 from ADHD glycan pool following digestion with  $\alpha(1-3,4)$  fucosidase. Comparison of panels B and C clearly reveals that in ADHD there is an increase of the relative ratio of A2FG2 compared with M7.

the total number of 46 analyzed glycan variables. Basic descriptive parameters for these 46 glycan features are shown in Table I.

TABLE II

Descriptive parameters of plasma glycome in children with ASD and controls. Glycan structures present in each glycan peaks (GP1–16, DG1–13) are shown in Supplementary Table I, and the way other glycans traits were calculated is described in the Materials and Methods section. Associations between ASD and *N*-glycans were calculated using a linear mixed effects model with *N*-glycans as the criterion and ASD as the predictor (fixed effect). Additionally, sex and age were used as covariates (both fixed effects).

	ASD				Controls				Linear regression with ASD as predictor
	N	Median	Percentiles		N	Median	Percentiles		
			25th	75th	Valid		25th	75th	
Age	78	11.00	9.00	14.00	48	12.50	9.25	15.75	
Glycan feature									p
GP1	78	0.06	0.05	0.07	48	0.05	0.04	0.07	5.03E-01
GP2	78	2.93	2.53	3.43	48	2.55	2.09	3.19	3.76E-01
GP3	78	1.66	1.55	1.84	48	1.61	1.40	1.82	6.39E-02
GP4	78	5.50	4.89	5.95	48	5.21	4.55	6.03	7.81E-01
GP5	78	2.34	2.20	2.49	48	2.29	2.13	2.52	6.90E-02
GP6	78	4.16	3.77	4.48	48	4.27	3.74	4.80	7.22E-01
GP7	78	9.65	9.14	10.35	48	9.57	9.03	10.36	6.87E-01
GP8	78	10.53	9.85	11.08	48	10.78	10.01	11.24	8.88E-01
GP9	78	40.28	38.37	41.58	48	40.38	38.25	41.64	9.87E-01
GP10	78	6.62	5.81	7.25	48	6.58	6.04	7.02	5.86E-01
GP11	78	1.62	1.48	1.76	48	1.59	1.41	1.74	1.00E+00
GP12	78	1.56	1.42	1.66	48	1.56	1.42	1.71	8.71E-01
GP13	78	5.52	4.97	6.14	48	5.76	5.01	6.62	6.22E-01
GP14	78	5.38	4.63	6.47	48	5.56	4.93	6.08	6.79E-01
GP15	78	0.76	0.60	0.87	48	0.77	0.65	0.91	6.05E-01
GP16	78	1.30	1.16	1.47	48	1.27	1.12	1.49	5.41E-01
DG1	78	0.07	0.05	0.10	48	0.06	0.04	0.09	9.72E-01
DG2	78	3.53	3.08	4.20	48	3.25	2.76	3.69	7.49E-01
DG3	78	2.50	2.28	2.71	48	2.44	2.09	2.69	1.06E-01
DG4	78	6.48	5.80	6.99	48	6.27	5.21	6.91	7.64E-01
DG5	78	51.71	50.20	53.22	48	51.52	50.04	53.05	9.60E-01
DG6	78	14.80	13.75	16.06	48	14.88	13.84	15.95	7.76E-01
DG7	78	1.50	1.37	1.71	48	1.54	1.37	1.73	3.38E-01
DG8	78	10.74	9.93	11.95	48	11.09	10.12	12.33	7.17E-01
DG9	78	3.03	2.37	3.94	48	3.32	2.69	3.75	3.84E-01
DG10	78	0.98	0.90	1.06	48	0.98	0.92	1.03	4.64E-01
DG11	78	2.65	2.29	2.93	48	2.59	2.36	2.98	9.46E-01
DG12	78	0.76	0.61	0.96	48	0.79	0.69	0.96	4.89E-01
DG13	78	0.75	0.62	0.91	48	0.77	0.66	0.93	9.63E-01
Monosialo	78	22.15	21.04	23.79	48	22.29	20.82	23.60	6.27E-01
Disialo	78	60.14	58.94	61.34	48	59.94	58.74	61.11	6.05E-01
Trisialo	78	13.55	12.64	15.06	48	13.72	12.66	16.07	6.78E-01
Tetrasialo	78	3.47	3.10	4.00	48	3.52	3.05	3.89	1.67E-01
FUC-C	78	22.27	20.45	23.94	48	22.56	20.82	24.05	8.52E-01
FUC-A	78	2.84	2.56	3.28	48	2.88	2.64	3.31	3.38E-01
BAMS	78	29.73	28.20	31.21	48	29.76	28.50	31.04	6.80E-01
BADS	78	70.78	69.21	72.46	48	71.41	68.68	72.38	5.99E-01
BA	78	80.60	79.29	82.17	48	80.73	78.23	81.73	4.32E-01
TRIA	78	15.18	13.93	16.28	48	15.17	14.40	16.96	3.02E-01
TA	78	4.16	3.72	4.65	48	4.27	3.85	4.66	9.95E-01
A2	78	0.07	0.05	0.09	48	0.06	0.04	0.08	8.13E-01

Some glycans significantly change with age (36), thus associations between ADHD and *N*-glycans were analyzed using a standard linear fixed effects model with *N*-glycans as the criterion, ADHD as the predictor, and sex and age as covariates. Following correction for multiple testing ( $0.05/46 = 0.001$ ) four associations of glycan levels were found to be significant, whereas further five were suggestively close to statistical significance (Table I).

The most prominent changes in plasma glycans in ADHD were the increases in glycan groups GP11 and DG7 and a decrease in GP12 (Fig. 1). Examples of control and ADHD chromatograms are presented in the Supplemental Fig. 1.

Although GP11 partly overlapped with GP10 and could not have been collected following chromatographic separation, the DG7 group was clearly separated from other peaks which enabled us to collect it for more detailed structural analysis. Main glycan structures present in GP7 are M7 (oligomannose glycan composed of two core *N*-acetylglucosamine and seven mannose residues) and A2FG2 (complex biantennary glycan with two core *N*-acetylglucosamine, three mannose, two antennary *N*-acetylglucosamine, one antennary fucose, and two antennary galactose residues). To determine which of the two structures is associated with ADHD, we pooled desialylated labeled glycans from 16 ADHD and 16 control sam-

ples, separated glycans by HILIC chromatography and collected eluates corresponding to DG7 peaks. These two eluates (ADHD and control) were then treated with an enzyme ( $\alpha$ -1-3,4 *almond meal* fucosidase), which removes antennary fucose. Defucosylated ADHD and control glycan pools were then reanalyzed by HPLC to separate A2G2 (A2FG2 following the removal of fucose) from M7. Results presented in Fig. 2 clearly demonstrate that although M7 is the major component of DG7 in controls, A2FG2 is the major component of DG7 in ADHD samples, and that it is the increase in A2FG2 that associates DG7 with ADHD. Confirmation for this observation was provided by treating the same pair of samples with the enzyme  $\alpha$ (1-2,3,6) *Jack bean* mannosidase that specifically cleaves mannose residues. This experiment (data not shown) showed that whereas in control samples the major glycan structure in DG7 is M7, in ADHD A2FG2 is increased and becomes the main glycan structure in DG7. This conclusion was further supported by digestion of the complete plasma glycome with  $\alpha$ (1-2,3,6) *Jack bean* mannosidase, which confirmed that there are no changes in the proportion of oligomannose structures (including M7) in ADHD (Supplemental Fig. 2).

The same type of glycan analyses were also performed on 81 autistic children and their 168 healthy siblings and parents. Basic descriptive parameters for the 46 measured glycan features are presented in Table II. Because this study sample was clustered in families, a linear mixed effects model was used to calculate statistical significance of the observed differences. No significant associations between glycan levels and ASD were observed.

#### DISCUSSION

We have performed the first analysis of human plasma *N*-glycome in patients with ADHD and ASD. Although we have found no differences between children with ASD and their healthy siblings, several significant associations between plasma glycans and ADHD were identified (Table I). By performing exoglycosidase sequencing, we have convincingly demonstrated that the observed association between ADHD and increased DG7 (and consequently also increased FUC-A, which is calculated from DG7) derived from the increase in relative levels of biantennary glycans with antennary fucose (A2FG2). In addition, ADHD was associated with a regular pattern of changes in GP11, GP12, GP14, GP16, tetrasialo-glycans, and trigalactosylated glycans. Two of these changes (GP11 and GP12) were statistically highly significant, whereas the remaining four were only slightly below the preset significance level of  $p < 0.001$  ( $p$  values between 0.0012 and 0.0024). Because the Bonferroni correction is highly conservative, these results suggest that the levels of GP14, GP16, tetrasialo glycans, and trigalactosylated glycans are also altered in ADHD. All these changes were consistent with the association between ADHD and the decrease in branching of plasma glycans. The only group of glycans that was found to

be increased in ADHD was GP11 (which is composed of mostly biantennary glycans), whereas all glycan groups that were decreased in ADHD consisted of tri- and tetraantennary glycans.

Glycan branching is very important for the regulation of adaptive properties of the cell membrane (26) and it was reported that the increased branching promotes order in the cell membrane and prolongs membrane receptor half-life (37). In addition, the exact structure of glycans attached to individual membrane receptors significantly affects their functional properties (25), thus it is easily imaginable that differences in glycosylation efficiency could result in altered neural signaling and represent a predisposition for the development of ADHD. It is important to note that we have analyzed levels of individual glycans in plasma. Each glycan can originate from several different proteins, and because glycosylation is cell- and protein-specific, relatively small difference in plasma levels could have important local functional consequences.

Recently we completed the first comprehensive population study of human plasma *N*-glycome, which revealed variability that by far exceeds the variability of proteins and DNA (33). However, within a single individual the composition of plasma glycome is remarkably stable (38) and environmental factors have rather limited impact on the majority of glycans (36). Specific altered glyco-phenotypes that can be associated with specific pathologies were also identified to exist in a population (39). Here we have described a new glyco-phenotype composed of increased A2FG2 and decreased tri- and tetraantennary glycans that associates with ADHD, whereas plasma *N*-glycome was not found to be significantly altered in ASD. We would speculate that the decrease in glycan branching could be one of the predisposing factors for ADHD because it was already reported to be associated with the activity of membrane receptors (26). If this proves to be correct, this would open a new direction for the development of potential therapeutics for ADHD.

\* This work was supported by the Croatian Ministry of Science, Education and Sport grants #309-0061194-2023 (to G. L.), #098-0982522-2455 (to N. P.) and #216-1080315-0302 (to I. R.); by European Commission EUROPARM and EUROGLYCOARRAYS grants, and by a grant from GOA 2006/12 Leuven Belgium and a grant from Cure Autism Now (USA). H. P. is postdoctoral researcher of the Fund for Scientific Research-Flanders FWO, Belgium.

§ This article contains supplemental Figs. 1-2 and Table I.

<sup>c</sup> To whom correspondence should be addressed: University of Zagreb, Faculty of Pharmacy and Biochemistry, Ante Kovačića 1, 10000 Zagreb, Croatia. Tel.: +385 1 639 4467; Fax: +385 1 639 4400; E-mail: glauc@pharma.hr.

<sup>d</sup> These two authors contributed equally.

Conflict of interest: None declared.

#### REFERENCES

1. Faraone, S. V., and Khan, S. A. (2006) Candidate gene studies of attention-deficit/hyperactivity disorder. *J. Clin. Psychiatry* 67 Suppl. 8, 13-20
2. Levy, S. E., Mandell, D. S., and Schultz, R. T. (2009) *Autism. Lancet*. 374, 1627-1638
3. American Psychiatric Association (2000) *Diagnostic and statistical manual of mental disorders, 4th edn, Text Revision*, American Psychiatric Association.

- ciation, Washington, DC.
4. Rommelse, N. N., Franke, B., Geurts, H. M., Hartman, C. A., and Buitelaar, J. K. (2010) Shared heritability of attention-deficit/hyperactivity disorder and autism spectrum disorder. *Eur. Child Adolesc. Psychiatry*. **19**, 281–295
  5. Biederman, J. (2005) Attention-deficit/hyperactivity disorder: a selective overview. *Biol. Psychiatry*. **57**, 1215–1220
  6. Wall, D. P., Esteban, F. J., Deluca, T. F., Huyck, M., Monaghan, T., Velez, de, Mendizabal, N., Goñi, J., and Kohane, I. S. (2009) Comparative analysis of neurological disorders focuses genome-wide search for autism genes. *Genomics* **93**, 120–129
  7. Nedic, G., Pivac, N., Hercigonja, D. K., Jovancevic, M., Curkovic, K. D., and Muck-Seler, D. (2010) Platelet monoamine oxidase activity in children with attention-deficit/hyperactivity disorder. *Psychiatry Res.* **175**, 252–255
  8. Hercigonja Novkovic, V., Rudan, V., Pivac, N., Nedic, G., and Muck-Seler, D. (2009) Platelet serotonin concentration in children with attention-deficit/hyperactivity disorder. *Neuropsychobiology* **59**, 17–22
  9. Sharp, S. I., McQuillin, A., and Gurling, H. M. (2009) Genetics of attention-deficit hyperactivity disorder (ADHD). *Neuropharmacology* **57**, 590–600
  10. Franke, B., Neale, B. M., and Faraone, S. V. (2009) Genome-wide association studies in ADHD. *Hum. Genet.* **126**, 13–50
  11. Tartaglia, N., Davis, S., Hench, A., Nimishakavi, S., Beauregard, R., Reynolds, A., Fenton, L., Albrecht, L., Ross, J., Visootsak, J., Hansen, R., and Hagerman, R. (2008) A new look at XYY syndrome: medical and psychological features. *Am. J. Med. Genet. A* **146A**, 1509–1522
  12. Miller, D. T., Shen, Y., Weiss, L. A., Korn, J., Anselm, I., Bridgemohan, C., Cox, G. F., Dickinson, H., Gentile, J., Harris, D. J., Hegde, V., Hundley, R., Khwaja, O., Kothare, S., Luedke, C., Nasir, R., Poduri, A., Prasad, K., Raffalli, P., Reinhard, A., Smith, S. E., Sobeih, M. M., Soul, J. S., Stoler, J., Takeoka, M., Tan, W. H., Thakuria, J., Wolff, R., Yusupov, R., Gusella, J. F., Daly, M. J., and Wu, B. L. (2009) Microdeletion/duplication at 15q13.2q13.3 among individuals with features of autism and other neuropsychiatric disorders. *J. Med. Genet.* **46**, 242–248
  13. Weerapana, E., and Imperiali, B. (2006) Asparagine-linked protein glycosylation: from eukaryotic to prokaryotic systems. *Glycobiology* **16**, 91R–101R
  14. Calo, D., Kaminski, L., and Eichler, J. (2010) Protein glycosylation in Archaea: Sweet and Extreme. *Glycobiology* **20**, 1065–1076
  15. Cummings, R. D. (2009) The repertoire of glycan determinants in the human glycome. *Mol. Biosyst.* **5**, 1087–1104
  16. Lee, R. T., Lauc, G., and Lee, Y. C. (2005) Glycoproteomics: protein modifications for versatile functions. *EMBO Rep.* **6**, 1018–1022
  17. Marek, K. W., Vijay, I. K., and Marth, J. D. (1999) A recessive deletion in the GlcNAc-1-phosphotransferase gene results in peri-implantation embryonic lethality. *Glycobiology* **9**, 1263–1271
  18. Crocker, P. R., Paulson, J. C., and Varki, A. (2007) Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* **7**, 255–266
  19. Marth, J. D., and Grewal, P. K. (2008) Mammalian glycosylation in immunity. *Nat. Rev. Immunol.* **8**, 874–887
  20. Ohtsubo, K., and Marth, J. D. (2006) Glycosylation in cellular mechanisms of health and disease. *Cell* **126**, 855–867
  21. Lauc, G. (2006) Sweet secret of the multicellular life. *Biochim. Biophys. Acta.* **1760**, 525–526
  22. Royle, L., Campbell, M. P., Radcliffe, C. M., White, D. M., Harvey, D. J., Abrahams, J. L., Kim, Y. G., Henry, G. W., Shadick, N. A., Weinblatt, M. E., Lee, D. M., Rudd, P. M., and Dwek, R. A. (2008) HPLC-based analysis of serum N-glycans on a 96-well plate platform with dedicated database software. *Anal. Biochem.* **376**, 1–12
  23. Huhn, C., Selman, M. H., Ruhaak, L. R., Deelder, A. M., and Wührer, M. (2009) IgG glycosylation analysis. *Proteomics* **9**, 882–913
  24. Selman, M. H., McDonnell, L. A., Palmblad, M., Ruhaak, L. R., Deelder, A. M., and Wührer, M. (2010) Immunoglobulin G glycopeptide profiling by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry. *Anal. Chem.* **82**, 1073–1081
  25. Skropeta, D. (2009) The effect of individual N-glycans on enzyme activity. *Bioorg. Med. Chem.* **17**, 2645–2653
  26. Dennis, J. W., Lau, K. S., Demetriou, M., and Nabi, I. R. (2009) Adaptive Regulation at the Cell Surface by N-Glycosylation. *Traffic* **10**, 1569–1578
  27. Barisic, K., Lauc, G., Domic, J., Pavlovic, M., and Fogel, M. (1996) Changes of glycoprotein patterns in sera of humans under stress. *Eur. J. Clin. Chem. Clin. Biochem.* **34**, 97–101
  28. Bauer, D., Haroutunian, V., Meador-Woodruff, J. H., and McCullumsmith, R. E. (2010) Abnormal glycosylation of EAAT1 and EAAT2 in prefrontal cortex of elderly patients with schizophrenia. *Schizophr. Res.* **117**, 92–98
  29. Baysal, B. E., Willett-Brozick, J. E., Badner, J. A., Corona, W., Ferrell, R. E., Nimgaonkar, V. L., and Detera-Wadleigh, S. D. (2002) A mannosyltransferase gene at 11q23 is disrupted by a translocation breakpoint that co-segregates with bipolar affective disorder in a small family. *Neurogenetics* **4**, 43–53
  30. Maguire, T. M., Thakore, J., Dinan, T. G., Hopwood, S., and Breen, K. C. (1997) Plasma sialyltransferase levels in psychiatric disorders as a possible indicator of HPA axis function. *Biol. Psychiatry*. **41**, 1131–1136
  31. Sáez-Valero, J., Sberna, G., McLean, C. A., Masters, C. L., and Small, D. H. (1997) Glycosylation of acetylcholinesterase as diagnostic marker for Alzheimer's disease. *Lancet* **350**, 929
  32. Conners, C. K. (1998) Rating scales in attention-deficit/hyperactivity disorder: use in assessment and treatment monitoring. *J. Clin. Psychiatry.* **59 Suppl 7**, 24–30
  33. Knezević, A., Polasek, O., Gornik, O., Rudan, I., Campbell, H., Hayward, C., Wright, A., Kolcic, I., O'Donoghue, N., Bones, J., Rudd, P. M., and Lauc, G. (2009) Variability, Heritability and Environmental Determinants of Human Plasma N-Glycome. *J. Proteome Res.* **8**, 694–701
  34. R Development Core Team (2009) R: A Language and Environment for Statistical Computing. Vienna, Austria. Retrieved from <http://www.R-project.org>.
  35. Almasy, L., and Blangero, J. (1998) Multipoint quantitative-trait linkage analysis in general pedigrees. *Am. J. Hum. Genet.* **62**, 1198–1211
  36. Knezevic, A., Gornik, O., Polasek, O., Pucic, M., Redzic, I., Novokmet, M., Rudd, P. M., Wright, A. F., Campbell, H., Rudan, I., and Lauc, G. (2010) Effects of aging, body mass index, plasma lipid profiles, and smoking on human plasma N-glycans. *Glycobiology* **20**, 959–969
  37. Partridge, E. A., Le Roy, C., Di Guglielmo, G. M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I. R., Wrana, J. L., and Dennis, J. W. (2004) Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis. *Science* **306**, 120–124
  38. Gornik, O., Wagner, J., Pucić, M., Knezević, A., Redzic, I., and Lauc, G. (2009) Stability of N-glycan profiles in human plasma. *Glycobiology* **19**, 1547–1553
  39. Pucic, M., Pinto, S., Novokmet, M., Knezevic, A., Gornik, O., Polasek, O., Vlahovick, K., Wang, W., Rudd, P. M., Wright, A. F., Campbell, H., Rudan, I., and Lauc, G. (2010) Common aberrations from normal human N-glycan plasma profile. *Glycobiology* **20**, 970–975