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Changes in plasma and IgG *N*-glycome during childhood and adolescence

Maja Pučić[2], Ana Mužinić[2], Mislav Novokmet[2], Marijana Škledar[3], Nela Pivac[4], Gordan Lauc[2,5], and Olga Gornik[1,5]

[2]Genos Ltd, Glycobiology Laboratory, 10000 Zagreb, Croatia; [3]Institute of Public Health of Zagreb County, Zaprešić, Croatia; [4]Division of Molecular Medicine, Ruđer Bošković Institute, 10000 Zagreb, Croatia; and [5]Faculty of Pharmacy and Biochemistry, University of Zagreb, Ante Kovačića 1, 10000 Zagreb, Croatia

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Despite the importance of protein glycosylation in all physiological and pathological processes and their potential as diagnostic markers and drug targets, the glycome of children is still unexplored. We analyzed *N*-linked plasma and IgG glycomes in 170 children and adolescents between 6 and 18 years of age. The results showed large biological variability at the population level as well as a large number of associations between different glycans and age. The plasma *N*-glycome of younger children was found to contain a larger proportion of large complex glycan structures ($r = -0.71$ for tetrasialylated glycans; $r = -0.41$ for trisialylated glycans) as well as an increase in disialylated biantennary structures ($r = 0.55$) with age. Core fucosylation and the level of agalactosylated plasma and IgG glycans decreased while digalactosylated glycans increased with age. This pattern of age-dependent changes in children differs from changes reported in adult population in both, direction and the intensity of changes. Also, sex differences are much smaller in children than in adults and are present mainly during puberty. These important observations should be accounted for when glycan-based diagnostic tests or therapeutics are being developed or evaluated.

Keywords: ageing / children plasma and IgG glycome / glycan analysis / *N*-glycans / protein glycosylation

Introduction

Contrary to proteins which are defined by a single gene, glycans are a result of an interplay between hundreds of genes and their products and are therefore inherently sensitive to all changes in the physiology of the cell. Many pathological conditions are associated with changes in glycan structures and these changes are promising candidates for novel diagnostic and prognostic tools (Freeze 2006; Lebrilla and An 2009; Jaeken 2010; Reis et al. 2010). Understanding of the role of glycan in various pathological conditions is also a good foundation for the development of novel therapeutics. The knowledge about variability of glycosylation in healthy individuals and the influence of some normal physiological and environmental factors are an essential prerequisite for any further study. However, this knowledge is still limited and first comprehensive population studies of human plasma and IgG *N*-glycomes were performed only recently (Knežević et al. 2009; Ruhaak et al. 2010; Pučić et al. 2011). Large variability in glycome composition between individuals was observed in human populations, but within a single healthy individual, the composition of plasma glycome was found to be very stable (Gornik et al. 2009) and environmental factors had only a limited impact on the majority of glycans (Knežević et al. 2010). Specific altered individual glyco-phenotypes that can be associated with specific pathologies were also identified (Pučić et al. 2010).

Previous glycan analyses were performed exclusively on adult population and glycosylation in children, especially healthy children, is still nearly unexplored. In this work, we analyzed *N*-glycosylation profiles of plasma and IgG samples taken from children and adolescents of school age in order to determine the levels of specific glycans in healthy children and to establish sex- and age-dependent changes.

Results and discussion

Plasma *N*-glycans were quantified in plasma of 170 children (84 boys, 86 girls, median age 11 years) by hydrophilic interaction high-performance liquid chromatography (HPLC) and weak anion exchange (WAX) chromatography. Additionally, IgG was isolated and IgG *N*-glycans quantified in 164 children (68 boys, 96 girls, median age 13) by hydrophilic interaction ultra performance liquid chromatography. Main glycome features were deduced from ratios of different chromatographic peaks as described previously (Knežević et al. 2010; Pučić et al. 2011). Descriptives and sex differences of *N*-linked glycan structure characteristics of plasma proteins and IgG are shown in Table I (for full descriptive statistics, see Supplementary data, Table SI).

[1]To whom correspondence should be addressed: Tel: +385 1 639 4467; Fax: +385 1 639 4400; e-mail: ogornik@pharma.hr

Table I. Descriptive statistics and sex difference of the plasma and IgG *N*-glycomes in healthy children and adolescents (6–18 years of age)

	6–10 years			11–14 years			15–18 years		
	Girls [*N* = 45; median (IQR)]	Boys [*N* = 40; median (IQR)]	Sex difference (*P*-value)	Girls [*N* = 18; median (IQR)]	Boys [*N* = 24; median (IQR)]	Sex difference (*P*-value)	Girls [*N* = 23; median (IQR)]	Boys [*N* = 20; median (IQR)]	Sex difference (*P*-value)
Plasma glycan feature									
Total plasma glycans (neutral + charged)									
Sialylation									
Monosialylated	21.81 (2.91)	21.52 (3.76)	0.958	22.23 (3.89)	20.73 (3.15)	0.084	22.32 (3.89)	21.34 (2.82)	0.465
Disialylated	58.73 (3.42)	58.64 (2.44)	0.996	59.35 (1.87)	62.12 (2.5)	0.003	60.1 (2.98)	62.41 (1.97)	**<0.001**
Trisialylated	16.43 (2.53)	16.115 (2.34)	0.631	15.24 (3.35)	14.84 (2.46)	0.809	15.48 (3.92)	13.54 (2.75)	0.007
Tetrasialylated	3.41 (1.38)	3.54 (0.99)	0.311	2.63 (0.83)	2.43 (0.60)	0.500	2.33 (0.65)	2.23 (0.58)	0.488
Fucosylation									
Core fucose	22.77 (5.18)	24.81 (5.38)	0.100	21.24 (5.0)	20.67 (4.45)	0.170	22.67 (5.68)	21.58 (3.78)	0.032
Antennary fucose	2.57 (0.76)	2.52 (0.69)	0.579	3.18 (1.06)	2.64 (1.08)	0.147	2.87 (1.08)	2.81 (0.96)	0.422
Branching									
Biantennary	78.73 (2.75)	78.57 (2.55)	0.721	79.87 (3.49)	80.49 (2.68)	0.347	79.85 (3.98)	82.14 (1.76)	0.002
Triantennary	16.35 (1.4)	16.43 (1.93)	0.570	16.36 (2.96)	15.64 (2.00)	0.354	16.1 (2.78)	14.43 (1.99)	**0.001**
Tetraantennary	4.59 (1.48)	4.9 (1.01)	0.283	4.12 (1.01)	3.72 (0.84)	0.263	3.97 (0.82)	3.48 (0.85)	0.053
Sialylation of biantennary glycans									
Monosialylated	29.33 (2.56)	29.44 (2.77)	0.867	28.67 (3.21)	27.91 (3.15)	0.121	28.2 (2.37)	27.86 (2.63)	0.827
Disialylated	69.56 (3.67)	69.50 (3.85)	0.653	70.92 (5.02)	72.36 (3.49)	0.109	69.96 (4.29)	72.20 (3.95)	0.119
Galactosylation									
Agalactosylated (G0)	3.59 (1.27)	3.83 (1.23)	0.337	2.50 (1.11)	3.69 (1.37)	**0.001**	2.89 (1.08)	3.34 (1.69)	0.088
Monogalactosylated (G1)	8.87 (1.61)	8.88 (1.51)	0.535	8.47 (2.25)	9.02 (1.33)	0.360	9.05 (1.99)	9.32 (2.73)	0.527
Digalactosylated (G2)	66.01 (3.41)	65.74 (3.87)	0.546	68.12 (3.12)	67.79 (2.99)	0.559	68.28 (3.58)	69.27 (2.46)	0.111
Trigalactosylated (G3)	14.47 (2.55)	14.79 (2.30)	0.895	13.36 (3.36)	13.01 (2.25)	0.731	13.8 (2.91)	11.63 (2.17)	**0.001**
Tetragalactosylated (G4)	2.46 (0.87)	2.86 (0.59)	0.140	2.12 (0.79)	2.05 (0.23)	0.576	1.87 (0.58)	1.72 (0.55)	0.197
	Girls [*N* = 28; median (IQR)]	Boys [*N* = 14; median (IQR)]		Girls [*N* = 34; median (IQR)]	Boys [*N* = 28; median (IQR)]		Girls [*N* = 34; median (IQR)]	Boys [*N* = 26; median (IQR)]	
IgG glycan feature									
Total IgG glycans (neutral + charged)									
Sialylation									
FGS/(FG + FGS)	23.67 (25.56)	25.58 (13.69)	0.762	24.52 (20.81)	25.06 (24.61)	0.240	27.46 (25.14)	26.04 (16.63)	0.421
FBGS/(FBG + FBGS)	36.33 (36.15)	28.98 (20.0)	0.208	34.46 (36.66)	39.01 (36.21)	0.141	41.51 (39.07)	40.22 (31.52)	0.811
FGS/(F + FG + FGS)	15.79 (22.95)	18.02 (10.67)	0.607	16.98 (20.93)	18.14 (22.06)	0.343	22.68 (25.11)	19.07 (17.89)	0.239
FBGS/(FB + FBG + FBGS)	25.60 (31.62)	19.67 (17.36)	0.284	24.53 (31.13)	27.35 (32.93)	0.235	31.27 (35.14)	29.89 (27.38)	0.623
FG1S1/(FG1 + FG1S1)	8.78 (8.08)	8.83 (3.82)	0.989	8.44 (8.47)	8.97 (8.32)	0.641	9.2 (9.20)	9.49 (4.58)	0.502
FG2S1/(FG2 + FG2S1 + FG2S2)	36.95 (21.35)	38.54 (10.62)	0.553	35.7 (19.68)	37.2 (15.59)	0.315	37.0 (20.55)	38.13 (11.18)	0.395
FG2S2/(FG2 + FG2S1 + FG2S2)	8.83 (10.07)	7.17 (5.39)	0.308	6.89 (12.20)	10.08 (13.72)	0.014	8.36 (9.6)	9.67 (11.19)	0.071
FBG2S1/(FBG2 + FBG2S1 + FBG2S2)	36.12 (13.18)	33.11 (3.18)	0.004	34.38 (15.46)	34.49 (11.29)	0.955	34.27 (15.11)	35.84 (7.5)	0.007
FBG2S2/(FBG2 + FBG2S1 + FBG2S2)	35.77 (28.57)	30.5 (16.32)	0.484	33.74 (27.41)	37.89 (26.84)	0.045	37.46 (32.81)	38.70 (25.5)	0.363
$F^{total}S1/F^{total}S2$	3.4 (4.45)	4.42 (2.22)	0.085	4.11 (5.33)	3.31 (4.03)	0.087	3.32 (4.62)	3.32 (4.22)	0.387
FS1/FS2	5.32 (8.01)	6.85 (3.25)	0.452	7.18 (11.34)	5.11 (6.88)	0.010	5.51 (7.84)	5.07 (6.58)	0.200
FBS1/FBS2	0.97 (1.02)	1.02 (0.55)	0.927	1.22 (1.36)	1.0 (0.75)	0.049	0.96 (1.62)	0.96 (1.01)	0.754
Bisecting *N*-GlcNAc									
FBS^{total}/FS^{total}	0.27 (0.26)	0.18 (0.07)	**<0.001**	0.27 (0.27)	0.23 (0.19)	0.054	0.25 (0.31)	0.25 (0.2)	0.403
FBS1/FS1	0.16 (0.16)	0.1 (0.03)	**<0.001**	0.17 (0.17)	0.13 (0.13)	0.007	0.14 (0.18)	0.15 (0.12)	0.493
FBS1/(FS1 + FBS1)	0.14 (0.12)	0.09 (0.02)	**<0.001**	0.14 (0.12)	0.11 (0.1)	0.007	0.13 (0.13)	0.13 (0.08)	0.493
FBS2/FS2	0.93 (0.91)	0.65 (0.24)	0.005	0.94 (0.99)	0.73 (0.82)	**<0.001**	0.88 (0.94)	0.84 (0.49)	0.31
FBS2/(FS2 + FBS2)	0.48 (0.27)	0.39 (0.09)	0.005	0.49 (0.26)	0.42 (0.26)	**<0.001**	0.47 (0.24)	0.46 (0.14)	0.31

Neutral IgG glycans									
Galactosylation									
$G0^n$	38.53 (29.0)	37.68 (7.01)	0.571	35.14 (31.56)	35.77 (24.05)	0.601	29.04 (36.5)	32.96 (22.49)	0.021
$G1^n$	43.68 (15.93)	43.51 (3.09)	0.802	44.41 (12.19)	43.98 (11.99)	0.591	45.45 (12.28)	45.17 (8.39)	0.917
$G2^n$	17.83 (16.56)	19.44 (4.70)	0.284	19.94 (22.01)	20.13 (17.38)	0.621	24.92 (28.58)	20.68 (16.03)	0.008
Core fucosylation and bisecting GlcNAc									
$F^{n\ total}$	97.61 (3.60)	97.13 (1.37)	0.147	96.75 (4.14)	97.01 (4.3)	0.396	96.65 (5.07)	96.55 (3.68)	0.561
$FG0^{n\ total}/G0^n$	98.44 (3.40)	98.13 (1.16)	0.26	98.15 (5.02)	98.20 (5.56)	0.989	98.27 (3.75)	97.95 (3.57)	0.216
$FG1^{n\ total}/G1^n$	98.77 (1.68)	98.54 (0.67)	0.101	98.43 (3.03)	98.52 (2.97)	0.799	98.53 (3.51)	98.52 (1.96)	0.296
$FG2^{n\ total}/G2^n$	94.18 (8.02)	93.07 (4.06)	0.390	92.63 (13.16)	92.83 (6.51)	0.854	92.95 (11.83)	93.09 (6.33)	0.531
F^n	85.57 (12.51)	85.58 (5.03)	0.947	81.95 (10.29)	85.33 (10.33)	**<0.001**	83.79 (9.35)	83.59 (6.04)	0.665
$FG0^n/G0^n$	85.74 (17.26)	84.19 (7.55)	0.722	81.7 (12.78)	85.02 (13.58)	0.007	82.58 (15.57)	82.17 (8.80)	0.788
$FG1^n/G1^n$	85.51 (11.98)	86.15 (5.39)	0.802	83.1 (8.64)	86.57 (10.87)	**<0.001**	84.16 (9.53)	84.55 (5.48)	0.114
$FG2^n/G2^n$	84.91 (12.73)	84.91 (5.51)	0.885	82.99 (15.86)	84.03 (9.72)	0.095	83.85 (16.98)	84.52 (8.96)	0.823
FB^n	11.98 (10.86)	12.60 (3.84)	0.664	14.66 (7.3)	11.64 (8.93)	**<0.001**	13.39 (7.31)	13.1 (4.8)	0.387
$FBG0^n/G0^n$	12.89 (14.46)	14.32 (7.03)	0.762	16.18 (11.73)	13.13 (10.58)	0.003	15.72 (13.82)	15.11 (6.78)	0.355
$FBG1^n/G1^n$	13.25 (11.19)	13.0 (4.73)	0.518	15.32 (8.82)	12.06 (10.35)	**<0.001**	14.35 (7.98)	13.55 (6.15)	0.064
$FBG2^n/G2^n$	9.42 (7.31)	7.9 (1.52)	0.04	9.48 (7.84)	7.97 (5.86)	**0.001**	9.08 (6.89)	8.34 (5.56)	0.474
FB^n/F^n	0.14 (0.15)	0.15 (0.05)	0.782	0.18 (0.11)	0.14 (0.12)	**<0.001**	0.16 (0.1)	0.16 (0.06)	0.379
$FB^n/F^{n\ total}$	12.30 (11.24)	12.79 (4.08)	0.782	15.06 (7.92)	12.03 (9.32)	**<0.001**	13.7 (7.78)	13.52 (4.87)	0.379

Significant differences are highlighted in bold. For description of glycan features, see Supplementary data, Table SIII. IQR, interquartile range.

Significant sex associated differences in plasma glycan levels have been shown to exist for many glycan groups in the adult population (Knežević et al. 2010). However, in this study, we did not observe any significant differences in plasma glycans between boys and girls before puberty (Table I). Only with the onset of puberty boys and girls started to differ in the level of agalactosylated (G0) plasma glycans (Figure 1A). When the glycosylation of IgG alone was evaluated, sex differences were revealed even before puberty with girls showing higher levels of fucosylated sialylated structures with bisecting GlcNAc (Table I). At the onset of puberty boys and girls differed in several glycan features expressing mainly levels of fucosylated neutral IgG glycans with and without bisecting GlcNAc (Figure 1B–D). Compared with boys, girls showed higher levels of IgG glycans with bisecting GlcNAc which has been reported before in a large scale study of IgG glycosylation in adults where levels of glycans with bisecting GlcNAc were found to be more prevalent in females than in males (Ruhaak et al. 2010).

Changes in the glycosylation of plasma proteins with hormonal status were reported to be associated with pregnancy (van de Geijn et al. 2009), oral hormonal therapies (Brinkman-Van der Linden et al. 1996; Saldova et al. 2012) and menopausal age (Knežević et al. 2010); thus, the observed differences in children at the onset of puberty are not unexpected.

Since the majority of plasma glycans did not differ between boys and girls, further analyses of plasma glycosylation were performed on the entire study population. In contrast, since several IgG glycan features showed sex difference, all the analyses of IgG glycosylation were performed separately for boys and girls. A number of significant correlations between age and plasma and IgG glycans were observed (Table II).

The observed correlations in plasma glycome indicated that many glycan features change with child's growth and development, including the decrease in glycan branching, galactosylation (tri- and tetragalactosylated glycans) and sialylation (tri- and tetrasialylated glycans) with age (Table II). The highest correlation coefficient was observed for tetrasialylated glycans ($r = -0.71$); showing a significant decrease in the complexity of glycan structures in children with age which was accompanied by an increase in disialylated biantennary glycans ($r = 0.27$). Scatter diagrams together with regression lines and coefficients of correlations of glycan complexity with age are shown in Figure 2.

Core fucosylation ($r = -0.35$) and the level of agalactosylated plasma glycans ($r = -0.36$) significantly decreased while the level of digalactosylated glycans ($r = 0.59$) increased with age. Since core fucosylated biantennary glycans come predominantly from IgG (Arnold et al. 2008; Royle et al. 2008), it is not surprising that the core fucosylation of IgG also significantly decreased ($r = -0.34$) and galactosylation (digalactosylated: $r = 0.49$) increased with age as observed in girls (Table II). Age-dependence of IgG glycans in boys revealed a significant increase in the incidence of bisecting GlcNAc in core fucosylated sialylated structures with age ($r \geq 0.44$, Table II).

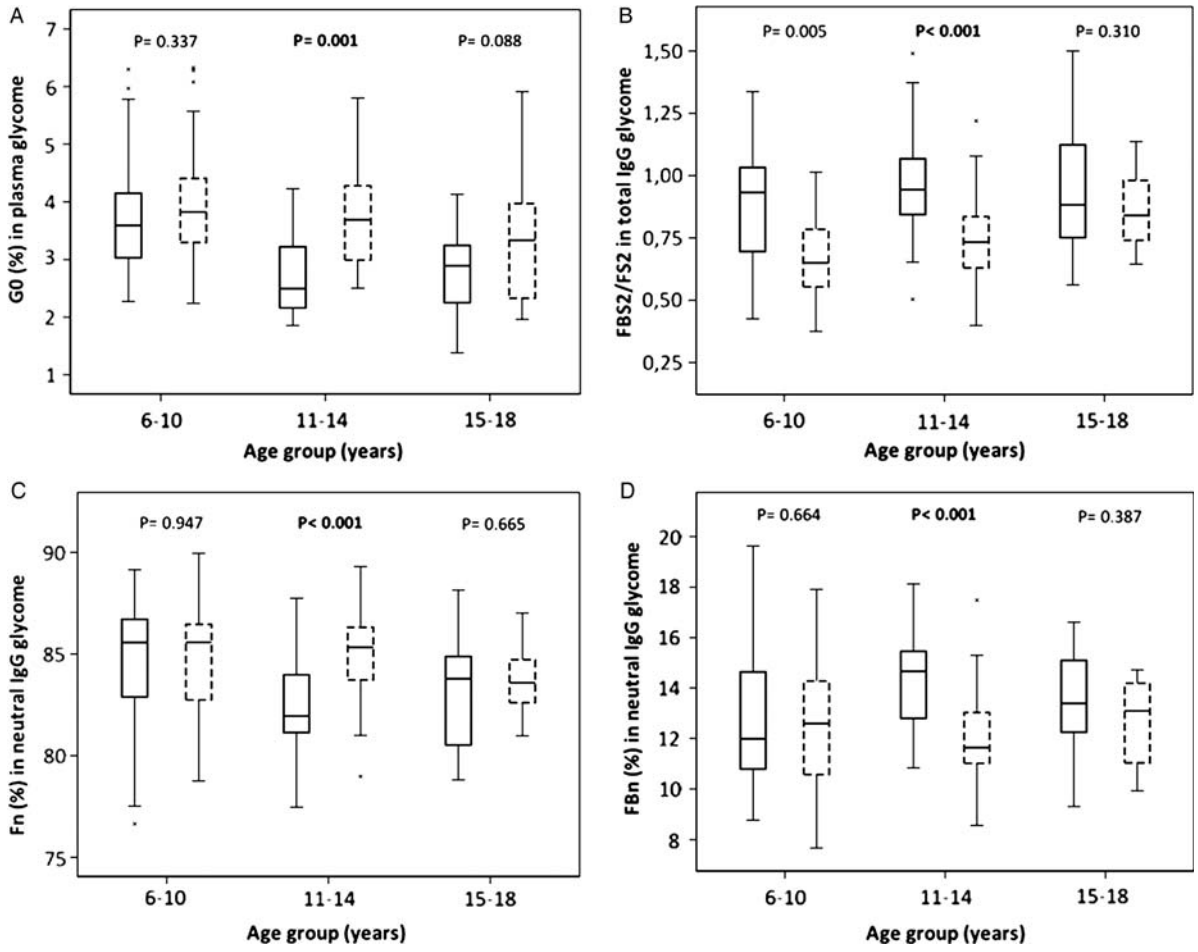

Fig. 1. Levels of plasma and IgG glycan features in three age groups (6–10, 11–14 and 15–18 years) in girls (continuous line) and boys (dashed line). G0 (%), the percentage of agalactosylated glycans in plasma glycome; FBS2/FS2, the ratio of fucosylated disialylated glycans with and without bisecting GlcNAc in the total IgG glycome; F^n (%), the percentage of fucosylated glycans without bisecting GlcNAc in the neutral IgG glycome; FB^n (%), the percentage of fucosylated glycans with bisecting GlcNAc in the neutral IgG glycome. Results are presented as box and whisker plots (median, middle lines; 25–75th percentiles, rectangles; range, lines; outliers, markers).

When the results of the plasma and IgG glycan changes during growing up in children were compared with those in adults, they differed significantly. The behavior of almost all glycan features changed trend or the rate of change in adulthood. An example of the opposite trends in plasma and IgG glycans between children and adults is presented in Figure 3. The increase in agalactosylated glycans with age is the most frequently reported change in glycans with age in adults (Yamada et al. 1997; Vanhooren et al. 2007; Klein 2008; Pučić et al. 2011). Our results revealed that agalactosylated glycans actually decrease with age in children and that the minimal level of agalactosylated glycans is reached in early adulthood (Figure 3). For many other glycan features that showed a significant correlation with age in children, in adulthood levels of glycans either stagnate or change with much lesser rate with age.

Children were then divided into three age groups of 6–10, 11–14 and 15–18 years. Results presented in Supplementary data, Table SII, show that plasma glycans in children significantly changed with the onset of puberty (differences between groups of 6–10 and 11–14 years). As for IgG glycans, significant difference related to the onset of puberty was only observed in boys for the levels of fucosylated sialylated structures with bisecting GlcNAc (Supplementary data, Table SII). In girls, the only significant difference was between the youngest (6–10 years) and oldest group (15–18 years) in the levels of galactosylation and core fucosylation (Supplementary data, Table SII).

Our study revealed that the compositions of the plasma and IgG *N*-glycome in childhood significantly differ from the plasma and IgG *N*-glycome in adulthood and, even more, changed with a great rate during child's growth. This is a very important observation, knowing the fact that changes in glycan structures are often studied as diagnostic biomarkers. Due to the involvement of glycans in many pathophysiological processes, carbohydrate-based therapeutics (such as Tamiflu, Relenza etc.) are being developed, aimed to interfere with, or modify, glycan–receptor bindings, which are parts of pathological cascades. However, the responsible use of these drugs in children requires a careful evaluation of different

Table II. Coefficients of correlation (*r*) between plasma and IgG glycans and age in healthy children and adolescents (6–18 years of age)

Plasma glycan feature	Both sex [*r* (*P*)]	IgG glycan feature	Girls [*r* (*P*)]	Boys [*r* (*P*)]	IgG glycan feature	Girls [*r* (*P*)]	Boys [*r* (*P*)]
Sialylation		*Sialylation*			*Galactosylation*		
Monosialylated	0.05 (0.514)	FGS/(FG + FGS)	0.17 (0.093)	0.01 (0.928)	$G0^n$	**−0.48** (<0.001)	−0.19 (0.130)
Disialylated	**0.55** (<0.001)	FBGS/(FBG + FBGS)	0.08 (0.445)	0.21 (0.081)	$G1^n$	0.28 (0.006)	0.28 (0.023)
Trisialylated	**−0.41** (<0.001)	FGS/(F + FG + FGS)	0.29 (0.005)	0.06 (0.649)	$G2^n$	**0.49** (<0.001)	0.11 (0.374)
Tetrasialylated	**−0.71** (<0.001)	FBGS/(FB + FBG + FBGS)	0.17 (0.099)	0.23 (0.064)	*Core fucosylation and bisecting GlcNAc*		
Fucosylation		FG1S1/(FG1 + FG1S1)	0.11 (0.281)	0.10 (0.400)	$F^{n\ total}$	**−0.34** (0.001)	−0.25 (0.039)
Core fucose	**−0.35** (<0.001)	FG2S1/(FG2 + FG2S1 + FG2S2)	0.02 (0.856)	−0.03 (0.797)	$FG0^{n\ total}/G0^n$	−0.14 (0.168)	−0.21 (0.082)
Antennary fucose	0.19 (0.014)	FG2S2/(FG2 + FG2S1 + FG2S2)	−0.08 (0.413)	0.13 (0.293)	$FG1^{n\ total}/G1^n$	−0.17 (0.097)	−0.18 (0.146)
Branching		FBG2S1/(FBG2 + FBG2S1 + FBG2S2)	−0.22 (0.029)	**0.42** (<0.001)	$FG2^{n\ total}/G2^n$	−0.07 (0.469)	−0.09 (0.452)
Biantennary	**0.48** (<0.001)	BG2S2/(FBG2 + FBG2S1 + FBG2S2)	0.00 (0.985)	0.10 (0.425)	F^n	−0.17 (0.102)	−0.31 (0.011)
Triantennary	**−0.32** (<0.001)	$F^{total}S1/F^{total}S2$	0.00 (0.986)	−0.22 (0.074)	$FG0^n/G0^n$	−0.26 (0.012)	−0.26 (0.034)
Tetraantennary	**−0.63** (<0.001)	FS1/FS2	0.03 (0.796)	−0.13 (0.307)	$FG1^n/G1^n$	−0.13 (0.208)	−0.21 (0.087)
Sialylation of biantennary glycans		FBS1/FBS2	−0.09 (0.402)	0.03 (0.805)	$FG2^n/G2^n$	−0.03 (0.805)	−0.17 (0.177)
Monosialylated	**−0.26** (0.001)	*Bisecting N-GlcNAc*			FB^n	0.08 (0.433)	0.23 (0.063)
Disialylated	**0.27** (<0.001)	FBS^{total}/FS^{total}	−0.04 (0.673)	**0.55** (<0.001)	$FBG0^n/G0^n$	0.25 (0.012)	0.21 (0.088)
Galactosylation		FBS1/FS1	−0.09 (0.381)	**0.59** (<0.001)	$FBG1^n/G1^n$	0.10 (0.339)	0.19 (0.120)
Agalactosylated (G0)	**−0.36** (<0.001)	FBS1/(FS1 + FBS1)	−0.09 (0.381)	**0.59** (<0.001)	$FBG2^n/G2^n$	−0.09 (0.397)	0.26 (0.032)
Monogalactosylated (G1)	0.00 (0.952)	FBS2/FS2	−0.02 (0.82)	**0.44** (<0.001)	FB^n/F^n	0.09 (0.366)	0.23 (0.055)
Digalactosylated (G2)	**0.59** (<0.001)	FBS2/(FS2 + FBS2)	−0.02 (0.82)	**0.44** (<0.001)	$FB^n/F^{n\ total}$	0.09 (0.366)	0.23 (0.055)
Trigalactosylated (G3)	**−0.47** (<0.001)						
Tetragalactosylated (G4)	**−0.68** (<0.001)						

Significant differences are highlighted in bold. For description of glycan features, see Supplementary data, Table SIII.

glycosylation profiles between children and adults, as well as the knowledge of the significant changes occurring in glycan structures with age in young population.

Materials and methods

Study population

Blood samples were collected from healthy children of preschool and school age during their regular medical examination at primary care units in Croatia. Blood samples were collected on anticoagulant; plasmas were immediately separated by centrifugation and stored at −20°C. The study was approved by the Medical School Osijek Ethical Committee and performed in conformance to the ethical guidelines of the Declaration of Helsinki. Plasma *N*-glycosylation was analyzed in 170 children (84 boys, 86 girls, median age 11 years, range 6–18 years) and IgG *N*-glycosylation was analyzed in 164 children (68 boys, 96 girls, median age 13, range 6–18 years).

IgG purification

Immunoglobulin G was isolated from plasma by affinity chromatography using 96-well protein G monolithic plates as described previously (Pučić et al. 2011). Briefly, 50 µL of plasma was diluted 10× with PBS, applied to the protein G plate and instantly washed. IgGs were eluted with 1 mL of 0.1 M formic acid and immediately neutralized with 1 M ammonium bicarbonate.

Glycan analysis

Glycan release and labeling was performed essentially as reported by Royle et al. (2008). Proteins were immobilized in a block of sodium dodecyl sulfate–polyacrylamide gel and *N*-glycans were released by digestion with recombinant *N*-glycosidase F. This was done in a 96-well microtiter plate to achieve the best throughput of sample preparation. After extraction, glycans were fluorescently labeled with 2-aminobenzamide.

Hydrophilic interaction chromatography

Released plasma glycans were separated by HPLC on a 250 × 4.6 mm i.d. 5 µm particle packed TSKgel Amide 80 column (Tosoh Bioscience, Stuttgart, Germany) at 30°C with 50 mM formic acid adjusted to pH 4.4 with ammonia solution as solvent A and acetonitrile as solvent B in a 48-min analytical run (Knežević et al. 2009). Released IgG glycans were separated by ultra performance liquid chromatography on a Waters BEH glycan column, 100 × 2.1 mm i.d., 1.7 µm BEH particles, at 60°C with 100 mM ammonium formate, pH 4.4, as solvent A and acetonitrile as solvent B in a 20-min analytical run (Pučić et al. 2011). In both cases, a fluorescence detector was set with excitation and emission wavelengths of 330 and 420 nm, respectively. The systems were calibrated using an external standard of hydrolyzed and 2AB-labeled glucose oligomers from which the retention times for the individual glycans were converted to glucose units (Royle et al. 2008).

Plasma chromatograms obtained were all separated in the same manner to 16 chromatographic peaks and 13 for

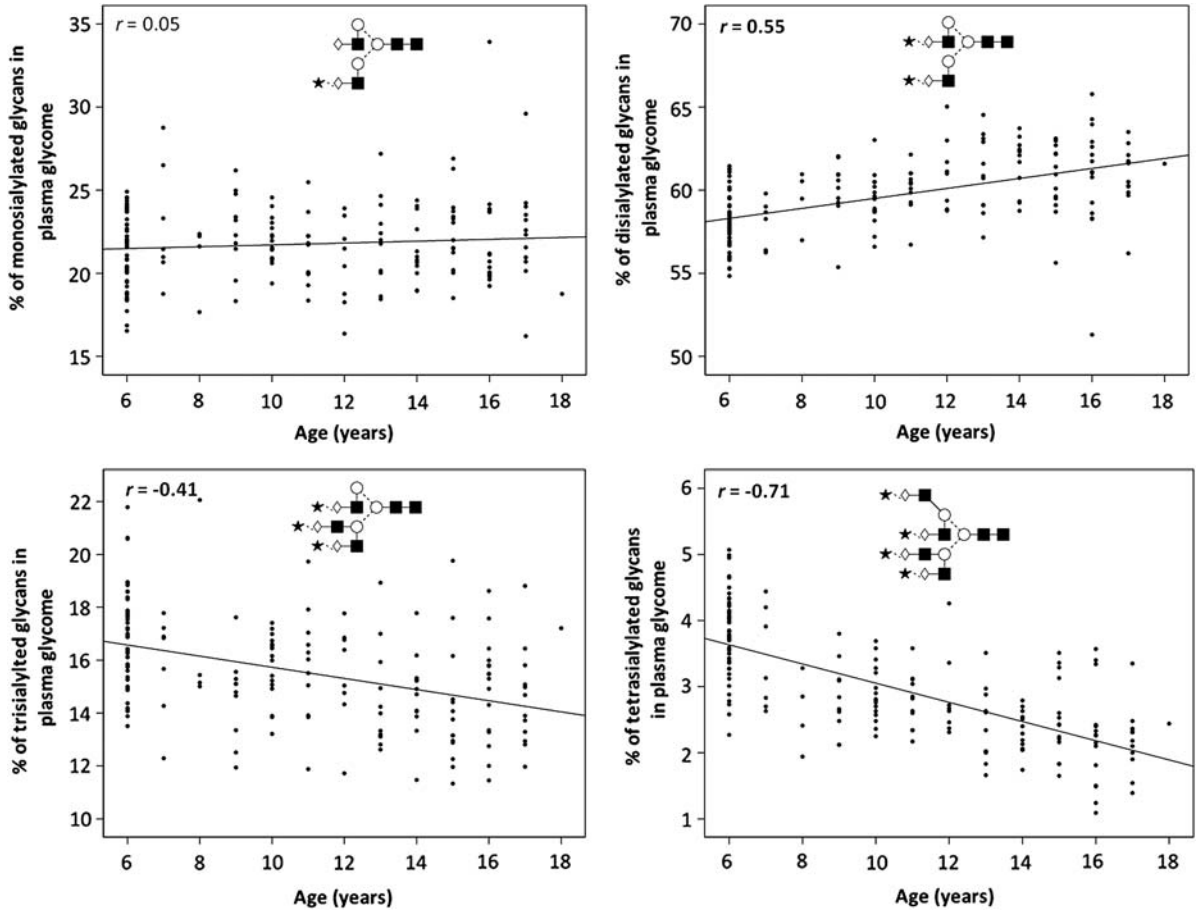

Fig. 2. Age-dependent decrease in branching and sialylation of plasma *N*-linked glycans in children (glycan scheme: square, *N*-acetylglucosamine; circle, mannose; romb, galactose; star, sialic acid). Significant correlation coefficients (*r*) in bold.

desialylated glycans, whereas the IgG chromatograms were separated to 24 peaks. The amounts of glycans present in each peak were expressed as % of the total integrated area.

Weak anion exchange HPLC

Additionally, plasma glycans were separated according to the number of sialic acids by WAX HPLC. The analysis was performed using a Prozyme GlycoSep C 75 mm × 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. Glycans were quantified from WAX profiles according to the level of sialylation (monosialylated, disialylated, trisialylated and tetrasialylated).

Sialidase digestion

Aliquots of the 2AB-labeled plasma glycan pool were dried down in 96-well polymerase chain reaction 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 *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 the AcroPrep™ 96 Filter Plates, 350 µL well, 10 K (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 structural characteristics (Supplementary data, Table SIII). Plasma glycans structural features were derived from either hydrophilic interaction chromatography [HILIC; total plasma glycans (GP) and total plasma glycans after sialidase treatment (DG)] or WAX integrated glycan profiles (Knežević et al. 2010). IgG glycan structural features were derived from HILIC integrated glycan profiles as described previously (Pučić et al. 2011). Individual glycan structures present in each plasma or IgG glycan peak were reported previously and are shown in Supplementary data, Table SIV.

Statistical analysis

The descriptive part of the statistical analysis was aimed at showing the basic characteristics of the population. The

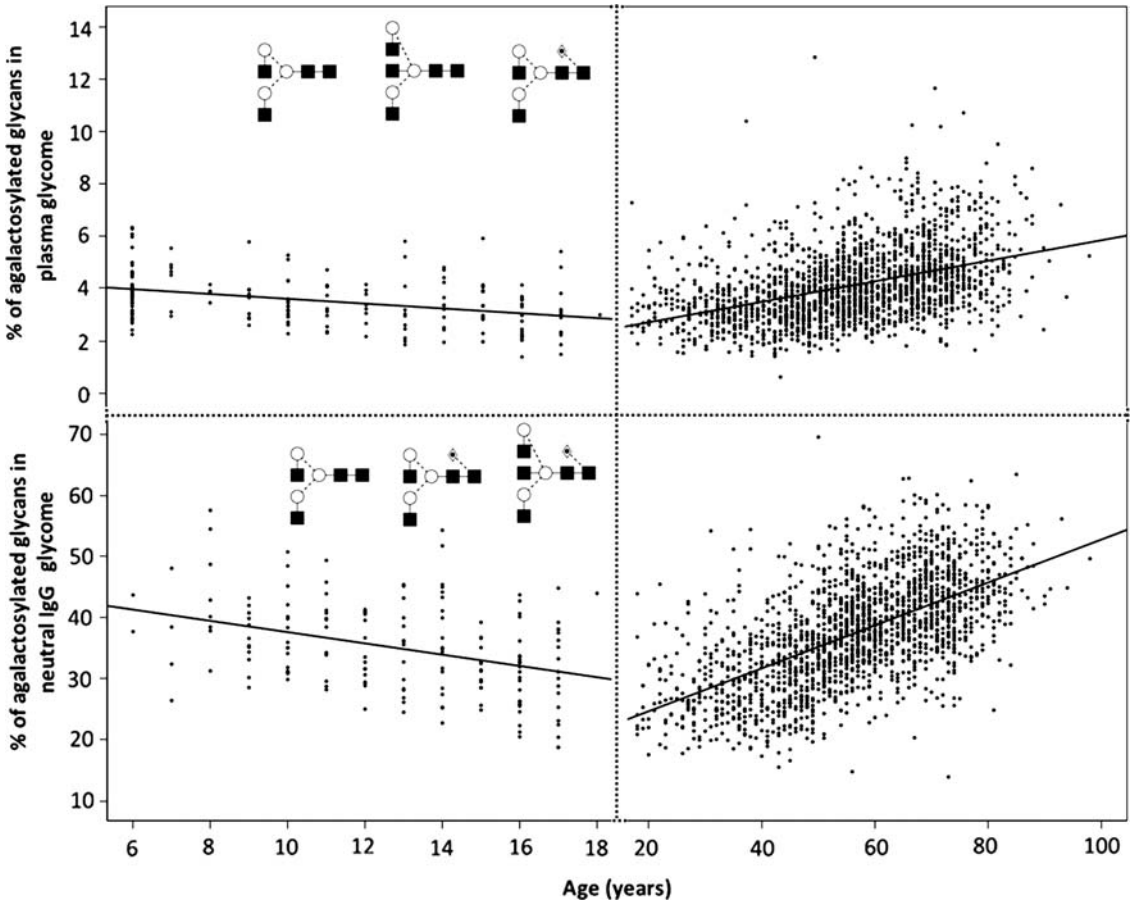

Fig. 3. Levels of agalactosylated glycans in plasma and IgG of children and adults. Changes in agalactosylated plasma and IgG glycans in adults were reported previously (Knežević et al. 2010; Pučić et al. 2011) and are here shown only for a comparison. Glycan scheme: square, *N*-acetylglucosamine; circle, mannose; romb with a dot, fucose.

population was then tested for normality using the Smirnov–Kolmogrov test and non-parametric statistical tests were further used. The Mann–Whitney test was used to analyze differences in levels of glycans between groups, whereas the Spearman's rank correlation was calculated for the association of glycan structures and age. The significance level was set at $P \leq 0.001$ to account for multiple testing. All reported *P*-values are two-tailed if not stated otherwise. Statistical analyses were performed with SPSS 13 (SPSS, Chicago, IL).

Supplementary data

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

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Conflict of interest

None declared.

Abbreviations

G0, agalactosylated glycans; G1, monogalactosylated glycans; G2, digalactosylated glycans; G3, trigalactosylated glycan; G4, tetragalactosylated glycans; GlcNAc, *N*-acetylglucosamine; HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography; WAX, weak anion exchange.

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