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Multiple juvenile idiopathic arthritis subtypes demonstrate pro-inflammatory IgG glycosylation

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Abstract

OBJECTIVES—Rheumatoid arthritis is associated with an excess of hypogalactosylated (G0) IgG that is considered relatively pro-inflammatory. Assessment of this association in juvenile idiopathic arthritis (JIA) is complicated by age-dependent IgG glycan variation. We undertook the first large-scale survey of IgG glycans in normal children and in patients with JIA, with a focus on early childhood, the time of peak JIA incidence.

METHODS—IgG glycans from healthy children and DMARD-naïve JIA patients were characterized using high-performance liquid chromatography (HPLC). Pro-inflammatory G0 glycans were quantitated with reference to monogalactosylated (G1) species. Associations were sought between G0/G1 and disease characteristics.

RESULTS—Among healthy children aged 9 months-16 years (n=165), G0/G1 was highly age-dependent, peaking in children <3 years old at 1.19 and declining to a nadir of 0.83 after age 10 years (Spearman ρ=0.60, $p<0.0001$). In patients with JIA (n=141), G0/G1 was elevated compared with controls (G0/G1 1.32 vs. 1.02, $p<0.0001$). Corrected for age, G0/G1 was abnormally high in all JIA subtypes (enthesitis-related arthritis not assessed), most strikingly in systemic JIA. Glycosylation aberrancy was comparable in patients with or without ANA and in both early- and late-onset disease, and exhibited at most a weak correlation with inflammatory markers.

CONCLUSIONS—IgG glycosylation is skewed toward pro-inflammatory G0 variants in healthy children, in particular during the first few years of life. This deviation is exaggerated in patients with JIA. The role for IgG glycan variation in immune function in children, including the predilection of JIA for early childhood, remains to be defined.

Juvenile idiopathic arthritis (JIA) contains a heterogeneous mixture of arthritis phenotypes of unknown etiology. Human leukocyte antigen (HLA) associations have been observed for

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multiple subtypes, suggesting a role for adaptive immune mistargeting (1, 2). The participation of antibodies in disease pathogenesis is supported by several observations, including the prevalence of autoantibodies (such as ANA and anti-DEK), circulating immune complexes, and complement consumption in blood and joint fluid in some patients (3-6).

One remarkable feature of the epidemiology of JIA is age of onset. The peak of JIA incidence occurs around 2-3 years of age (1, 7, 8). This peak is composed primarily of patients in the oligoarticular subgroup, but is evident also in seronegative polyarticular JIA, psoriatic JIA, and potentially systemic JIA (7, 9-11). By contrast, onset before age 1 year is unusual. The basis for this epidemiological pattern is unknown. One possibility is that it reflects the point of first contact between a genetically susceptible host and a specific environmental trigger. Supporting this concept, younger and older JIA patients exhibit different HLA associations, even within the same ILAR subtype (1, 2).

A complementary possibility is that some feature of early childhood immunity may favor initiation of arthritis. Children and adults differ immunologically in multiple respects (12). For example, the proportion of circulating lymphocytes that are naïve is initially high and falls gradually. Circulating IgG nadirs at approximately 3-4 months of age with the decay of maternal antibodies, rising to 60% of adult levels by 1 year and to adult levels by 10 years of age (13). Humoral immunity is also functionally immature in young children, evident most strikingly in poor responses to polysaccharide antigens in the first 18-24 months of life (14).

Another potentially important difference between pediatric and adult immunity concerns IgG glycosylation. Approximately 3% of the mass of IgG is carbohydrate, representing principally 2 branched glycans that attach to a canonical asparagine (Asn297) in each heavy chain (Figure 1A) (15). These oligosaccharides reside within the Fc region, where they help to maintain its three-dimensional conformation (16). Polymorphisms in IgG glycans modulate its ability to bind Fc receptors and fix complement, and thus are highly determinative of antibody effector function (15, 17). In particular, IgG glycans lacking galactose (G0) bind mannose-binding lectin, thereby facilitating activation of complement (Figure 1B) (18-20). Interestingly, one analysis of IgG glycans in healthy individuals found that children, like older adults, exhibited an excess of pro-inflammatory G0 IgG glycoforms (21), though a smaller survey did not identify this pattern (22).

Hypogalactosylation of IgG may be particularly relevant for arthritis. Studies over the last 30 years have demonstrated that adult rheumatoid arthritis (RA) is associated with an increased prevalence of circulating G0 IgG (23-29). Further, elevation in G0 IgG can predate the diagnosis of RA by more than 3 years, varies directly with disease activity, and is particularly pronounced for antibodies directed against citrullinated peptides (28, 30). Finally, animal studies have suggested that antibodies rendered G0 by enzymatic techniques are especially arthritogenic (31). Together, these studies support a role for aberrant IgG glycosylation in the pathogenesis of RA.

A similar mechanism may be operative in JIA. Several groups have studied IgG glycans in patients with arthritis beginning before the age of 16 years (24-26, 32, 33). Using diverse methodologies, these series have generally (though not invariably (32)) come to the conclusion that IgG hypogalactosylation is a feature of childhood arthritis. Yet the data have important limitations. First, the number of patients studied was relatively small (total n=100), divided among multiple phenotypic classes using diverse nomenclatures. Second, most patients were sampled while receiving treatment, known to alter IgG glycosylation (27, 29). Finally and most critically, patients were not always matched to controls by age. Indeed, only a very few normal samples have been characterized from children below the

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age of 6 years, the peak of JIA onset, rendering IgG glycan data from young JIA patients exceptionally difficult to interpret (21, 22).

In the present study, we set out to characterize IgG hypogalactosylation in JIA, with a particular focus on early childhood. To achieve this goal, we collected samples from healthy children and patients with JIA naïve to disease modifying anti-rheumatic drug (DMARD) treatment, enabling age-normalized analysis of IgG glycan variation by disease subtype, laboratory features and age of onset. Our results establish the association of JIA with aberrant IgG glycosylation, and demonstrate marked elevation in hypogalactosylated IgG in healthy young children, suggesting a possible mechanism favoring onset of JIA in early childhood.

PATIENTS and METHODS

Patients

Healthy pediatric samples were obtained from 4 cohorts: (I) patients without inflammatory disease evaluated in the Rheumatology Program at Children's Hospital Boston (CHB); (II) patients recruited from the primary care clinic at CHB; (III) discard specimens from patients undergoing lead screening at primary care practices referring to the CHB clinical laboratory, in whom measured lead levels were normal (<6 μg/dl); (IV) healthy children recruited for a biorepository of pediatric biosamples at Cincinnati Children's Hospital Medical Center (CCHMC). For some normal patients, age data available for review were limited to integer year of age (e.g. 1y, 2y). For these patients, an age of integer+0.5 years (e.g. 1.5y, 2.5y) was assumed in calculations and plots. JIA samples were obtained from biorepositories at CHB and CCHMC. Entry criteria for JIA patients included: (a) a definitive diagnosis of JIA according to ILAR criteria (34); (b) no DMARD therapy within 6 months of sampling; (c) no concurrent systemic illness. Almost all JIA patients had recent onset disease (mean interval from symptom onset to sample 0.5y). Healthy adult samples were drawn from consented volunteers. These studies were performed in accordance with the requirements of the respective Institutional Review Boards.

Glycan characterization

Glycans were analyzed as described previously (28, 35). Briefly, N-glycans were liberated enzymatically from 5μL of whole serum or plasma, labeled with a fluorescent tracer, and analyzed by normal-phase high-performance liquid chromatography (NP-HPLC), which provides precise relative quantitation of molecular species separated by size and charge. Area under the glycan elution peaks was calculated, and G0 was normalized to the monogalactosylated (G1) fraction which is independent of age (21, 22), including in the pediatric age range (AE, PAN, data not shown). Since the large majority of neutral biantennary glycans in serum are from IgG, the G0/G1 ratio in whole serum or plasma is a proxy for IgG G0/G1 (R^2=0.83) (35).

Inflammatory markers

CRP and ESR studies were performed by the clinical labs of each institution. S100A12 and MRP8/14 were assayed in 69 CCHMC patients by ELISA (36).

Statistical analysis

Population means were compared with Student's t test (two-tailed) using GraphPad Prism Version 4.0. Indicated errors reflect standard deviation. Linear regression to correct for age was performed using PASW Statistics 18 (release 18.0.0).

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RESULTS

Equivalence of sampling conditions

To ensure comparability of G0/G1 data obtained under different sampling conditions, blood was drawn from healthy adult volunteers and processed in duplicate across a range of conditions mirroring the study samples. As expected, given the known stability of IgG glycans, no difference among conditions was apparent (Figure 2). In 2 of 100 tests, one replicate diverged by > 10% while the second was in line with all other results from the same donor, an error rate (2%) concordant with our previous data on the reproducibility of this technique (28). We concluded that all pediatric sources could be employed together in our analysis.

Age-dependence of normal IgG glycosylation in childhood

165 samples were available from children without known inflammatory disease, ranging in age from 9 months to <16 years. G0/G1 ratios demonstrated a clear age-dependent decrease over time, from 1.19 ± 0.22 in children <3 years of age to a nadir at 0.83 ± 0.14 after age 10 (Figure 3, Spearman ρ=0.60, $p<0.0001$). Comparing these with our published glycan data from healthy adult blood donors (28), children under 3y of age exhibited mean G0/G1 ratios equivalent to adults aged 60-70y, while older children (≥10 years) exhibited glycosylation equivalent to adults <40y. These results confirm the U-shaped distribution of G0 with age, extending the observation with high resolution into early childhood (21). No overall difference between genders was evident, although the highest (and lowest) G0/G1 values in young children were all female.

IgG glycosylation in JIA

Samples were obtained from 141 patients with JIA, representing all ILAR subtypes except enthesitis-related arthritis. Overall, IgG glycosylation differed markedly from controls (Figure 4A). JIA G0/G1 was comparable to adult RA (JIA 1.32 ± 0.40 vs. RA 1.36 ± 0.43) (28). Aberrant IgG glycosylation was observed across the age spectrum, and indeed was particularly marked in older children with JIA (Figure 4B).

ILAR criteria and alternate classification methods

We next divided patients according to ILAR criteria (34). Compared to pediatric controls as a whole, elevation of G0/G1 was evident in all subtypes analyzed with the exception of extended oligoarthritis, for which few samples were available (Figure 5A). Given divergent age distributions among cases and controls, we employed linear regression to adjust for age, confirming that elevated G0/G1 is a feature of JIA overall and of all JIA subtypes tested (Table 1).

However, classification of JIA remains a work in progress. While rheumatoid factor (RF) positive polyarticular JIA and systemic JIA are readily distinguished, substantial uncertainty surrounds the categories of oligoarthritis (persistent and extended), RF negative polyarthritis, and psoriatic JIA (37). We therefore grouped these patients together and assessed whether age of onset (<6y vs. ≥6y) or ANA status were informative with regard to IgG glycan status in JIA. We found similar elevation of G0/G1 in all groups (Figure 5B and C). Of note, when analysis was restricted to JIA patients presenting during the early-childhood G0 peak (<3 years, n=27, G0/G1 1.27 +/- 0.40), no clear difference could be identified from controls aged 1 to <3years (n=38, G0/G1 1.18 +/- 0.24).

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Inflammatory markers

Whereas systemic JIA patients were the most deviant in G0/G1, we considered the possibility that G0/G1 might be a proxy for systemic inflammation. A weak correlation could be identified between G0/G1 and CRP (n=34, ρ=0.17, p=0.03), but significance was borderline with ESR (n=120, ρ=0.17, p=0.06) and absent with S100A12 (n=69, ρ=0.10, p=0.42) and MRP8/14 (n=69, ρ=0.11, p=0.37).

DISCUSSION

Juvenile idiopathic arthritis exhibits a remarkable predilection for early childhood, a tendency that is most pronounced in oligoarticular JIA but also evident in other subtypes. Our results identify one potential contributor to this epidemiology: an elevation in pro-inflammatory IgG G0 glycoforms in normal young children, simultaneous with the peak incidence of this disease. Further, we demonstrate conclusively that hypogalactosylation of IgG, corrected for age, is evident in JIA generally and across specific JIA subtypes.

The basis for the elevation of hypogalactosylated IgG in early childhood is unknown. Glycosylation of IgG occurs in the Golgi apparatus, and variation in glycan structure is believed to reflect expression of enzymes that control the sequential addition of sugar residues to the growing oligosaccharide. However, the regulation of this process is poorly understood. It is possible that different populations of B cells elaborate antibodies with different glycosylation patterns (38). Alternately, glycosylation may reflect the cytokine environment in which B cells mature (39). Since the subset distribution, surface phenotype, and effector activity of T and B cells all vary with age, it will be an interesting challenge to identify the specific factors involved in the pediatric IgG glycan phenotype (12).

The “skew” toward G0 IgG in young children is likely to have physiological relevance. Hypogalactosylation of IgG may be a mechanism by which young children compensate for immature humoral immunity. Conversely, enhanced effector potency might predispose young children to IgG-mediated inflammatory diseases, potentially including JIA. If confirmed, this novel hypothesis could have important implications for the understanding of pediatric immune function and dysfunction.

We found that elevation of G0 IgG was relatively similar in all JIA subtypes tested, and that the G0/G1 of JIA overall was essentially identical to that previously determined for RA (28). This result is consistent with several possible explanations.

First, aberrant IgG glycosylation could be an epiphenomenon of synovitis. Our data do not exclude this possibility, but there are several reasons to doubt that it is the case. Direct experimental evidence supports the potentially enhanced arthritogenicity of G0 antibodies (20, 31, 40, 41). In adult RA, glycosylation becomes aberrant more than 3 years prior to diagnosis, suggesting that glycosylation precedes overt joint disease rather than resulting from it (28). Finally, we found correlations between G0/G1 and markers of systemic inflammation to be weak or absent.

A second basis for parallel G0/G1 elevation across JIA subtypes may lie with classification. ILAR criteria imperfectly delineate homogeneous patient groups, as assessed by HLA associations, peripheral blood mRNA patterns, and clinical and laboratory phenotypes (1, 2, 10, 42-44). Similarity of G0/G1 among JIA subtypes could therefore reflect scrambled classification of patients with respect to optimal biological subgroups.

A final explanation for our results could be termed the “threshold hypothesis.” Hypogalactosylation may push a pool of potentially pathogenic IgG across a threshold

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required to engender synovitis, and this threshold may be similar on average across individuals. This hypothesis makes the interesting prediction that the incidence of arthritis should vary with average G0/G1 in the healthy population, and indeed inflammatory arthritis peaks both in early childhood and in the 7th decade (21, 22, 45). Further, in the youngest children with JIA (aged <3y at onset), we found no significant difference in G0/G1 compared with age-matched controls. An intriguing corollary of the threshold hypothesis is that a decline in G0/G1 with age might in some cases favor amelioration of disease in young children. Indeed, the subset of JIA most prevalent in this age group, persistent oligoarticular JIA, is the only type of idiopathic inflammatory arthritis with a spontaneous remission rate in excess of 50% (46). These speculations raise interesting opportunities for hypothesis-directed investigation.

Our work has several limitations. Our G0/G1 method measures total serum N-glycans, and while serum G0 and G1 are primarily derived from IgG it is possible that other serum proteins could influence our results. Our technique does not address other variable IgG residues that could also affect IgG function, including core fucose, an additional N-acetylglucosamine at the junctional mannose, or sialic acid (Figure 1A). A further technical limitation is that VDJ recombination and somatic mutation result in the appearance of a Fab region N-glycan attachment site in approximately 20% of IgG molecules, contributing to "background" signal and reducing the specificity of our findings for Fc glycans (15). However, these exposed glycans are generally sialylated and therefore highly charged, migrating outside the HPLC peaks used for our G0/G1 calculations (28, 35).

Most importantly, we have not directly examined the ability of JIA IgG to fix complement, engage Fc receptors, form immune complexes, or otherwise promote inflammation. Additional study is needed to determine the net effect of aberrant galactosylation and other IgG glycan changes on the effector potency of the IgG pool, and the role of these changes as contributory to the pathogenesis of JIA.

Taken together, our data provide the first detailed examination of glycan variation as a function of age in children, in particular in young children during the window of highest vulnerability for JIA. They suggest that the antibody pool of young children may be more potent at triggering effector responses than that of older children and younger adults. This is an observation with important ramifications for pediatric immunity, potentially including susceptibility for JIA, a disease family we have confirmed to be characterized by marked elevation in hypogalactosylated IgG. Further work will be required to define the physiological and pathophysiological importance of these observations.

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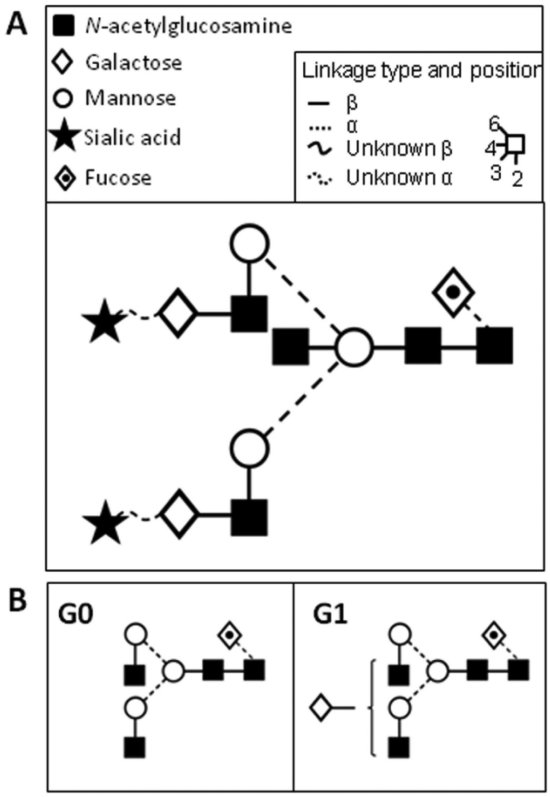


Figure 1. Structure of IgG-associated N-glycans

A. Bi-antennary (2-armed) structure of IgG Fc asparagine-linked glycans, with core pentasaccharide (stem composed of 2 N-acetylglucosamines (GlcNAc) and 3 mannoses) elaborated variably with additional sugars as noted, drawn using Oxford nomenclature (47). **B**. Major structures present in agalactosylated (G0) and monogalactosylated (G1) fractions. Note core fucosylation but absence of bisecting GlcNAc or terminal sialic acid, while a single galactose (if present) may be attached to either the 2,3 or the 2,6 arm.

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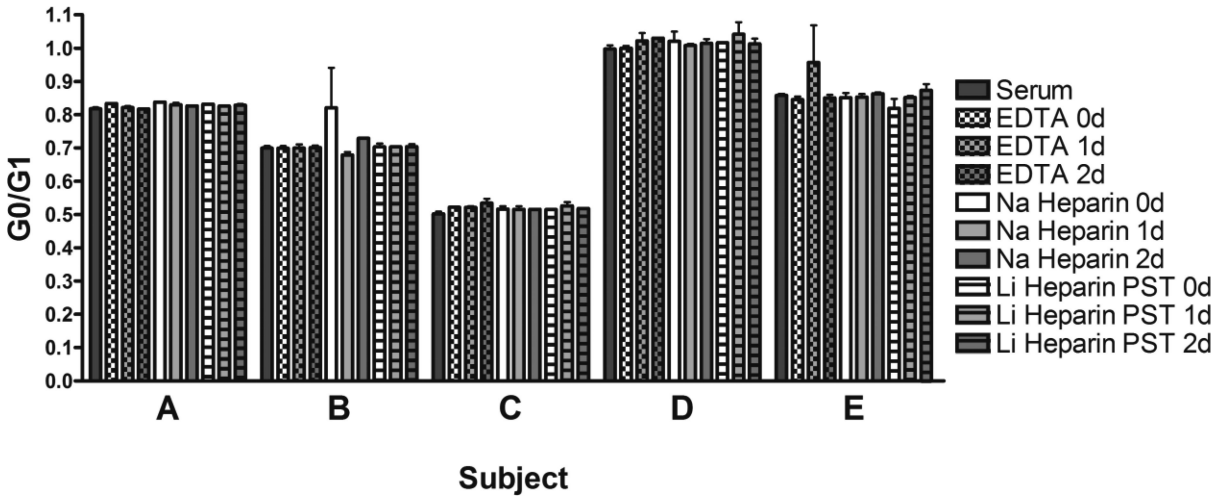


Figure 2. IgG glycans are stable across different collection conditions

Blood was drawn from 5 healthy adult donors into the collection tubes employed for our control and arthritic patients, including plain glass, EDTA, Na heparin, and Li heparin plasma separator tube (PST). Plain glass tubes were allowed to clot × 2h RT followed by centrifugation of contents to separate serum. Plasma was obtained similarly from each of the other tubes, either from separation after 2h (0d) or after room temperature storage for 24h (1d) or 48h (2d). All samples were frozen at -80C and thawed together for simultaneous glycan analysis by HPLC.

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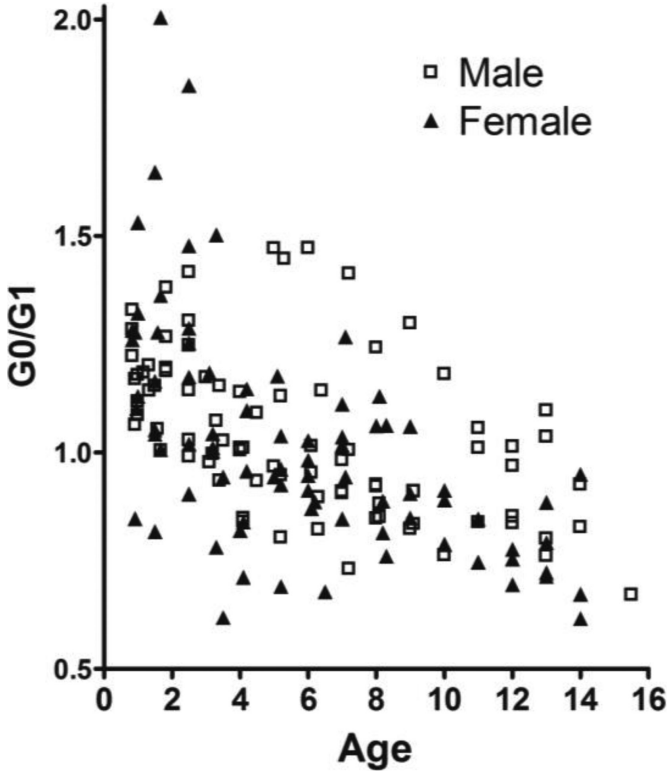


Figure 3. Hypogalactosylation of IgG glycans in healthy children

Data depict G0/G1 determined by HPLC from 165 healthy children, demonstrating variation with age (Spearman ρ=0.60, $p<0.0001$).

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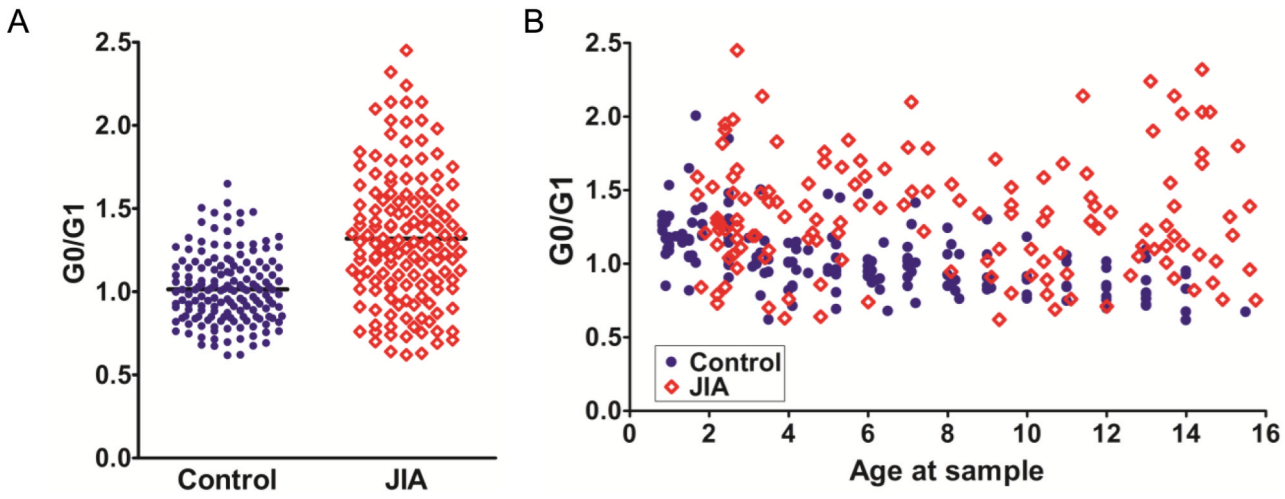


Figure 4. IgG glycosylation in JIA

A. G0/G1 in all JIA patients (n=141) vs. all controls aged 1y to <16y (n=156). Controls younger than 1 year were excluded because no case of JIA began before 1y. Healthy G0/G1=1.02 +/- SD 0.21 vs. JIA 1.32 +/- SD 0.40, p<0.0001. **B**. Distribution of G0/G1 among patients with JIA across the age spectrum. In JIA patients, age reflects time at sample, averaging 0.5y after disease onset in this cohort.

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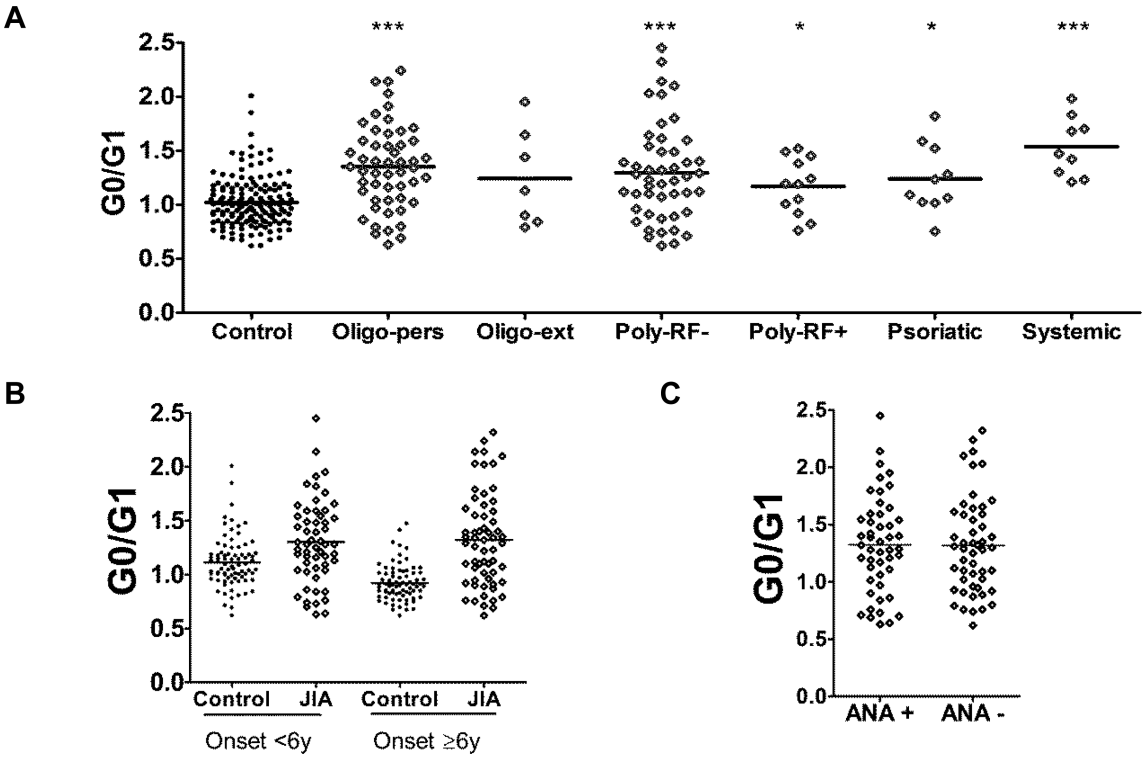


Figure 5. IgG glycosylation in JIA by classification system

A. G0/G1 among JIA patients as per ILAR subset (ERA not included). * <0.05, *** <0.001 by Mann-Whitney. **B**. G0/G1 as a function of age of onset of JIA among patients with oligoJIA, poly RF-JIA, and psoriatic JIA, compared with controls aged 1-<6y or ≥6y. Both subgroups of JIA are distinct from control groups and the control groups differ from each other (all P<0.001) but older and younger JIA patients exhibit similar G0/G1 (p=ns). **C**. Pooled analysis of patients with oligoJIA, poly RF- JIA, and psoriatic JIA with documented ANA status (ANA defined as above normal range for assay, n=98, P=ns).

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Table 1

Association of JIA and JIA subtypes with elevated G0/G1 after adjustment for age by linear regression

Data reflect unstandardized β coefficients. OligoJIA-extended omitted for insufficient sample size. Ages used are those at sample acquisition.

JIA subset	*β*	*95% CI*	P
All JIA	**0.317**	**0.245-0.389**	**<0.001**
JIA subtypes			
OligoJIA-persistent	0.337	0.225-0.389	<0.001
PolyJIA RF neg	0.338	0.241-0.435	<0.001
Poly JIA RF pos	0.325	0.203-0.447	<0.001
Psoriatic JIA	0.242	0.116-0.368	<0.001
Systemic JIA	0.467	0.331-0.599	<0.001