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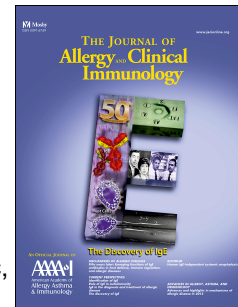


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Identification of dominant anti-glycan IgE responses in school-children by glycan microarray

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Identification of dominant anti-glycan IgE responses in school-children by glycan microarray

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Capsule Summary

In rural areas in many helminth-endemic areas, elevated levels of anti-glycan IgE are often seen. We used synthetic glycan microarray technology to characterize anti-glycan IgE antibody responses among rural and urban children in Ghana, West Africa. Glycan microarray technology presents an innovative approach to the exploration and dissection of antibody responses to glycan structures in population-based studies.

Key Words

Immunoglobulin E; Sub-Saharan Africa; Anti-glycan IgE; Cross-reactive carbohydrate determinants; Urban-rural; Glycan microarray; Core α -1,3-fucose; Core xylose; Galactose- α -1,3-galactose; sensitization

Abbreviations

α -1,3-gal: galactose- α -1,3-galactose
BSA: bovine serum albumin
CCD: cross-reactive carbohydrate determinants
GlcNAc: N-Acetylglucosamine
IQR: interquartile range
MFI: Median Fluorescence Intensity

To the Editor:

In helminth-endemic areas, elevated levels of cross-reactive IgE to environmental and food allergens are often seen that do not translate into positive skin prick tests or allergy symptoms.¹ Among Ghanaian children, such cross-reactivity was shown to be associated with *Schistosoma haematobium* infection and dominated by high IgE against cross-reactive carbohydrate determinants (CCDs).² The specific carbohydrate motifs involved in this IgE recognition were not determined.²

Recently, glycan microarrays have been developed that allow detailed characterization of IgE binding to glycan motifs.^{3, 4} Here we report the use of a microarray with 126 synthetic N-glycans and short oligosaccharides to identify specific glycan motifs associated with IgE cross-reactivity among urban and rural Ghanaian children.

Study methodology details are provided in the Online Repository. Sera from children attending schools we classified as rural (n=20), urban low socio-economic status (SES) (n=20) and urban high SES (n=20) were assessed. We also included sera from Italian pollen allergic controls with anti-CCD IgE (n=5) and meat allergic controls from the United States with anti-galactose- α -1,3-galactose (α -1,3-gal) IgE (n=4).

The characteristics of the Ghanaian subjects are shown in Table E1. Rural participants had high burdens of intestinal helminthiasis (50%) and malaria parasitemia (40%) compared to urban subjects. *S. haematobium* infection was found in 26.3% of urban low SES children compared to 5% in the other schools. ImmunoCAP-determined IgE sensitization (≥ 0.35 kU/L) to all allergens was most prevalent in the rural group and lowest in the urban high SES school.

A heatmap identified glycan motifs preferentially bound by IgE in each area (Fig. E2). High responses to structures with core α -1,3-fucose without core xylose (G73 and G75) were observed in rural children compared to urban high SES children (Fig.1A and Table E2). Although rural children had low responses to core xylose alone (structure G34), a few individuals in both urban groups had elevated IgE to this structure. Responses to structures that had both core xylose and core α -1,3-fucose (G37 and G39) were similar to those seen to core α -1,3-fucose alone (G73). Interestingly, in Italian

controls, elevated responses were seen to structures with core xylose (G34, G37 and G39) and less to structures with core α -1,3-fucose without core xylose (G73 and G75).

IgE responses to structure G113 containing the α -1,3-gal motif were most elevated in rural children and lowest in urban high SES children (Fig.1B). Weaker responses to structure G112 (gal α -1,3-gal attached to glucose instead of N-Acetylglucosamine) were observed among rural children. For US meat allergic patients, MFIs for structures G112 and G113 were higher than those seen for rural Ghanaian children.

IgE reactivities to core xylose and to core α -1,3-fucose found in bromelain (measured by ImmunoCAP) were compared to the same motifs on the microarray (Fig. 2). Although a weak correlation was observed between IgE to bromelain and to core xylose alone (structure G34), there were strong correlations between IgE to bromelain and to core α -1,3-fucose alone (structure G73, Spearman's $\rho = 0.74$, $P < .001$) as well as to structure G37 with both core xylose and α -1,3-fucose (Spearman's $\rho = 0.74$, $P < .001$). Correlations between IgE to α -1,3-gal (measured by ImmunoCAP) and IgE to the two α -1,3-gal motifs on the array were relatively weak (for G113, Spearman's $\rho = 0.52$, $P < .001$ and for G112, Spearman's $\rho = 0.29$; $P = .03$).

To examine whether *S. haematobium* infection played a role in anti-glycan IgE responses, motifs preferentially bound by IgE among *S. haematobium* positives were identified by heatmap (Fig. E3 – Online Repository). IgE responses to core xylose alone were elevated in most *S. haematobium* positives but not in negatives (Fig. E4A - Online Repository). By contrast, responses to structures that included core α -1,3-fucose were elevated in both *S. haematobium* positives and negatives (Fig. E4 B-D Online Repository). No significant associations were found between intestinal helminthiasis or malaria parasitemia and IgE responses to microarray glycans.

Our study is the first to use a synthetic glycan microarray to elucidate specific motifs associated with carbohydrate-related IgE cross-reactivity (CCDs as well as α -1,3-gal). Overall, higher anti-glycan IgE responses were observed in the rural area compared to both urban groups. Strikingly, the prevalence of IgE sensitization to α -1,3-gal among rural subjects as determined by ImmunoCAP and glycan

microarray was very high. The relatively poor correlation between IgE titers in both assays may be explained by the use of different antigen sources representing α -1,3-gal: bovine thyroglobulin with a heterogeneous glycan composition⁵ on ImmunoCAP versus synthetic glycans on the microarray.

Relatively high percentages of IgE sensitization to α -1,3-gal have also been observed in rural areas of Kenya (76%) and Ecuador (37%).⁶ It has been proposed (but yet to be demonstrated) that besides ticks, other ectoparasites or helminths may be involved.⁶ In our small study, we observed no association between helminth infection and α -1,3-gal sensitization. Although α -galactobiose has been found in some helminths, this is mostly in α -1,4 or β -1,6 linkages. Only for *Fasciola hepatica* has α -1,3-gal been demonstrated to be expressed on glycolipids.⁷ In-depth studies are needed to explore whether other helminths express α -1,3-gal and associations with IgE sensitization.

The N-linked glycans containing core α -1,3-fucose and β -1,2-linked core xylose are the main plant and insect glycoprotein residues associated with carbohydrate-related IgE cross-reactivity.⁸ IgE reactivity in Ghanaian children was higher to structures with core α -1,3-fucose compared to core xylose alone although a few individuals in the urban groups had elevated IgE to the latter. This observation may reflect the source of primary sensitization. N-glycans with core xylose together with core α -1,3-fucose and modifications have been identified in the egg stage of schistosomes.⁹ In our study, *S. haematobium* infection, mainly in urban low SES subjects, was significantly associated with raised IgE to core xylose. Although some *S. haematobium* positives had elevated IgE to structures with core α -1,3-fucose only, similarly raised levels to these structures were observed among *S. haematobium* negatives suggesting that these may not have been driven by schistosome infection. Therefore, our observations link schistosome infection in IgE cross-reactivity involving core xylose.

Overall, our findings suggest that cross-reactive IgE among Ghanaian children may be directed against both core xylose and core α -1,3-fucose independently. Further investigations are needed to explore other factors aside from helminths such as insects that may drive reactivity to these motifs. Recently, protein microarray technology has revolutionized allergy diagnostics by allowing the simultaneous assessment of specific IgE to multiple allergens with a small amount of serum. Our

153 investigation illustrates how glycan microarrays can further improve molecular diagnosis of specific
154 IgE to allergenic motifs by providing additional information on IgE profiles of patients.

155

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Figure Legends

Fig. 1A: IgE responses to selected N-glycan motifs.

N-glycan structures eliciting the greatest IgE binding among Ghanaian children were identified by heatmap. Responses to these structures are shown among Ghanaian children (stratified by school category) and Italian pollen allergic controls. Four additional structures with key variants were included for comparison and are indicated on the figure.

*Kruskal-Wallis Test of between-area differences in IgE response among Ghanaian subjects only, $P < .05$.

Structures included for comparison

**Response missing for one Italian pollen allergic control for Structure G37 due to smear on the specific microarray slide that obstructed the reading of microarray spots for this structure only.

Fig. 1B: IgE responses to α -1,3-gal motifs.

IgE reactivities to α -1,3-gal motifs are shown among Ghanaian children (stratified by school category) and US meat allergic controls. Lewis X saccharide (G127) was included as a control for small synthetic oligosaccharides.

* Kruskal-Wallis Test of between-area differences in IgE response among Ghanaian subjects only, $P < .05$.

Structure included for comparison.

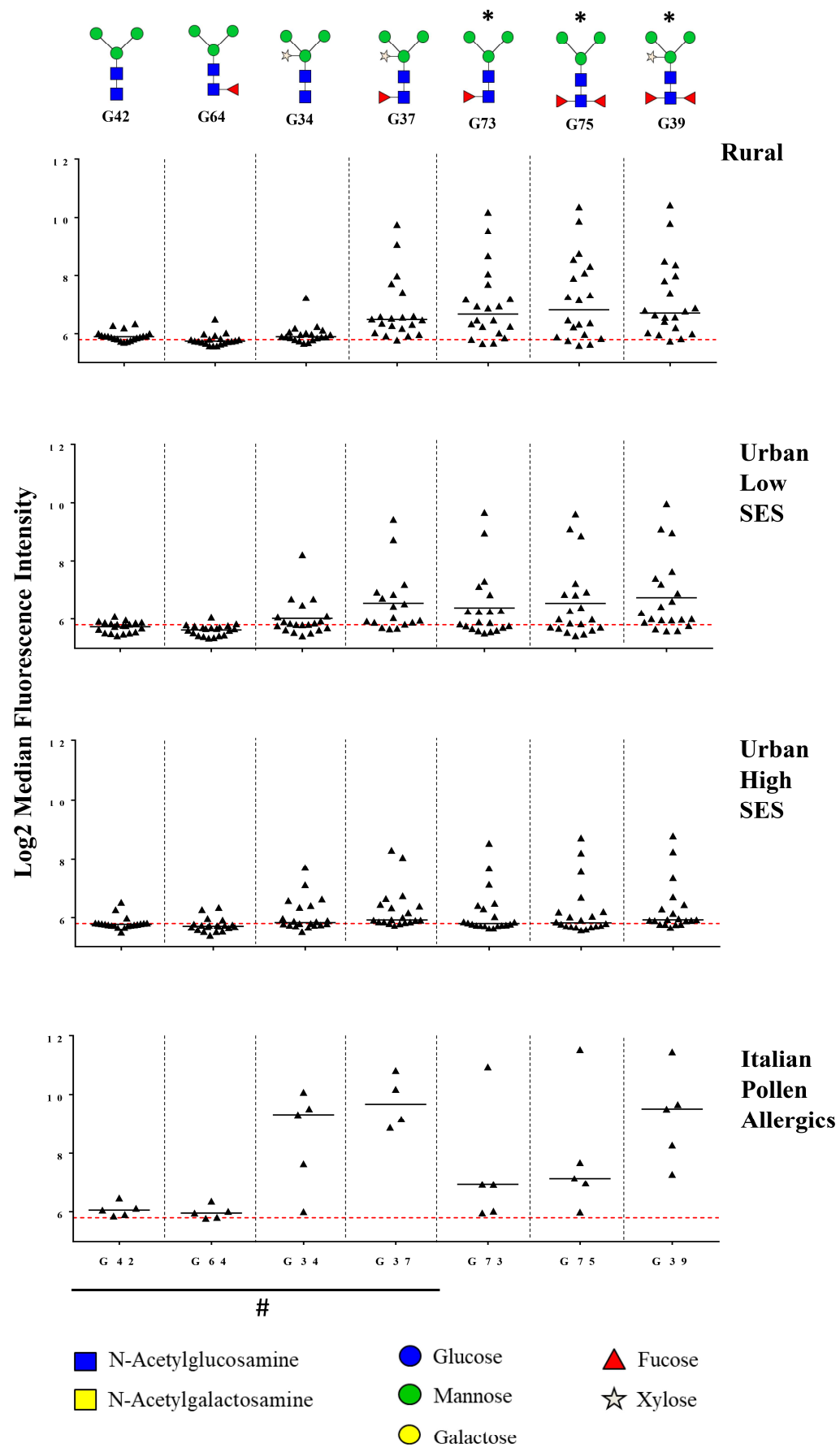
Fig. 2: Correlations between IgE to bromelain measured by ImmunoCAP and IgE to N-glycans measured by glycan microarray in Ghanaian children.

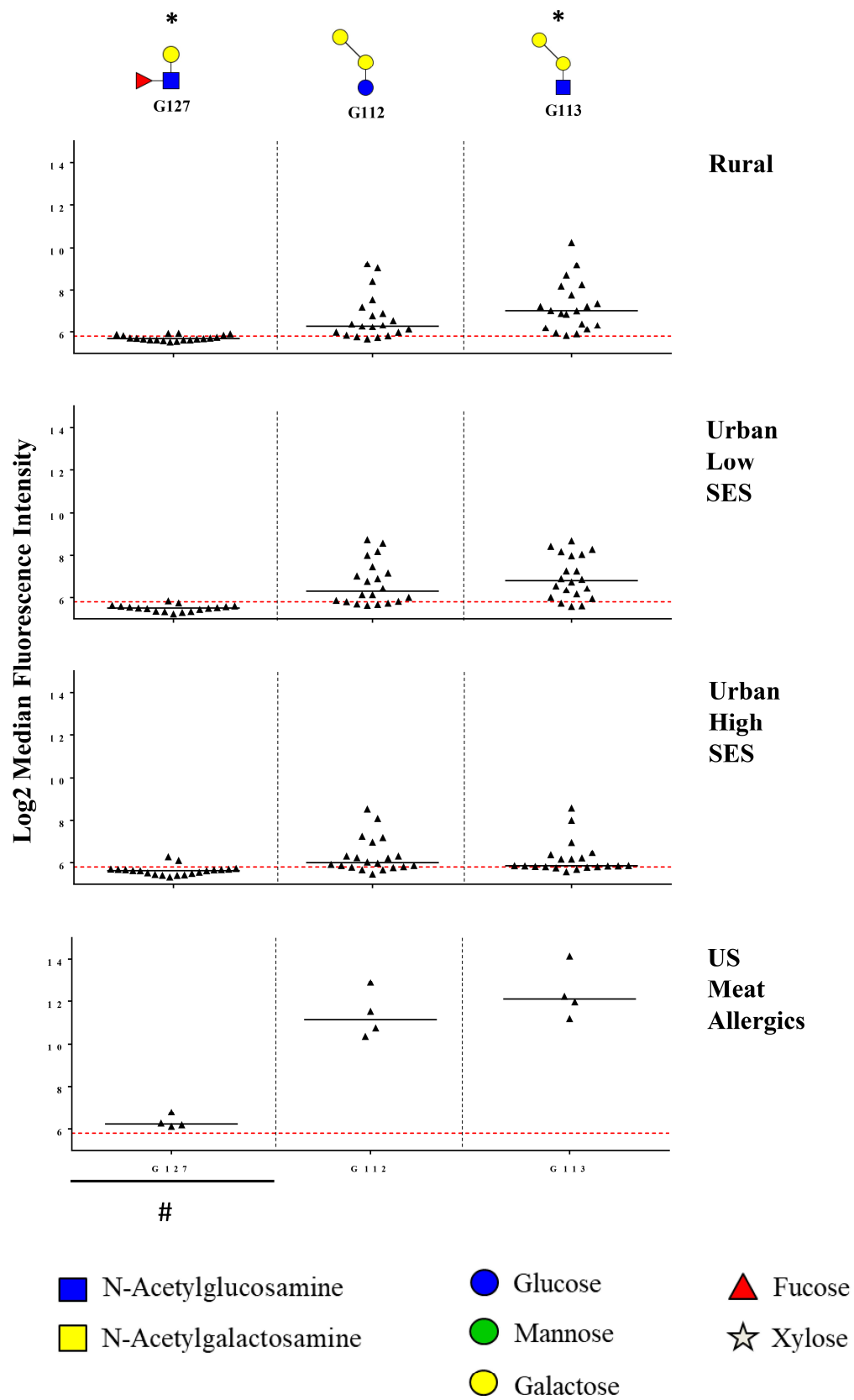
Correlation between IgE to bromelain and

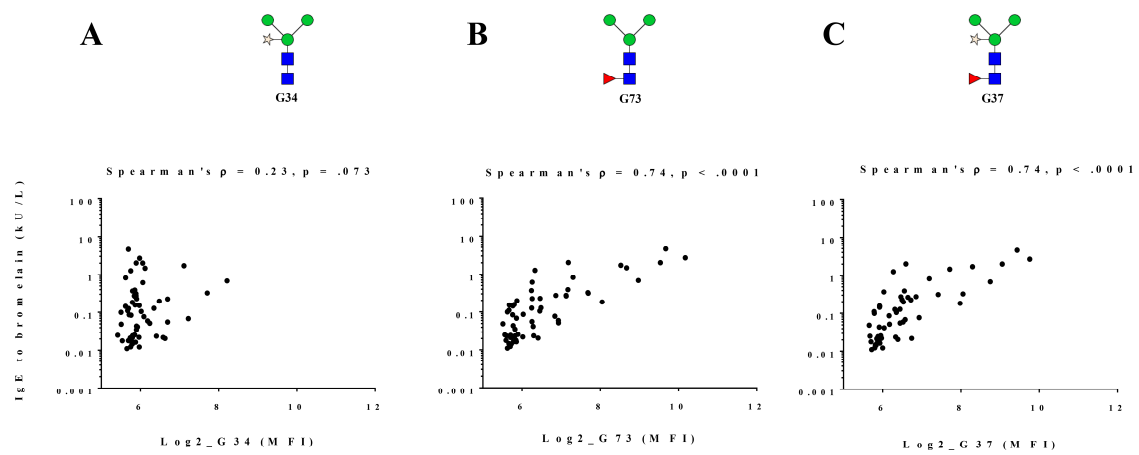
A. Core xylose only (Structure G34),

B. Core α -1,3 fucose only (Structure G73)

C. Core α -1,3 fucose + core xylose (Structure G37).







Identification of dominant anti-glycan IgE responses in school-children by glycan microarray

Online Repository

Materials and methods

Study design and population

A subset of subjects from a larger cross-sectional study on allergic sensitization and parasitic infections among schoolchildren in Ghana^{E1} were included in this investigation. Briefly, the study was conducted between March 2006 and March 2008 among children aged between 5 and 16 years attending schools in the Greater Accra Region. European^{E2, E3} and African^{E4, E5} studies have observed a protective effect of exposure to microbe-rich rural environments on allergy outcomes in children as well as distinct within-urban differences based on socioeconomic status (SES). Therefore, our study population consisted of children from rural, urban low SES and urban high SES areas that reflected the dynamic environmental changes associated with urbanization in Ghana. The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board, Ghana (NMIMR-IRB CPN 012/04-05).

Sera (n=60) were randomly selected from three schools: 1) a rural school (Toflokpo Presbyterian) located in the Dangme West district endemic for *S. haematobium*, intestinal helminths and malaria, 2) an urban low SES public school (Nii Okine Basic) in the city of Accra where approximately 18% of recruited participants were found to be infected with *Schistosoma haematobium*^{E6} and 3) an urban high SES private school (Greenhill International) also in Accra. There were significantly more males randomly selected from the rural school (15 out of 20) compared to the urban schools. Given this over-representation, we later examined the role of gender on anti-glycan IgE responses stratifying by each area. No consistent independent effect of gender on responses was observed.

Sera from 5 Italian grass pollen allergic patients with elevated IgE to bromelain and horseradish peroxidase (HRP) were included in our investigation as reference sera for IgE positivity to cross-reactive carbohydrate determinants (CCDs).^{E7} The Italian pollen allergic controls (3 males and 2 females) had median IgE to bromelain of 13.2 kU/L (IQR: 10.8 – 30.1) and to HRP of 6.0 kU/L (IQR: 2.3 to 7.6).^{E7} Samples from 4 meat allergic patients from the United States were also included as reference sera for IgE reactivity to galactose- α -1,3-galactose (α -1,3-gal). The US meat allergics (3 males, 1 female) had median IgE to α -1,3-gal of 44.1 kU/L (IQR: 7.4 – 178.4) and IgE to beef of 16.0 kU/L (IQR: 1.7 – 108.8) both measured by ImmunoCAP.

Glycan microarray binding assays

A microarray of 126 synthetic N-glycans and short oligosaccharides^{E8} was used to assess IgE responses in sera. This microarray is currently not commercially available. Fig. E1 (Online Repository) shows 126 of the synthetic glycan structures on the microarray. The array also included fluorescently-labelled bovine serum albumin (BSA) as a control for the printing process and Gal α -1,3-Gal β -1,4-GlcNAc-BSA (Dextra Laboratories Ltd, Reading, United Kingdom) as an additional structure representing α -1,3-gal.

Synthesized glycan structures were immobilized on a Nexterion H N-hydroxysuccinimide-coated glass slide (Schott AG, Mainz, Germany). Each slide consisted of seven sub-arrays containing the 128 structures in quadruplicates. The synthesis of the glycan structures, slide printing procedures and binding studies to evaluate the array have been described in detail elsewhere.^{E8} Printed glass slides were blocked with 50 mM of ethanolamine in 50 mM sodium borate buffer, pH 9.0 for one hour, washed, dried and stored at -20°C.

Microarray slides stored at -20°C were thawed and dried at room temperature. Arrays were incubated while shaking with 300 μ l of 1:30 diluted serum in PBS with 1% BSA and 0.01% Tween 20 (T₂₀) for one hour at room temperature, then washed in PBS with 0.05% T₂₀ and then PBS alone. This was followed by a 30-minute incubation in the dark with PromoFluor 647-labelled anti-human IgE (Sanquin reagents, Amsterdam, The Netherlands) diluted 1:400. The PromoFluor 647 labelling of IgE was carried out using a Promokine kit (PromoCell GmbH, Heidelberg, Germany) per manufacturer's

instructions. The slides were then processed through final washing steps, spin-dried and kept in the dark until scanning.

Microarray slides were scanned using a G2565BA scanner (Agilent Technologies, Santa Clara California, USA) at 10 μm resolution using 2 lasers (532nm and 633nm). GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, California) aligned the spots using circular features with a composite pixel intensity threshold of 10. For each spot, the ratio of the median fluorescence intensity (MFI) of the spot and local background MFI was calculated and then multiplied by the average background MFI for all glycan spots. For each structure, the average over 4 spots was determined and then log-transformed. The MFI value for all arrays combined was 5.8. This value was used as a cut-off for IgE responsiveness in our study.

IgE measurements to allergen extracts, a CCD marker and α -1,3-gal

ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) measurements were carried out according to the manufacturer's instructions to determine serum-specific IgE levels to house dust mite extract (*Dermatophagoides pteronyssinus*), cockroach extract (*Blattella germanica*), peanut extract (*Arachis hypogaea*), the CCD marker bromelain and α -1,3-gal (source material: bovine thyroglobulin).

Parasitological examinations

The Kato-Katz method was used to detect intestinal helminth eggs (hookworm - *Necator americanus* and *Ancylostoma duodenale*, *Ascaris lumbricoides*, *Trichuris trichiura* and *S. mansoni*) in one 25 mg stool sample.^{E9} Ten ml of urine was filtered through a nylon nucleopore filter (pore size, 12 μm) for the detection of *S. haematobium* infection.^{E10} A small quantity of blood was used to prepare a Giemsa-stained thick smear slide to detect malaria parasites.

Statistical Analysis

Analysis was performed using IBM SPSS version 20.0 (IBM Corp, Armonk, NY, USA). Area differences in the distribution of subject characteristics were examined by Pearson's χ^2 test (two degrees of freedom) and the Kruskal-Wallis test for continuous variables. A p-value of less than .05 was taken as the level for statistical significance. Heatmaps were constructed to identify glycan

79 structures preferentially bound by IgE based on raw MFI data according to school category as well *S.*
80 *haematobium* infection status. For each structure, the average MFI was calculated for each school
81 category as well as *S. haematobium* infection category (positive versus negative). Most IgE responses
82 were not normally distributed following log transformation (base 2) so nonparametric tests were used
83 for further analysis. Kruskal-Wallis tests were used to examine anti-glycan IgE differences between
84 the three areas. Correlations between IgE measured by ImmunoCAP and IgE to glycan motifs on the
85 microarray were examined using Spearman's rank correlation coefficient.

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Figure Legends – Supplementary material (Online Repository)

Fig. E1: Glycan microarray with 126 synthetic glycan motifs (Source: Brzezicka *et al*, *ACS Chem Biol*. 2015 May 15; 10 (5):1290-302). Two additional structures containing BSA were included on the array but are not shown in this figure.

Fig. E2: Heatmap of average MFIs for each structure used to visualize IgE binding to microarray glycans according to school category. Structures eliciting the highest responses are indicated by red and the lowest by dark blue. The motif compositions of these structures are shown in the figure.

Fig. E3: Heatmap of average MFIs for each structure used to visualize IgE binding to microarray glycans according to *Schistosoma haematobium* infection status. Analysis was restricted to the urban low SES group where the infection was most prevalent. Structures eliciting the highest responses are indicated by red and the lowest by dark blue. The motif compositions of these structures are shown in the figure.

Fig. E4: IgE responses to major N-glycan motifs eliciting the greatest responses among *S. haematobium* positives. Each motif is compared to structure G42 without modifications with lines connecting responses for the same individuals. Responses are shown to A) core xylose alone, B) core α -1,3-fucose alone C) core xylose + core α -1,3-fucose and D) core xylose, core α -1,3-fucose + core α -1,6-fucose.

1 **Tables - Online Repository**2 **Table E1:** Characteristics of Study Participants

Factor	AREA				#P value
	ALL, n/N (%)	Rural, n / N (%)	Urban low SES, n/N (%)	Urban High SES, n/N (%)	
Age (years) §					
Median (IQR)	11.8 (10.0 – 13.1)	11.1 (9.9 – 12.4)	13.2 (9.6 – 14.0)	11.9 (11.0 – 12.8)	.26
Gender					
Male	31 / 60 (51.7)	15 / 20 (75.0)	8 / 20 (40.0)	8 / 20 (40.0)	.04
Female	29 / 60 (48.3)	5 / 20 (25.0)	12 / 20 (60.0)	12 / 20 (60.0)	
Parasitic infections (positive) §					
<i>Schistosoma haematobium</i>	7 / 59 (11.9)	1 / 20 (5.0)	5 / 19 (26.3)	1 / 20 (5.0)	.06
Intestinal Helminth**	11 / 58 (19.0)	10 / 20 (50.0)	1 / 20 (5.0)	0 / 18 (0.0)	<.001
<i>Plasmodium sp.</i>	11 / 60 (18.3)	8 / 20 (40.0)	3 / 20 (15.0)	0 / 20 (0.0)	.004
Specific IgE levels (≥0.35kU/L)					
Bromelain (MUXF3)	12 / 60 (20.0)	7 / 20 (35.0)	4 / 20 (20.0)	1 / 20 (5.0)	.06
House dust mite (Der p)	12 / 60 (20.0)	8 / 20 (40.0)	4 / 20 (20.0)	0 / 20 (0.0)	.007
Cockroach (Bla g)	23 / 60 (38.3)	13 / 20 (65.0)	6 / 20 (30.0)	4 / 20 (20.0)	.009
Peanut (Ara h)	9 / 60 (15.0)	5 / 20 (25.0)	3 / 20 (15.0)	1 / 20 (5.0)	.21
Galactose-α-1,3-galactose (α-1,3-gal)	18 / 60 (30.0)	14 / 20 (70.0)	4 / 20 (20.0)	0 / 20 (0.0)	<.001

3

4 # P value calculated for the association between factors and area using Pearson's χ^2 test (two degrees of freedom) or Kruskal-Wallis test for continuous variables5 **Intestinal helminth = *Ascaris lumbricoides*, *Trichuris trichiura*, *Schistosoma mansoni* and hookworm

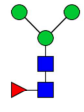
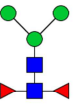
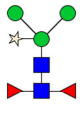
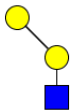
6 § Age of one rural subject was missing

7 §§ Missing information for *S. haematobium* = 1 Rural and 1 Urban Low SES subject. Intestinal helminth = 2 Urban high SES subjects

8 Significant associations are shown in bold.

9 **Table E2:** Median IgE responses to glycan structures preferentially bound by IgE according to the heatmap. Responses stratified by school category are
 10 shown.

11

Glycan Structure	Rural	Urban low SES	Urban high SES	Kruskal-Wallis Test
	Median MFI (IQR)	Median MFI (IQR)	Median MFI (IQR)	p-value
 G73	6.66 (6.08 - 7.57)	5.88 (5.68 - 6.70)	5.80 (5.71 - 6.38)	.013
 G75	6.81 (5.91 - 8.25)	5.99 (5.68 - 6.91)	5.82 (5.68 - 6.20)	.036
 G39	6.70 (6.07 - 7.94)	6.11 (5.90 - 7.35)	5.93 (5.77 - 6.44)	.03
 G113	7.03 (6.23 - 8.08)	6.82 (6.05 - 8.03)	5.86 (5.81 - 6.33)	.002

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