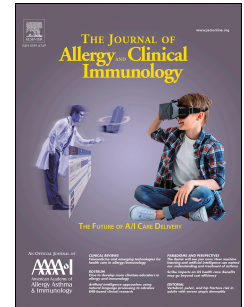


Journal Pre-proof

α -Gal specific-IgE prevalence and levels in Ecuador and Kenya: relation to diet, parasites and IgG4

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Journal Pre-proof α -Gal sensitization patterns in children from rural Ecuador and Kenya versus USA



Ecuador

Kenya

Virginia, USA

Virginia, USA

Population studied:

Reference cohorts of control and asthmatic children

α -Gal syndrome

α -Gal sIgE prevalence:

32%

54%

25%

100%

α -Gal sIgE levels:

Low-moderate
(GM: 1.8 IU/mL)

Low-moderate
(GM 3.3 IU/mL)

Low-moderate
(GM: 2.0 IU/mL)

Moderate-high
(GM: 9.1 IU/mL)

Total IgE levels:

High
(GM: 450 IU/mL)

High
(GM: 330 IU/mL)

Normal
(GM: 90 IU/mL)

Normal-high
(GM: 146 IU/mL)

α -Gal sIgE as
% of total IgE:

Low
(GM: 0.2%)

Low
(GM: 0.4%)

Moderate
(GM: 1.8%)

High
(GM: 6.2%)

Agent suspected
to be major cause
of sensitization:



Ascaris
and/or other
helminths



Tick bites, mostly
from lone star ticks
(*A. americanum*)

Abbreviations

α -Gal: galactose- α -1,3-galactose
GM: geometric mean
sIgE: specific IgE

Ascaris image courtesy of CDC - Henry Bishop;
Tick image courtesy of CDC - Dr. Amanda Loftis,
Dr. William Nicholson, Dr. Will Reeves and Dr. Chris Paddock

α -Gal specific-IgE prevalence and levels in Ecuador and Kenya: relation to diet, parasites and IgG4

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ABSTRACT:

Background: IgE to α -Gal is a cause of mammalian meat allergy and has been linked to tick bites in North America, Australia and Eurasia. Reports from the developing world indicate that α -Gal sensitization is prevalent but has been little investigated.

Objective: To seek evidence for the cause(s) of α -Gal sensitization and lack of reported meat allergy among children in less developed settings in Ecuador and Kenya.

Methods: IgE to α -Gal and total IgE were assessed in children from Ecuador (n=599) and Kenya (n=254) and compared to children with (n=42) and without known (n=63) red meat allergy from the Southeastern USA. Information on diet, potential risk factors and helminth infections were available for children from Ecuador. IgG4 to α -Gal and antibodies to regionally-representative parasites were assessed in a subset of children.

Results: In Ecuador (32%) and Kenya (54%) α -Gal specific-IgE was prevalent, but levels were lower than children with meat allergy from the USA. Sensitization was associated with rural living, antibody markers of *Ascaris* exposure and total IgE, but not active infections with *Ascaris* or *Trichuris* species. In Ecuador 87.5% reported consuming beef ≥ 1 x per week, including 83.9% of those who had α -Gal specific-IgE. Levels of α -Gal specific-IgG4 were not high in Ecuador, but were greater than in children from the USA.

Conclusion: These results suggest that in areas of the developing world with endemic parasitism that α -Gal sensitization is: i) common, ii) associated with *Ascaris* exposure, and iii) distinguished by a low percentage of specific/total IgE compared to individuals with meat allergy in the USA.

Key Messages

- The prevalence of α -Gal specific-IgE in areas of the developing world with endemic parasitism can exceed the prevalence reported in areas of the USA and Europe where tick-acquired mammalian meat allergy is commonly recognized.
- The lack of cases of reported mammalian meat allergy in Ecuador and Kenya is likely explained by the fact that levels of IgE specific for α -Gal are low, particularly in relation to total IgE.
- An association between antibodies to *Ascaris* and α -Gal specific-IgE, and also higher levels of IgG4 to α -Gal, suggests that chronic exposure to *Ascaris* contributes to sensitization in areas where this helminth is endemic.

Capsule Summary

In tropical areas of the developing world IgE to α -Gal is prevalent and associated with *Ascaris* exposure, but represents a minor fraction of total IgE in comparison to meat allergic patients from the USA.

Key words

IgE; α -Gal; mammalian meat allergy; *Ascaris*; parasite; sensitization

Abbreviations

α -Gal – galactose- α -1,3-galactose

AGS - α -Gal syndrome

ELISA – enzyme-linked immunosorbent assay

IgE – immunoglobulin E

IgG – immunoglobulin G4

IRB – Institutional Review Board

UVA – University of Virginia

INTRODUCTION

Galactose- α -1,3-galactose (α -Gal) is an oligosaccharide of non-primate mammals and the target of IgE antibodies in subjects with the “ α -Gal syndrome” (AGS)¹. The syndrome, which commonly manifests with delayed reactions to mammalian meat, has been well described in the southeastern USA and also parts of Australia, Europe and Asia. In these regions the dominant cause of sensitization has been related to tick bites, though other ectoparasites such as chiggers could be involved². Although there has been little information on the incidence or prevalence of the syndrome, population-based screening has revealed that the frequency of sensitization to α -Gal can approach 20% in some areas of the USA and Europe where certain species of ticks are endemic^{3,4}. Interestingly, we and others have reported striking rates of IgE sensitization to α -Gal in studies conducted in developing countries such as Kenya, Zimbabwe and Ecuador^{3,5}. For example, 76% of children from a village in rural Kenya and 47% of children from a rural area in Esmeraldas province of Ecuador were reported to have specific-IgE (sIgE) to α -Gal³. Because those studies were originally designed to study asthma, information about food allergy is lacking in these two studies. Nonetheless, we are unaware of an epidemic of allergy to mammalian meat in these populations.

We have considered several possibilities that could explain the high rates of α -Gal sensitization without a corresponding high rate of mammalian meat allergy. As compared to children with symptomatic mammalian meat allergy, IgE positive children from Ecuador or Kenya could have: i) lower levels of α -Gal sIgE, ii) similar absolute α -Gal sIgE levels, but lower as a percentage of total IgE, and/or iii) higher levels of IgG4, an antibody subclass that is often associated with tolerance. An additional question relates to the factors that could contribute to the high rate of

106 α -Gal sensitization reported in developing regions. A role for endoparasitic exposures is
107 suggested by the fact that: i) α -Gal has now been identified in species of *Plasmodium*⁶ and
108 *Leishmania*⁷, and also members of the nematode and platyhelminth phyla⁸⁻¹⁰ and ii) malaria
109 and geohelminths, such as *Ascaris*, *Trichuris* and hookworm, are endemic in many of these
110 areas. Thus, here we sought to determine whether the high prevalence of α -Gal sensitization in
111 Ecuador and Kenya could be explained by geohelminth or *Plasmodium* exposures. We
112 hypothesized that endoparasite exposure(s) are associated with low levels of α -Gal sIgE and
113 concomitant high levels of α -Gal IgG4 – an immune signature often associated with tolerance.
114 To address these questions we compared serologic responses in children from Ecuador and
115 Kenya with responses in age-comparable reference populations, including children in the USA
116 with the α -Gal-related mammalian meat allergy.

METHODS

Study samples

Ecuadorian schoolchildren: Children living in riverine communities in rural Districts of Eloy Alfaro and San Lorenzo (n=376) and the city of Esmeraldas (n=223), Esmeraldas Province in northwest Ecuador, were recruited in a case-control study of asthma/wheeze, nested within a larger cross-sectional survey, as previously described^{11, 12}. Esmeraldas is at sea level, equatorial, and with a tropical rainforest climate¹³. Cases (n=251) were defined by a parental report of recent wheeze (i.e., wheeze within the past 12 months) while controls (n=348) had no history of wheeze symptoms. Urban children were from neighborhoods on the periphery of the city of Esmeraldas to which immigrants from northern rural Districts of the province had migrated over the last 40 or so years. Questionnaires were administered to the parent or guardian of each child and included detailed questions about exposures and diet. Blood samples were drawn and plasma stored for later analysis. Stool samples were collected and examined for presence and intensity of geohelminth eggs and larvae using Kato Katz and formol-ether methods, as previously described¹². All children in the nested case-control study with complete data and sample collection were included in the analysis. Approval for this study was obtained locally in the area where subjects were enrolled and from the University of Virginia Institutional Review Board (UVA-IRB).

Kenyan schoolchildren: Children were enrolled from the 4th grade of elementary schools in the rural village of Kabati (n=131) and the industrialized town of Thika (n=123) which were in proximity to each other and ~50-80km north of Nairobi, as previously described¹⁴. This region is approximately 5000-6000 feet above sea level and is near the equator with a subtropical

highland climate¹³. Blood samples were drawn and plasma stored for later analysis. Approval for this study was obtained from the Kenya Medical Research Institute (KEMRI) ethical review board and from the UVA-IRB.

Children in southeastern United States with α -Gal meat allergy: This population included 42 children from central Virginia (age <18 years) who reported hives and/or anaphylaxis to mammalian meat and had specific IgE testing consistent with AGS (i.e., α -Gal sIgE > 0.35 IU/mL). 27 of the cases were part of a cohort of 261 children and adults with mammalian meat allergy that were prospectively enrolled and described in a recent report¹⁵. 15 of the children were identified through a retrospective chart review of AGS cases managed at UVA between 2015 and 2020. Both the prospective and retrospective arms were approved by the UVA-IRB.

Children from central Virginia unselected for food allergy: This population included children enrolled from the emergency department or a pediatrician's office in Charlottesville, VA as part of a case-control study of pediatric asthma, as previously described^{16, 17}. This area is at ~500 feet above sea level and has a humid subtropical climate¹³. Cases were defined as subjects who were seen for acute wheezing symptoms; control children were evaluated for problems such as trauma, gastrointestinal distress or fever but did not have acute respiratory symptoms or signs. No information about diet or food allergy was collected as part of this investigation. Of the 74 subjects in the parent study, sera were available from 63 for additional serologic investigation. This study was approved by the UVA-IRB.

ImmunoCAP IgE and IgG4 Assays: Sera were tested for total IgE, specific IgE and specific IgG4 using the ImmunoCAP 250 instrument (Thermo-Fisher/Phadia U.S., Portage, MI). The IgE results

are expressed as international units (IU) per milliliter with 1 IU equivalent to approximately 2.4 ng. A positive reaction to α -Gal was considered as 0.35 IU/ml or greater. Using a previously described technique, specific IgE to α -Gal was assayed using cetuximab on the solid phase and specific IgG4 to α -Gal was assayed with α -Gal-HSA (human serum albumin) on the solid phase¹⁸. All other IgE and IgG4 assays utilized commercial ImmunoCAPS (Thermo-Fisher/Phadia).

IgG assays to *Ascaris*, Malaria, *Toxocara* and *Strongyloides*: ELISA kits were purchased from commercial vendors and carried out according to manufacturer's instructions: *Ascaris* IgG and Malaria IgG/IgM kits were from IBL International, Hamburg, Germany; *Strongyloides* IgG/IgM kit was from IVD Research (Carlsbad, CA, USA); *Toxocara canis* IgG to secretory-excretory antigens of *T. canis* larvae were measured as described¹⁹. Positive responses were determined by comparison with an internal calibrator control or pre-determined OD cut-off.

Statistical Analysis: The central tendency of antibody levels was assessed with geometric means and 95% confidence intervals (95%CI). Comparisons between two groups were analyzed using Student's t-test for parametric data and Mann-Whitney U test for non-parametric data. Correlations between variables were analyzed using Spearman rank correlation coefficients (r_s). Categorical variables were compared using χ^2 or Fisher's exact test, as appropriate. The Kruskal-Wallis statistic was used for multiple comparisons of continuous non-parametric data and χ^2 for trend was used for multiple comparisons of categorical data. Statistical analysis was performed using GraphPad Prism, version 7. Logistic regression was conducted using SPSS v25.

RESULTS

Characteristics of study samples

The median age of the children in Ecuador (11 years, range 6-19) and Kenya (11 years, range 8-15) was little different than the age of children who had AGS in the USA (12 years, range 5-15) (**Table 1**). The children who had AGS were predominantly male (74% male and 26% female), whereas the children from Ecuador (56% male and 44% female) and Kenya (51% male and 49% female) had a more balanced representation of girls and boys. In Ecuador 99% of the children identified as Afro-Ecuadorian. In Kenya all of the children were indigenous Africans. The children with AGS were predominantly Caucasian (88%). The unselected children from the USA trended younger than their counterparts with AGS (median age 8 years, range 4-18), were more frequently girls and 56% were African American and 35% Caucasian. Among the different samples, recurrent wheeze/asthma was present in 29% from Ecuador, 7% from Kenya, 19% with AGS and 40% among children from the USA not selected on the basis of food allergy.

Comparison of α -Gal sensitization across samples

In regards to α -Gal sensitization, 32% of the Ecuadorian children, 54% of the Kenyan children and 25% of control children from the southeastern USA were positive for IgE to α -Gal (**Fig 1A**). The levels of α -Gal sIgE were lower in all three of these populations compared to the children from Virginia who had symptomatic AGS (**Fig 1B**)³. Not surprisingly, total IgE was higher in the serum of children from Kenya and Ecuador than in the sera of children from the USA (**Table 1**). Accordingly, α -Gal sIgE as a percentage of total IgE was markedly lower in children from Kenya and Ecuador as compared to children from the USA with symptomatic α -Gal syndrome (**Fig 1C**).

Frequency of beef consumption in relation to α -Gal sensitization

Among study samples, dietary information was available only for children from Ecuador. Of the 594 children in Ecuador whose parents or guardians answered a question about frequency of beef (“carne”) consumption, 524 (87.5%) reported consuming at least once a week. Although information about allergic reactions to beef or other mammalian products was not addressed in the questionnaire, only 3 (0.5%) children reported never consuming beef. The frequency of children who consumed beef at least once a week was lower in children who were sensitized (83.9%) versus non-sensitized (90.3%), but even among those with α -Gal sIgE levels of class 4 or above (i.e., >17.5 IU/mL) 77.8% reported eating beef at least once a week (**Fig 1D**).

Factors associated with α -Gal sensitization

In the cohort from Ecuador detailed information was available to investigate risk factors associated with α -Gal sensitization including exposures to geohelminths and other relevant parasites. Where complementary information was available we compared findings from Ecuador with results from Kenya and other reference samples.

Demographic, clinical and socioeconomic characteristics: Male sex, rural living and contact with farm animals were associated with α -Gal sensitization in Ecuadorian children (**Table II**). The association between rural living and α -Gal sIgE prevalence was also seen in children from Kenya (**Fig 2A and Table E1**). Levels of α -Gal sIgE were higher in the children from Kenya who lived in the rural village of Kabati as compared to their counterparts who lived in the industrial town of Thika (**Fig 2B**); a similar trend in α -Gal sIgE levels in relation to rural living was observed in Ecuador. Interestingly, α -Gal sIgE as a percentage of total IgE was lower in the rural versus

urban Kenyan children, but this was not true in Ecuador (**Fig 2C**). Monthly income and presence of cat or dog in the house were not associated with sensitization (**Table II**). Subjects with histories of wheeze were neither more likely to be positive, nor have higher antibody levels, for α -Gal sIgE (**Fig E1**).

Exposure to parasites: *Ascaris* and *Trichuris* were present in the stool of ~50% of Ecuadorian children at the time of the blood draw. These geohelminths were found in similar frequency among α -Gal sensitized and non-sensitized subjects (**Table II**) and there was no correlation between parasite burden of *Ascaris* or *Trichuris* with α -Gal sIgE (**Fig 3A**). The frequency of samples with hookworm was higher in α -Gal sensitized subjects (χ^2 , $p < 0.01$), but hookworm was present in only 6% of the sample. Accordingly, the correlation between hookworm burden and α -Gal sIgE was weak ($r_s = 0.14$, $P < 0.001$). In a subset of the Ecuadorian children we also measured specific IgG and IgG4 as markers for previous exposures to *Ascaris*. The association of α -Gal sIgE with *Ascaris* sIgG was moderate ($r_s = 0.31$, $P < 0.001$). Among 15 children from the USA with AGS only a single serum had detectable IgG to *Ascaris* (**Fig 3B**). In the Ecuadorian children there was also a moderate association between *Ascaris* sIgG4 and α -Gal sIgE ($r_s = 0.43$, $P < 0.001$) (**Fig 3C**). Because malaria is present in Esmeraldas Province in Ecuador and there have been reports that *Plasmodium* species (which cause malaria) can express α -Gal, we also measured antibodies specific for *Plasmodium* as a marker of exposure. Serologic evidence of malaria exposure was present in 43.9% of the children but there was no association between IgG/IgM to *Plasmodium* species and α -Gal sIgE ($r_s = 0.02$, $P = 0.79$); none of the children with AGS tested positive for *Plasmodium* spp. antibodies (**Fig E2A**). We also assessed the relationship of α -Gal

slgE with serologic markers of *Strongyloides stercoralis* and *Toxocara canis* exposure, but no association was observed (**Fig E2B and C**).

IgE to *Ascaris* and Total IgE: IgE to *Ascaris* was more frequent in α -Gal sensitized than non-sensitized Ecuadorian children (80.5% vs 40.7%, $p < 0.001$) (**Table II**), with a correlation coefficient between *Ascaris* slgE and α -Gal slgE of 0.45 ($P < 0.001$) (**Fig 4A**). When *Ascaris* IgE was stratified into 5 groups based on level, there was a significant difference in α -Gal slgE prevalence, ranging from 7.3% in the group lacking *Ascaris* IgE up to 67% in the group with the highest *Ascaris* IgE levels (χ^2 for trend, $p < 0.001$), but median α -Gal slgE levels among positives were similar across all *Ascaris* IgE levels (**Fig 4B**). Consistent with the findings in Ecuador, the relationship between IgE to *Ascaris* and α -Gal slgE was moderately strong in the children from Kenya, with a correlation coefficient of 0.56 ($p < 0.001$) (**Fig E3**).

Total IgE was much higher in α -Gal sensitized children compared to non-sensitized children in children from Ecuador (**Table II**) and Kenya (**Table E1**). The correlation coefficient between total IgE and α -Gal slgE was 0.49 ($P < 0.001$) in Ecuador and 0.59 ($P < 0.001$) in Kenya (**Fig E4**). Not surprisingly, α -Gal slgE as a % of total IgE decreased as levels of total IgE titers increased in children in Ecuador and Kenya (Kruskal-Wallis, $P < 0.001$) (**Fig E5**). The correlation of α -Gal slgE with total IgE levels raised the possibility that non-specific binding could occur with the ImmunoCAP in situations with high total IgE. To investigate this possibility, we assessed α -Gal slgE prevalence in two reference populations in relation to total IgE levels – children from an urban area of Costa Rica where there are effective public health programs for geohelminth control and children from northern Sweden where the climate and social conditions are

unsuitable for geohelminth transmission. Additional details on these samples, which we have previously investigated in relation to asthma, are available in the online supplement^{20, 21}. In Costa Rica and Northern Sweden there were 63 children who had total IgE levels >1000 IU/mL, but only 6 of those were sensitized to α -Gal (**Table III**).

Levels of IgG4 to α -Gal are higher in children from Ecuador and Kenya compared to children in the USA.

Elevated levels of α -Gal sIgG4 could be expected: i) in a situation where allergic sensitization to α -Gal was driven by prolonged exposure(s), such as occurs with geohelminths and other endoparasites (as opposed to tick bite exposure, which is intermittent), and ii) to be associated with tolerance to mammalian meat. This hypothesis was addressed by assaying α -Gal sIgG4 in a subset of the sera from Ecuadorian children using ImmunoCAP. The sera were chosen to be representative of the overall sample with respect to sex, area of residence (urban vs. rural) and current wheeze. As shown in **Fig 5A**, levels of IgG4 to α -Gal were all less than 1 μ g/mL and were lower than levels of IgG4 to two allergens which children in Ecuador are commonly exposed - casein (in the form of cow's milk) and *Ascaris*. Moreover, among the children with detectable IgE to the respective allergens, the IgG4/IgE ratio was lower for α -Gal than casein (**Fig 5B**). Nonetheless, Ecuadorian children had higher levels of α -Gal sIgG4 than reference children from the USA (**Fig 5C**).

DISCUSSION

In contrast to those IgE responses that are important causes of allergic diseases in industrialized societies (e.g., dust mite, cat, peanut, etc.), α -Gal sensitization was positively associated with rural living and farm animal contact. Similar findings, including the higher rates in males than females, have also been reported in North America²². In the USA these variables lose significance when accounting for tick exposure²², which is consistent with a large body of evidence which supports the connection between tick bites and α -Gal sensitization in North America, Europe, Australia and Japan¹. Unfortunately, tick history was not available in the current studies and there are no validated serologic assays to determine prior tick exposure. While ticks, and also other ectoparasites such as chiggers², cannot be excluded as a cause of sensitization, there are good reasons to consider that endoparasites could contribute to induction of α -Gal sIgE in developing areas.

It is well established that chronic parasitism, particularly to geohelminths, is endemic among many children who reside in rural Ecuador and Kenya. In the current cohort, 67% of children from Ecuador had geohelminth larvae in their stool at the time of enrollment and IgG to *Ascaris*, a biomarker of prior *Ascaris* exposure, was detected in 113 of 123 screened (92%). Serologic markers reflecting prior exposure were also common for malaria (44%), *Strongyloides* (47%) and *Toxocara* (32%). Notably, α -Gal has been identified on several species of parasites, including *Plasmodium* spp. (causal agent of malaria)⁶, *Leishmania* spp.⁷ and helminths of the nematode, trematode and platyhelminth phyla⁸⁻¹⁰. Although the frequency of stool geohelminth burden was similar in children with and without α -Gal sensitization, α -Gal

sensitization was associated with each of IgG, IgG4 and IgE to *Ascaris*. By contrast, serologic markers of prior exposure to *Plasmodium*, *Toxocara* and *Strongyloides* were not associated with α -Gal sensitization. When the relationship between *Ascaris* sIgE and α -Gal sIgE was stratified by quintile of *Ascaris* sIgE, it was apparent that the frequency of α -Gal sensitization, but not the level of IgE to α -Gal, was strongly associated with the level of IgE to *Ascaris* (**Fig 4B**). Such a finding could reflect a situation where α -Gal is a minor allergen and/or where α -Gal is only present transiently during the parasite lifecycle. To date we are unaware that α -Gal has been identified in *Ascaris* species. Hodžić et al. recently probed for α -Gal in soluble crude extracts prepared from adult *A. suum* using the M86 monoclonal antibody but did not detect the oligosaccharide by ELISA or Western Blot¹⁰. Pörtl et al. carried out mass spectrometry using similar source material. While a number of glycans were identified, galactosyl groups were rare and were suggested to have β -linkages²³. Given that it is the larvae (rather than the adults) that transit through the lungs as part of the *Ascaris* lifecycle, we speculate that α -Gal may be selectively expressed during the larval stage. This would also be consistent with the idea that the respiratory mucosa may be more favorable for IgE induction than the gastrointestinal tract.

Despite the fact that 32% of the Ecuadorian children were sensitized to α -Gal, nearly 90% of the total cohort, and 83% of those that were sensitized, reported eating mammalian meat at least once a week. This finding is consistent with our clinical experience. We have seen many individuals who have positive α -Gal sIgE tests but nonetheless routinely consume mammalian meat without subjective symptoms. This is also supported by the work of Fischer et al. in Germany who investigated a cohort with high risk for tick bites, i.e. - forest workers and hunters. They found that >90% of those who were sensitized to α -Gal nonetheless tolerated

mammalian meat without overt allergic reactions⁴. A relevant question is whether the level of IgE to α -Gal can predict clinical reactivity to mammalian meat. Dr. Levin and colleagues reported on the utility of α -Gal sIgE levels in predicting clinical reactions to mammalian meat in a cohort of children and adults in the Eastern Cape of South Africa. They found a significant overlap in sIgE levels among individuals who reacted and tolerated mammalian meat, however α -Gal sIgE of 5.5 IU/mL and specific/total IgE ratio of 2.1% were associated with a 95% probability of clinical allergy to mammalian meat²⁴. In the current report, levels of IgE to α -Gal were lower, and IgE to α -Gal expressed as a percentage of total IgE strikingly lower, in children from Ecuador and Kenya as compared to age-similar children in the USA with AGS. The levels were also less than the cut-offs described in the study from South Africa. It is also important to consider that affinity/avidity of α -Gal sIgE could vary between individuals and/or different populations.

Tolerance is favored in situations with a relatively high ratio of specific IgG4 to specific IgE^{25, 26}. For example, we have previously shown that cow's milk tolerant children from an unselected birth cohort had IgG4/IgE ratios to cow's milk proteins which exceeded 1,000:1²⁷. In the current study, IgG4 to α -Gal was not present at high levels in children from Ecuador and the ratio of α -Gal specific IgG4/IgE was only ~20:1, as compared to a ratio of ~500:1 for casein. Hence, IgG4 to α -Gal is unlikely to explain tolerance to mammalian meat despite widespread IgE sensitization. Nonetheless, IgG4 to α -Gal was higher in the children from Ecuador than in age-similar children from the USA. A logical explanation, and one that is consistent with our understanding that IgG4 results from chronic allergen exposure, is that elevated IgG4 is an indicator of chronic/recurrent exposure to the relevant sensitizing agent²⁸. This contrasts with

the situation in North America, Europe, Japan and Australia, where the relevant exposure is from occasional tick bites and there is little or no IgG4 to α -Gal²⁹⁻³¹.

There are several limitations to this report. The study samples from Ecuador and Kenya were originally recruited to study asthma and information about allergic reactions to food were not collected. While we do not know how many, if any, subjects from these cohorts experienced allergic reactions to mammalian meat, the data from Ecuador indicates that the majority of children routinely consumed mammalian meat. The current data support our hypothesis that geohelminths contribute to α -Gal sensitization, but cannot exclude a role for tick bites. Additional information about local tick populations and prospective questioning about tick exposures would be required to better address this possibility. We hypothesize that the reason IgG4 levels to α -Gal were higher in children from Ecuador than the USA relates to differences in the nature of the sensitizing exposure, i.e., chronic exposure to a geohelminth in Ecuador versus intermittent exposure to tick bites in the USA, but this is indirect evidence. We did not carry out inhibition assays to confirm specificity of IgE to α -Gal, but the results from Sweden and Costa Rica indicate that high levels of total IgE are not sufficient to explain α -Gal sensitization. For the investigation into geohelminth exposures we focused on parasites in which serologic assays were available. A role for other geohelminths or parasites, especially those which could cross-react with the *Ascaris* assay, cannot be excluded. However, the relation of α -Gal sIgE was evident for *Ascaris* but not *Toxocara* or *Strongyloides*, which are also nematodes. There is some evidence that *Echinococcus* or *Schistosoma* may contain α -Gal glycans, but these helminths are not thought to be of relevance in our study populations¹⁰.

In conclusion, IgE to α -Gal was more prevalent among children from rural Ecuador and Kenya living in an area with endemic parasitism than children from an area in the USA where mammalian meat allergy is widely reported. Geohelminth eggs were present in stool samples at a similar frequency among sensitized and non-sensitized children, however IgE, IgG and IgG4 to *Ascaris* were all associated with α -Gal sIgE. To our knowledge, α -Gal has not been reported in *Ascaris* species, but nonetheless it is likely that *Ascaris* and/or other endoparasites which are endemic in developing regions contribute to sensitization to this blood-group-like glycan. An intriguing possibility is that IgE to α -Gal represents an adaptive response that evolved in higher primates as a means of recognizing and mounting rapid defense against ecto- and endo-parasites. This hypothesis would be consistent with emerging ideas on the evolutionary role of allergic immunity in defending against parasites and venomous organisms^{25, 32, 33}. This hypothesis would also distinguish α -Gal from most other forms of food allergy, where the adaptive benefit of IgE is not clear.

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TABLES

Table I. Characteristics of subjects in the major cohorts

Clinical and Immune Characteristics	Ecuador (n=599)	Kenya (n=254)	USA	
			α -Gal syndrome (n=42)	Controls (n=63)
Age, median (range) ^a	11 (6-19)	11 (8-15)*	12 (5-15)	8 (4-18)
Sex, male (%) ^b	337 (56%)	130 (51%)*	31 (74%)	28 (44%)*
Recurrent wheeze/asthma, n (%) ^b	170 (29%)	19 (7%)*	8 (19%)	25 (40%)*
Consume beef ≥ 1 x per week, n (%)	524 (87.5%)	NA	NA [†]	NA
Total IgE, GM, IU/mL (95%CI) ^c	450 (404-502)**	330 (261-418)*	146 (104-204)	90 (58-140)
α -Gal sIgE, n>0.35 (%) ^b	194 (32%)**	137 (54%)**	42 (100%)	16 (25%)**
α -Gal sIgE GM, IU/mL (95%CI) ^c	1.8 (1.5-2.1)**	3.3 (2.7-4.1)**	9.1 (6.1-13.5)	2.0 (1.1-3.6)**
α -Gal sIgE as % of total, GM (95%CI) ^c	0.2 (0.1-0.2)**	0.4 (0.3-0.5)**	6.2 (4.5-8.6)	1.8 (0.8-3.9)*

^a Student's T test, compared to AGS^b Fisher's exact test, compared to AGS^c Mann-Whitney U test, compared to AGS[†] Most of these children were not consuming mammalian meat, but detailed dietary history was not availablep value in relation to α -Gal syndrome - *<0.05, **<0.001

Table II. Characteristics of subjects in Ecuador (n=599) in relation to α -Gal sensitization.

Characteristic	α -Gal sIgE positive (n=194)	α -Gal IgE negative (n=405)	OR (95% CI)	p
<i>Demographic and Clinical Characteristics</i>				
Age, mean (range) ^a	11.3 (7-19)	11 (6-19)	na	0.18
Sex, male ^b	136 (70.1%)	201 (49.6%)	2.4 (1.6-3.4)	<0.001
Rural ^b	142 (73.2%)	234 (57.8%)	2.0 (1.4-2.9)	<0.001
Monthly income > \$250 ^b	33 (17.0%)	80 (19.8%)	0.8 (0.5-1.3)	0.42
Current wheeze ^b	57 of 192 (29.7%)	113 of 404 (28.1%)	1.1 (0.7-1.6)	0.69
<i>Diet and Exposures</i>				
Never consume beef ^b	2 of 192 (1.0%)	1 of 402 (0.3%)	4.2 (0.5-61)	0.20
Consume beef ≥ 1 x per week ^b	161 of 192 (83.9%)	363 of 402 (90.3%)	0.6 (0.3-0.9)	0.02
Never consume milk ^b	4 (2.1%)	4 (1.0%)	2.1 (0.6-7.3)	0.28
Consume milk ≥ 1 x per week ^b	156 (80.4%)	347 (85.7%)	0.7 (0.4-1.1)	0.1
Consume unpasteurized milk ^b	75 (38.7%)	153 (37.8%)	1.0 (0.7-1.5)	0.84
Cat in house ^b	72 of 193 (37.3%)	155 (38.3%)	1.0 (0.7-1.4)	0.82
Dog in house ^b	147 of 193 (76.2%)	324 (80%)	0.8 (0.5-1.2)	0.28
Farm animal contact ^b	56 of 193 (29.0%)	68 (16.8%)	2.0 (1.3-3.0)	<0.001
<i>Ascaris</i> in stool ^b	96 (49.5%)	178 (44.0%)	1.2 (0.9-1.8)	0.2
<i>Trichuris</i> in stool ^b	113 (58.2%)	224 (55.3%)	1.1 (0.8-1.6)	0.5
Hookworm in stool ^b	20 (10.3%)	17 (4.2%)	2.6 (1.4-5.2)	0.004
Any worm in stool ^b	140 (72.2%)	264 (65.2%)	1.4 (1.0-2.0)	0.09
<i>Immune markers</i>				
Total IgE, GM (95%CI) ^c	1141 (975-1335)	288 (255-326)	na	<0.001
Total IgE >500 IU/mL ^b	152 (78.4%)	141 (34.8%)	6.8 (4.6-10.1)	<0.001
<i>Ascaris</i> IgE ≥ 0.7 IU/mL ^b	162 (83.5%)	165 (40.7%)	7.4 (4.8-11.2)	<0.001
Positive skin test (any allergen) ^b	54 (27.8%)	71 (17.5%)	1.8 (1.2-2.7)	0.004

^a compared with Student's T test^b compared with χ^2 ^c compared with Mann-Whitney U test

496

497 **Table III. Prevalence of α -Gal sensitization in relation to total IgE levels in reference**
 498 **populations as compared to children from Ecuador.**

	Entire cohort			Total IgE: 1000-2000 IU/mL			Total IgE: >2000 IU/mL		
	n	α -Gal pos (%)	P*	n	α -Gal pos (%)	p*	n	α -Gal pos (%)	p*
Ecuador	599	194 (32.4%)	na	104	57 (55%)	na	89	65 (73%)	na
<i>Reference cohorts:</i>									
Costa Rica	277	13 (5%)	<0.001	28	3 (11%)	<0.001	18	2 (11%)	<0.001
N. Sweden	413	1 (0.2%)	<0.001	11	0 (0%)	<0.001	6	1 (17%)	0.01

499 * Comparison of reference population with Ecuador using Fisher's exact test

500

FIGURE LEGENDS

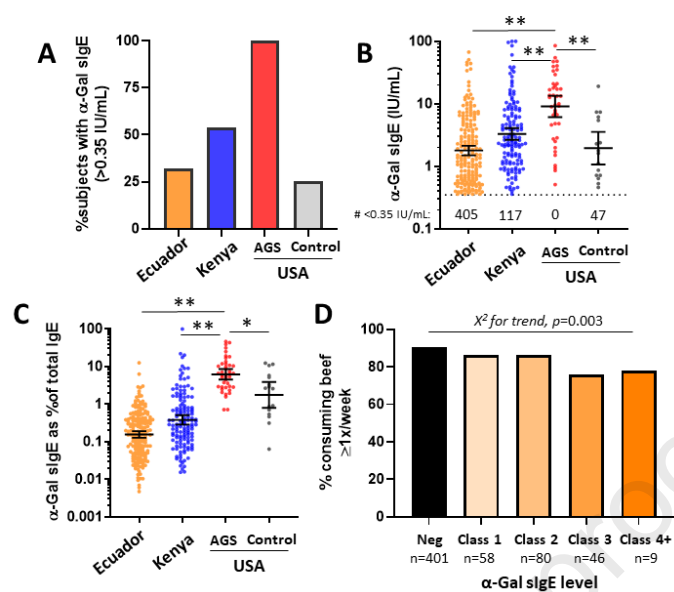
Fig 1. (A) Prevalence, **(B)** levels, and **(C)** levels in relation to total IgE, of α -Gal sIgE (>0.35 IU/mL) among children from population-based samples in Ecuador and Kenya as compared to control children or children with mammalian meat allergy in the Southeastern USA. **(D)** Beef consumption in children from Ecuador in relation to α -Gal sIgE level.

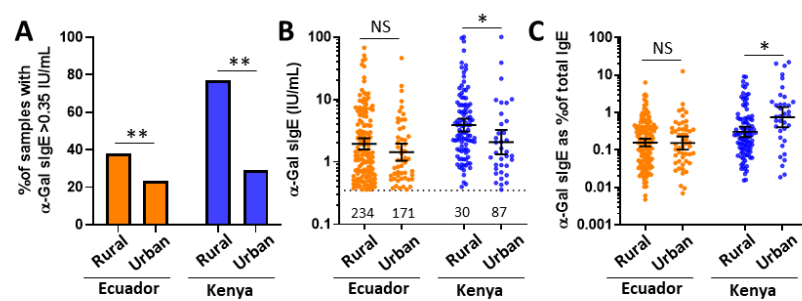
Fig 2. Comparison of **(A)** prevalence, **(B)** levels, and **(C)** levels in relation to total IgE, for α -Gal sIgE in children from rural and less rural areas (“urban”) of Esmeraldas province of Ecuador and the rural community of Kabati compared to the industrialized “urban” town of Thika, Kenya. The values in panel B reflect the number of samples that were < 0.35 IU/mL. Prevalence values were compared using χ^2 . Levels of α -Gal sIgE were expressed as geometric mean of positives (95% CI) and compared by the Mann-Whitney U test, $*p<0.05$, ns – $p>0.05$.

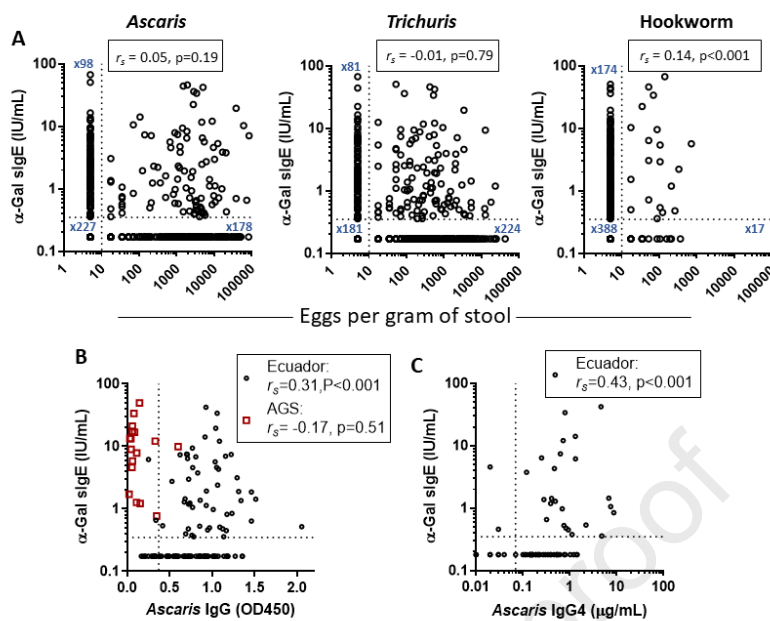
Fig 3. (A) Relationship between geohelminth parasite burden and α -Gal sIgE in children from Ecuador ($n=599$). Relationship of α -Gal sIgE with *Ascaris* IgG **(B)** and *Ascaris* IgG4 **(C)** in children from Ecuador ($n=123$) and children with α -Gal syndrome (AGS) in the USA ($n=18$). r_s – Spearman’s rank correlation. For α -Gal sIgE and eggs per gram of stool, dotted lines reflect threshold of detection. For *Ascaris* IgG and IgG4 the dotted line reflects approximation of calibrator cut-off (with minor inter-assay variation) for distinguishing positive and negative values.

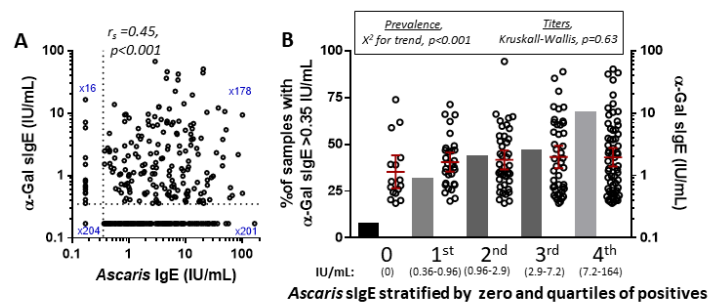
Fig 4. (A) *Ascaris* sIgE versus α -Gal sIgE in children from Ecuador ($n=599$). **(B)** Relationship between α -Gal sIgE prevalence (bars, left y-axis) and levels (scatter plot, right y-axis) in the cohort when stratified based on *Ascaris* sIgE status, where $n \leq 0.35$ IU/mL = 220 and $n > 0.35$ IU/mL = 379.

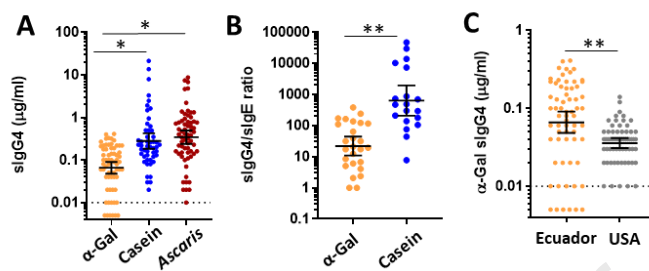
Fig 5. (A) IgG4 to α -Gal ($n=65$), Casein ($n=50$) and *Ascaris* ($n=65$) in children from Ecuador. **(B)** Ratio of sIgG4/sIgE (normalized to ng/ng) in children from Ecuador who had detectable sIgE to the respective IgG4 component. **(C)** IgG4 to α -Gal in children from Ecuador ($n=63$) compared to children from USA who were unselected for food allergy ($n=63$). Values expressed as geometric mean of positives (95% CI) and compared by the Mann-Whitney U test, $*p<0.05$, $**p<0.001$.

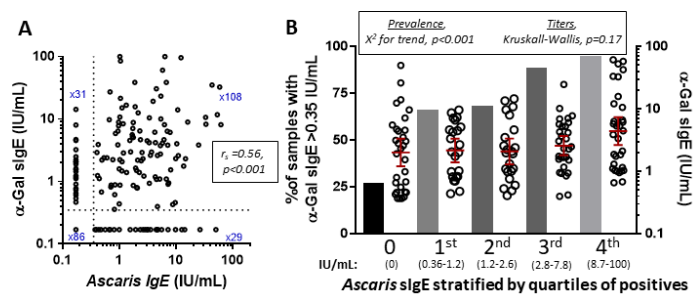


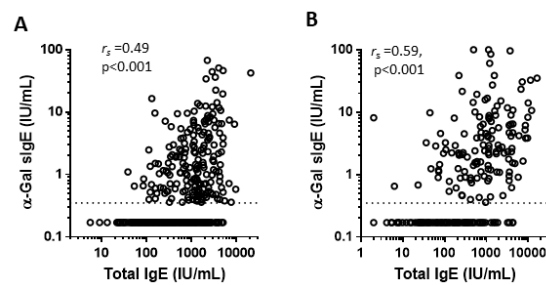


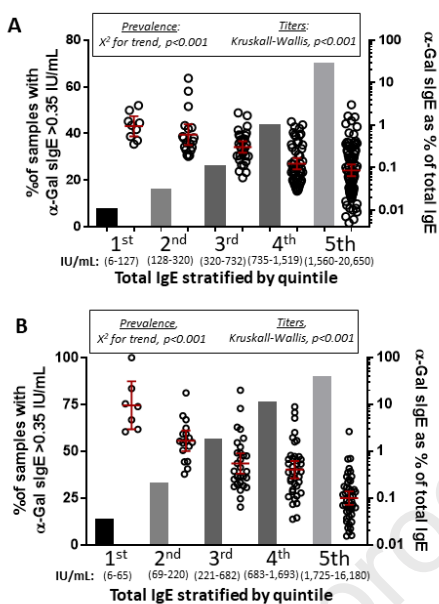


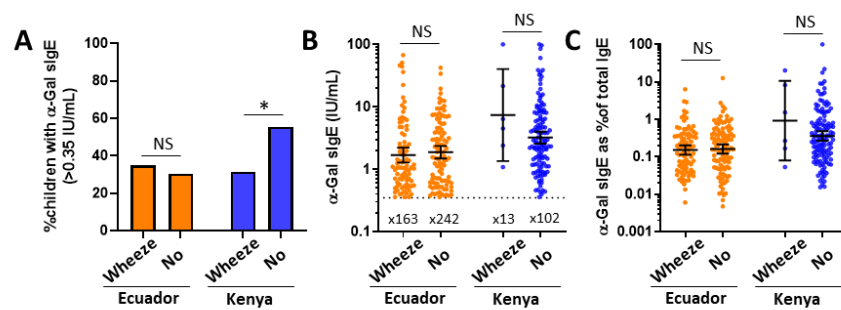


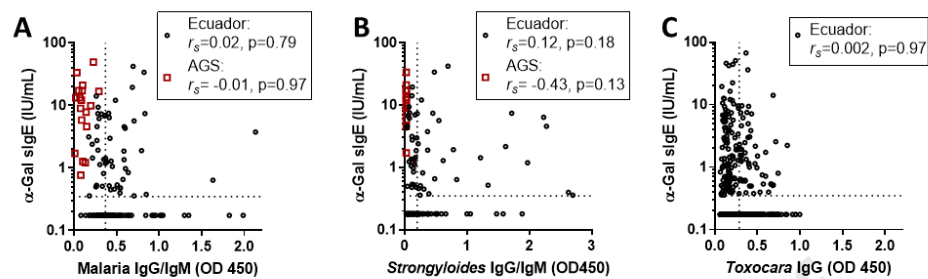












ONLINE REPOSITORY

Minor populations studied

- 1. Children from Costa Rica:** As part of a cross-sectional case-control study of asthma, 287 children aged 7-12 yrs (mean age 9.1 years, 53.2% male) were enrolled via the Emergency Department of the main tertiary hospital in San Jose, Costa Rica¹. San Jose has a Tropical savannah climate². Geohelminths are common in Costa Rica, but anti-helminths are administered to children annually starting at age 1 and active parasitic infections are infrequently identified³. There were 96 cases, defined by presentation for acute wheezing illness, and 191 controls who presented for evaluation of urgent medical problems unrelated to acute wheeze (most commonly trauma, gastrointestinal symptoms and/or fever). Of the 191, 65 had a history consistent with stable asthma. In the Costa Rica population dust mite sensitization is much more strongly associated with asthma than sensitization to *Ascaris*. For the current analysis blood samples were available for IgE testing in 277 of the subjects. The study was approved by the Ethics Committee at the Hospital Nacional de Niños and by the Institutional Review Board at the University of Virginia.
- 2. Subjects from northern Sweden:** Subjects from Kiruna and Luleå in northern Sweden were enrolled in a prospective, population-based study as previously reported⁴. This area has a subarctic climate² and thus is inhospitable to many allergenic species, such as dust mites, cockroaches, fungi and ticks. Of the 963 subjects who provided a blood sample at the age 19 year time point, 413 of these were selected for α -Gal sIgE testing. This included all 218 subjects who were sensitized to cat and an additional 195 randomly selected subjects. The study included detailed information about asthma and exposure history and was approved by the regional ethics committee of Umeå University and the Institutional Review Board at the University of Virginia.

Table E1. Characteristics of subjects in Kenya (n=254) in relation to α -Gal sensitization.

Characteristic	α -Gal IgE > 0.35 IU/mL (n=137)	α -Gal IgE $\leq$ 0.35 IU/mL (n=117)	p
Age, median (range)	11 (9-15)	10 (8-15)	<0.001 ^a
Sex, male, n (%)	71 (51.8%)	59/116 (50.9%)	0.90 ^b
Rural	101 (73.7%)	30 (25.6%)	<0.001 ^b
Asthma	6/133 (4.5%)	13/115 (11.3%)	0.06 ^b
Total IgE, GM (95%CI)	855 (657-1113)	109 (80-147)	<0.001 ^c
Total IgE >500 IU/mL	94 (68.6%)	19 (16.2%)	<0.001 ^b
<i>Ascaris</i> IgE $\geq$ 0.7 IU/mL	98 (71.5%)	20 (14.6%)	<0.001 ^b
Dust mite IgE $\geq$ 0.7 IU/mL	24 (17.5%)	10 (8.5%)	0.04 ^b
Cockroach IgE $\geq$ 0.7 IU/mL	68/134 (50.7%)	18/116 (15.5%)	<0.001 ^b

^a compared with Student's T test^b compared with Fisher's exact test^c compared with Mann-Whitney U test

FIGURE LEGENDS

Fig E1. Comparison of **(A)** prevalence, **(B)** levels, and **(C)** levels in relation to total IgE, for α -Gal sIgE in children with or without recurrent wheeze in Ecuador and Kenya. Prevalence values were compared using χ^2 . Levels of α -Gal sIgE were expressed as geometric mean of positives (95% CI) and compared by the Mann-Whitney U test, * $p < 0.05$, NS – $p > 0.05$.

Fig E2. Relationship of α -Gal sIgE with serologic markers of three parasites prevalent in Esmeraldas: **(A)** α -Gal sIgE and Malaria IgG/IgM in children from Ecuador ($n=123$) and children with α -Gal syndrome (AGS) in the USA ($n=15$). **(B)** α -Gal sIgE and *Strongyloides* IgG/IgM in children from Ecuador ($n=123$) and children with AGS in the USA ($n=15$). **(C)** α -Gal sIgE and *Toxocara* IgG in children from Ecuador ($n=516$) r_s – Spearman's rank correlation. For α -Gal sIgE dotted lines reflect threshold of detection. For the three representative parasites the dotted line reflects the calibrator cut-off distinguishing positive and negative values.

Fig E3. **(A)** *Ascaris* sIgE versus α -Gal sIgE in children from Kenya ($n=254$). **(B)** Relationship between α -Gal sIgE prevalence (bars, left y-axis) and levels (scatter plot, right y-axis) in the cohort when stratified based on *Ascaris* sIgE status, where $n \leq 0.35$ IU/mL = 117 and $n > 0.35$ IU/mL = 137

Fig E4. Total IgE versus α -Gal sIgE in children from **(A)** Ecuador ($n=599$) and **(B)** Kenya ($n=254$) with Spearman's correlations (r_s).

Fig E5. Relationship between α -Gal sIgE prevalence (bars, left y-axis) and α -Gal levels as a percentage of total IgE (scatter plot, right y-axis) in the cohort when stratified by quintiles of total IgE in **(A)** Ecuador and **(B)** Kenya.

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