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Conflict of interest

None

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Abstract

Background: IgE to galactose alpha-1,3 galactose (alpha-gal) causes alpha-gal syndrome (AGS) delayed anaphylaxis after ingestion of mammalian meat. Development of sensitization has been attributed to tick bites, however the possible role of other parasites has not been well studied.

Objectives

We assessed presence, relative abundances, and site of localisation of alpha-gal containing proteins in common ecto- and endo-parasites endemic in a high AGS prevalence area. We investigated the ability of ascaris antigens to elicit a reaction in a humanised rat basophil in-vitro sensitisation model.

Methods: Total IgE, *Ascaris*-specific IgE, and alpha-gal IgE were measured in sera of challenge-proven AGS patients and non-allergic controls. Presence, concentration and localisation of alpha gal was assessed in parasites by ELISA, Western blotting and

Immunohistochemistry (IHC). The ability of *A. lumbricoides* antigen to elicit IgE dependent reactivity was demonstrated using the RS-ATL8 basophil reporter system.

Results: Alpha-gal IgE correlated with *A. lumbricoides*-specific IgE. Alpha-gal protein at 70-130kDa was detected in *A. lumbricoides* at concentrations higher than those found in *Rhipicephalus evertsi* and *Amblyomma hebraeum* ticks. IHC localised alpha-gal in tick salivary acini and the helminth gut. Non-alpha-gal containing *A lumbricoides* antigens activated RS-ATL8 basophils primed with serum from AGS subjects.

Conclusion: We demonstrate the presence, relative abundances, and site of localisation of alpha-gal containing proteins in parasites. The activation of RS-ATL8 IgE reporter cells primed with serum from AGS subjects on exposure to non-alpha-gal containing A lumbricoides proteins indicates a possible role of exposure to *A. lumbricoides* for alpha-gal sensitisation and clinical reactivity.

Keywords

Alpha-gal, food allergy, Galactose-alpha-1,3-galactose, helminths, meat allergy, red meat allergy, ticks, *Ascaris lumbricoides*, anaphylaxis, *Rhipicephalus evertsi*, *Amblyomma hebraeum*,

Abbreviations used

Alpha-gal: Galactose-alpha-1,3-galactose

IHC: Immunohistochemistry

E/S: Excretory-secretory

BSA: Bovine Serum Albumin

GSI-B4: Griffionia simplicifolia isolectin B4

BTG: Bovine Thyroglobulin

ConA: ConcavaIlin

Capsule Summary

An abundance of alpha-gal is found in *A. lumbricoides, A. hebraeum* and *R. evertsi*. Basophils primed with serum from allergic subjects are activated by non-alpha-gal containing *A. lumbricoides* antigens.

Clinical Implications

These data indicate a possible causal role of exposure to *A. lumbricoides* for alpha-gal sensitisation and elicitation of clinical reactivity.

INTRODUCTION

Galactose-alpha-1,3-galactose (alpha-gal), an oligosaccharide antigen found in non-primate mammalian cells (1,2), causes delayed anaphylaxis to mammalian meat (3,4). Despite wide variation in alpha-gal-IgE levels in patients with clinical reactivity to meat and the presence of sensitisation to alpha-gal without clinical meat allergy in some populations (5), clinical reactivity to meat is associated with higher levels of specific-IgE (sIgE) against alpha-gal and a higher anti-alpha-gal sIgE: total IgE ratio. The values above which there is a 95% probability of having meat allergy are alpha-gal sIgE of >5.5kU/L and alpha-gal sIgE: total IgE ratio of 2.12% (6).

The source of IgE sensitisation to alpha-gal is currently regarded as being due to cross-sensitization after being bitten by hard ticks (7–11). Sensitization may be induced by ticks via the presence of mammalian blood in the tick midgut after a blood meal (12), the presence of endogenous tick alpha-gal antigens (including those found in non-fed larval ticks) (13) or alpha-gal producing tick-borne microbial pathogens (14). Recent studies have shown alpha-gal epitopes in the salivary glands of fed *Amblyomma americanum* and *Ixodes scapularis* (15), *Ixodes. ricinus* (9) and *Haemaphysalis longicornis* (16) and the mid-guts of ticks belonging to the *Ixodes* family, particularly *I. ricinus* (12) and *I. scapularis* (15). However, other organisms have not been rigorously examined for the presence of alpha-gal or their ability to induce IgE responses against alpha-gal.

There are other possible sensitising organisms for alpha-gal allergy. Occurrence of sIgE to alpha-gal in individuals in areas endemic to helminths (5,17) suggests sensitization may be a product of more than one candidate agent. Sera from patients with documented helminth infections, however, do not consistently contain IgE antibodies to alpha-gal (18). The complexity of helminth life cycles has led them to evolve life strategies that allow them to evade host immunity. The presence of alpha-gal on some helminth somatic and excretory-secretory (E/S) antigen glycans (19–21) suggests the use of the alpha-gal epitope as a form

of molecular mimicry. However, the role of helminths in sensitizing the human host to alpha-gal is not well defined.

In this study we examined local endo- and ecto-parasites for the presence, site and relative abundance of alpha-gal containing proteins, to assess the relationships between clinical reactivity and serological markers of *A. lumbricoides* sensitisation as well as the ability of *A. lumbricoides* antigens to induce a response in RS-ATL8 IgE reporter cells primed with serum from allergic subjects in comparison to non-allergic control serum.

METHODS

Participants

Subjects with alpha-gal allergy and controls were identified in the Mqanduli district, Eastern Cape province, South Africa as previously described.(22) Briefly, participants with a history of symptoms of adverse reactions to red meat were enrolled along with participants with no history of adverse reaction to red meat and who were regularly consuming meat. Investigators completed questionnaires assessing demographics of the participants, clinical symptoms, and a history of exposure to tick bite, scabies or parasites endemic to the area. Blood samples were collected from all participants to test for total IgE and specific IgE antibodies (ImmunoCAP® Phadia) to alpha-gal antigen and *A. lumbricoides* spp. Sera were analysed using an Immunocap100 (ThermoFisher Scientific) which has a lower detectable level of 0,1 kilounits per millilitre. Participants with a history of adverse reactions to meat and who were sensitised to alpha-gal were invited for food challenge to cooked beef sausage performed as described previously (22). Both subjective and objective symptoms were recorded as participants were observed for at least 8 hours from ingestion and, if a reaction occurred, for at least 2 hours after the reaction resolved. Subjective symptoms were defined as severe and persistent abdominal cramping only, and objective symptoms as abdominal pain, vomiting,

diarrhoea, scratching, hives, erythema, angioedema and severe reactions such as respiratory problems and hypoperfusion (23). Patients with subjective symptoms only, were classified as alpha-gal allergic (alpha-gal subjective) and those with any objective symptoms as alpha-gal allergic (alpha-gal objective). The study was approved by the Human Research Ethics Committee of the University of Cape Town (174/2017) and informed consent, parental consent, and assent were obtained from all participants.

Antigen acquisition and preparation

Ticks were collected from livestock (predominantly cows, sheep, pigs, donkeys, and mules) in Mqanduli district, Eastern Cape. Ticks were identified to species level using the standard veterinary taxonomy classification key for South Africa (24). 6 species of hard ticks (Ixodidae) from 3 genera of *Amblyomma* (1 species: *A. hebraeum*), *Rhipicephalus* (4 species: *R. appendiculatus, R. decoloratus, R. evertsi* and *R. microplus*) and *Haemaphysalis* (1 species: *H. elliptica*) were identified. *A. hebraeum* and *R. evertsi* were the most frequently detected species.

Adult *A. lumbricoides* worms and *Echinococcus. granulosus* cyst wall and cyst fluid were obtained from patients from the Red Cross War Memorial Children's Hospital (Cape Town, South Africa). All organisms were stored in sterile conditions at -80°C until used. Pork kidney procured from a commercial butchery was used as a positive control, and a boiled whole hen's egg as a negative control.

Somatic antigen was prepared from *A. lumbricoides* (adult worm)*, Nippostrongylus brasiliensis* (third larval stage - L3)*, Taenia crassiceps* (L3) *Echinococcus* sp., adult ticks, pork kidney, and boiled egg. Tissue was homogenized in RIPA buffer (Merck) and then centrifuged at 2000 x g for 30 mins at 4°C. Supernatants were then used for subsequent analysis.

175 *A. lumbricoides* E/S proteins were obtained by maintaining live adult *A. lumbricoides* in DMEM
176 with 1% Pen-strep (ThermoFisher Scientific), 1% L-glutamine (Merck), 1% Gentamycin
177 (ThermoFisher Scientific) and 1% Glucose (w/v). Live adult *A. lumbricoides* were maintained
178 at 37°C. Media were harvested every three days. E/S proteins were concentrated using an
179 Amicon ultraconcentrator (Merck) and re-suspended in 5 mL of PBS.

180 BCA protein estimation kit (Pierce, Rockford) determined protein concentration of all antigens
181 which were then stored at a standard concentration of 500 µg/mL at -80 °C.

182

183 **Detection of alpha-gal glycoprotein**

184 Alpha-gal was detected by incubating antigen coated ELISA plates with anti-alpha-gal chicken
185 single chain variable fragment (scFv) antibody (1:5000) (25). Biotin anti-6-His Epitope Tag
186 antibody (BioLegend) diluted in 1% BSA (Carl Roth; fraction V mol biol grade) in PBS/0.05%
187 Tween-20 (PBS-T) was added to plates followed by Streptavidin Horseradish Peroxidase
188 (Strep-HRP) (BioLegend) incubation. As validation, biotinylated *Griffonia simplicifolia* isolectin
189 B4 (GSI-B4) (Merck) at a dilution of 1:5000 was also added to another set of antigen coated
190 plates.

191 Forty µg/mL of each antigen preparation were separated on a 10% SDS-PAGE gel at a voltage
192 of 120-150V, and transferred to a 0.4µm nitrocellulose membrane (Bio-Rad) (80V for 2 hours).
193 Protein transfer was validated by Ponceau S staining. The nitrocellulose membranes were
194 blocked with 3% BSA (Carl Roth) in PBS at room temperature then incubated with anti-alpha-
195 gal chicken scFv antibody (1:5000). Detection of anti-alpha-gal chicken scFv binding to alpha-
196 gal was by Biotin anti-6His tag (1:5000) and Strep-HRP conjugate (1:5000). GSI-B4 lectin was
197 also used to detect alpha-gal and visualised using a Strep-HRP conjugate.

198 Quantification of alpha-gal glycosylated protein on 40 µg/mL of antigen was achieved by using
199 Galα(1,3)Galβ(1,4)GlcNAc-HSA (Dextra Laboratories, UK) as a standard. Densitometric

analysis of immunoblots was carried out and normalised with total protein on the loading control. Experimental data shown is representative of mean ± s.d. from 4 independent experiments.

Alpha-gal inhibition assay

Inhibition of IgE binding to *A. lumbricoides* somatic antigen by 2 mg/mL bovine thyroglobulin(26) (BTG; Merck) was determined by ELISA. F96 Maxisorp ELISA plates (ThermoFisher Scientific/Nunc Roskilde, Denmark) were coated overnight at 4°C with 50 µL of 50 µg/mL *A. lumbricoides* somatic antigen in 50 mM carbonate buffer pH 9.6. Plates were washed with PBS-T and blocked with 5% BSA (Carl Roth, Germany) in PBS. Plates were incubated with 50 µL 1:10 sera diluted in PBS in the presence or absence of 2 mg/mL BTG and incubated at 37°C for a further 90 min in triplicates. Fifty µL of anti-human-IgE-HRP (Abcam, UK), diluted 1:500 in PBS-T with 5% BSA were added and incubated at 4°C. Following the final washing step, 50 µL of 1-Step Ultra TMB-ELISA (ThermoFisher Scientific) were added and the plates incubated for 90 min at 37°C. Reactions were stopped by addition of 50 µL 2M sulfuric acid and absorbance measured at 450 nm in a spectrophotomer (CLARIOstar Plus, Germany).

Immunolocalization of alpha-gal

Ticks and *A. lumbricoides* were preserved in 4% formaldehyde prior to embedding in paraffin wax blocks for staining. Cut sections (5-7µm) were hydrated in varying concentrations of alcohol and blocked with 3% H_2O_2 for 15 minutes. 0,1M citrate buffer (pH 6) was used for antigen retrieval for 2 minutes in a pressure cooker. Sections were blocked with BSA. Additional blocking with biotin was required for *A. lumbricoides*. (Carl Roth, Germany). Staining for alpha-gal was performed by adding anti-alpha-gal chicken scFv antibody (1:200)

on wax embedded *A. lumbricoides* and ticks cut sections in an overnight incubation at 4°C. Detection of binding was performed by sequentially adding Biotin-labelled anti-6His tag as a secondary antibody (1:1000), Strep-HRP (1:400). For ticks, VIP substrate (Vector laboratories) was used for visible color development and methylene green for counterstaining whereas *A. lumbricoides* slides were treated with DAPI then counterstained with hematoxylin.

Cell Culture

RS-ATL8 cells (27) were cultured in Eagle's Minimum Essential Medium (Merck) supplemented with 10% heat inactivated Foetal Bovine Serum (Merck), 2 mM L-glutamine (Merck) and Penicillin/Streptomycin (100 U/mL and 100 µg/mL, respectively; both ThermoFisher Scientific) in a T-75 flask (Nunc Easy flask, ThermoFisher Scientific). Details can be found in Wan et al., (28).

Serum Samples

Serum samples were heat inactivated at 56°C for 5 minutes to avoid cytotoxicity and added to the cell suspension at 1:100 dilution factor. This heat treatment inactivates the complement without affecting the ability of IgE to bind to the FcεRI receptor.

Recombinant expression and purification of Ascaris allergens

Two *A. lumbricoides* antigens Asc l 1 (29) (UniProt O46207) and Asc l 3 (30) (UniProt. C0L3K2) were expressed in HEK293-6E suspension cell culture system. Both coding sequences were synthetically produced by GeneArt (ThermoFisher Scientific) with NheI/BamHI restriction sites flanking the CDS for direct cloning into pTT28 vector (National

Research Council - NRC Canada, NRC patent file 11266) for extracellular recombinant expression of C-terminal His-tagged proteins. Codons were optimized for expression in human cells. All final constructs were confirmed by DNA sequencing.

The transfection of the cells was performed according to the protocol described by Durocher et al., (31). Briefly, 2µg of purified vector (pTT28-Asc I antigens) per 10^6 cells were used for transfection of suspension HEK293-6E cells using 25 kDa branched polyethylenimine (PEI) from Polysciences (Warrington, PA) in 3:1 (PEI:DNA) ratio. 24 hours after transfection, the cells were stimulated with 0.5% (w/v) Tryptone N1 of the total volume of the culture and incubated at 37°C in a humidified incubator under constant shaking for three days. The cell culture was harvested and after centrifugation the medium supernatant was collected and filtered before purification by affinity chromatography using HisTRAP-HP column in ÄKTA Start (Cytiva). Purified proteins were quantified by BCA assay (Pierce BCA Protein Assay Kit). Purity was assessed by SDS-PAGE and in-frame expression by Western Blotting with an anti-HisTag antibody.

Humanised Rat Basophilic Leukaemia Cell Line RS-ATL8 Assay

Cell Sensitization: RS-ATL8 cells were resuspended in medium to a concentration of 1x10^6 cells/mL. The cells were sensitized with 5 min heat-inactivated serum at a 1:100 dilution. 50 µL of the sera and the cell suspension were added to NUNC white-96 well plate (ThermoFisher Scientific) and incubated in a humidified incubator at 37˚C and 5% CO_2 for 18-20 h.

Controls and Allergens Stimulation: The next day, the medium was removed, and the cells were washed once with PBS. Fifty µL of each of the following conditions using phenol red free MEM medium (ThermoFisher Scientific) were added to the appropriate wells in triplicates: negative control (cells sensitized with serum, but unstimulated); the positive controls 10 µg/mL Concanavalin A (Merck) and 1 µg/mL polyclonal goat anti-human IgE antibody (Merck); the

test samples contained 10 µg/mL *A. lumbricoides* E/S protein or the recombinant allergens of Asc l 1 and Asc l 3 both at 1 µg/mL and incubated for 3.5 hours in a humidified incubator at 37°C and 5% CO_2. Using the ONE-Glo Luciferase Assay System (Promega), 50 µL of ONE-Glo Reagent was added to each well. The luminescence was then measured using a CLARIOstar plus multimode microplate reader.

Statistical analysis

Analysis of antibody levels and a history of exposure to common parasites between alpha-gal allergy cases and controls as well as cases stratified by objective and subjective symptoms was done in Stata v15 (StatCorp, Texas, USA). Mann-Whitney tests were used to test the difference in the numerical exposure variables while Fisher's exact test was used for the categorical versions since the continuous data was skewed and the large sample size assumption was not met, respectively. Graphical presentation of the serum IgE levels was done using R core team (2020) using a log base 10 transformation to exclude all the zero values. Correlation analysis was done using Pearson correlation coefficient (r) to measure the linear correlation between serum IgE levels.

The differences between relative abundance of alpha-gal glycosylated proteins was carried out in Stata v15 by one-way analysis of variance (ANOVA) and post-hoc analysis with Bonferroni correction. A Kruskal-Wallis test followed by Mann Whitney U test was carried out so as to analyse luminescence data in GraphPad Prism version 8. A p-value of less than or equal to 0.05 was considered statistically significant for all analyses. In graphs, asterisks were used to denote significance as follows: * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$.

RESULTS

Exposure to *A. lumbricoides* associates with detection of alpha-gal sIgE

Allergic patients were identified predominantly by positive food challenge (81 positive cases, from 114 cases screened), and 3 by extremely high levels of alpha-gal specific IgE>150 kU/L and a history of recent severe reaction. Alpha-gal IgE ranged between 0.7 and 344.5 kU/L. Alpha-gal:total IgE ratio ranged from 0.1% to 67.6%. Of twenty-six control participants who were regularly consuming red meat, half had no alpha-gal sensitisation and did not undergo a food challenge. Half had positive alpha-gal IgE and underwent an oral food challenge showing no reaction.

Alpha-gal-specific IgE and *A. lumbricoides* -specific IgE (and Alpha-gal-specific IgE/ total IgE ratio and Ascaris-specific IgE/total IgE ratio) were both significantly higher in cases than controls (Table 1). *A. lumbricoides* sIgE also associated with alpha-gal sIgE with a moderate level correlation in cases (r=0.42; p=0.007) and in controls (r=0.53; p=0.091; Figure 1). The correlation co-efficient was slightly higher in controls than cases and the lower p-value is a function of lower numbers. Despite this, there are some subjects that have significant amounts of anti-alpha-gal IgE with no detectable serum activity against *A. lumbricoides* (Figure 1).

Alpha-gal is detected in *A. lumbricoides* and regionally endemic tick spp.

Detection of alpha-gal was established by Western blotting of tissue from *A. lumbricoides*, other parasitic helminths and regionally endemic ticks and positive (pork kidney) and negative (boiled egg) controls (Figure 2A and Supplementary Figure E1 A and B). This demonstrated the presence of alpha-gal in pork kidney, *A. lumbricoides*, *A. hebraeum,* and *R. evertsi*. Protein bands positive for alpha-gal when probed with anti-alpha-gal chicken scFv were detected between 70-200kDa in all positive samples (Figure 2A and Figure E1B) with bands >250kDa also detected in *A. lumbricoides* and male ticks. Fewer bands were detected in female ticks of both species, and these were between 70 and 130 kDa. In *A. lumbricoides* a prominent band around 100kDa was also detected along with multiple bands between 130 and >250kDa. Bands between 70 to 130kDa positive for alpha-gal were detected in pork kidney (32).

Semi-quantitative estimation of alpha-gal concentration established by densitometry (Figure 2B) and ELISA (Figure 2C) detected a higher concentration of alpha-gal in pork kidney and *A. lumbricoides* in comparison to tick extracts. Densitometric analysis identified a raised relative abundance of alpha-gal glycosylated protein in all sensitising organisms, and pork kidney (p-value=0.002) (Figure 2B). *A. lumbricoides* alpha-gal abundance was higher than that of both tick species (5.34±0.61 vs 3.38±2.19; p =0.10). There was a marked higher concentration of alpha-gal in males than female *A. hebraeum* ticks (p=0.006) and a smaller, non-significant difference in male vs female *R. evertsi* (Figure 2B and 2C).

Alpha-gal was detected only in somatic antigen, but not in E/S antigen of adult *A. lumbricoides* (Figure 2D and 2E), indicating any potential source of alpha-gal from *A. lumbricoides* is not secreted.

To identify whether binding by IgE to *A. lumbricoides* somatic antigen is dependent on the presence of alpha-gal in the *A. lumbricoides* somatic antigen, we used a bovine thyroglobulin inhibition assay (33). In most cases, co-incubation with 2 mg/mL BTG did not lead to pronounced changes of IgE binding to *A. lumbricoides* somatic extract (50 µg/mL). However, a small subset of sera showed strong inhibition of IgE binding in the presence of BTG to very close or below the background levels suggesting that in these patients, a large proportion of IgE was recognizing alpha-gal rather than other (protein) allergens in *A. lumbricoides* somatic extract (Figure 2F).

Immunolocalization of alpha-gal in *A. lumbricoides* and regionally endemic tick spp.

The anatomical sites where alpha-gal could be detected in *A lumbricoides,* male *A. hebraeum* and *R. evertsi,* and female *R. evertsi* ticks was established by immune-histochemical probing (Figures 3 and 4). Negative control, staining with secondary antibody only, showed no binding in *A. lumbricoides* (Figure 3E) or tick species (Figure 4 A-D). In *A. lumbricoides* tissue, alpha-

gal was detected along the intestinal lining after probing with either anti-alpha-gal chicken scFv or GSI-B4 lectin (Figures 3C and D). GSI-B4 lectin also detected signal in gonads, eggs, and the pseudocoel (Figure 3D).

In ticks, alpha-gal was detected by anti-alpha-gal chicken scFv in salivary acini close to the tick's mouthpiece (Figure 4A-D). Staining with GSI-B4 lectin showed staining of salivary acini and secreted alpha-gal in the lumen of the ticks (Figures 4A-C). In female *A. hebraeum* sections, alpha-gal was detected in the gastric lumen after probing with anti-alpha-gal scFv chicken antibody (Supplementary Figure E3).

RS-ATL8 Assay

To identify the potential for *A. lumbricoides* antigens to induce an allergic reaction we assessed the ability of both native *A. lumbricoides* E/S and recombinant *A. lumbricoides* allergens to induce luciferase activity in a humanized IgE basophil reporter system RS-ATL8 (27) (28) primed with serum from patients with and without alpha gal-allergy.

As expected, there were no differences between the groups in the negative control (Figure 5A) or the two positive controls (Figure 5B and C). However, luciferase responses were significantly higher to the rAsc l 1 antigen in the "alpha-gal objective" patient group compared to both the "alpha gal negative" patient group and the "alpha gal subjective" patient group (Figure 5E). With native *A. lumbricoides* E/S antigen and rAsc l 3 antigens, there was a significant higher response in the "alpha gal objective" group when compared to those without alpha gal allergy, but not between any other groups (Figure 5 D and F).

DISCUSSION

In this study, we identify a relationship between exposure to the common helminth *A. lumbricoides* and alpha-gal allergy in a well-defined cohort of individuals with challenge-proven alpha-gal syndrome in South Africa. We also detected higher concentrations of alpha-gal in *A. lumbricoides* compared to local tick species; a typical source of sensitization to alpha-gal.

Our identification of raised alpha-gal-specific IgE and *A. lumbricoides*-specific IgE (and Alpha-gal-specific IgE/total IgE ratio as well as *A. lumbricoides*-specific IgE/total IgE ratio) being significantly higher in subjects with alpha-gal allergy than subjects without, presented infection/ exposure to *A. lumbricoides* as source of sensitisation to alpha-gal. Frequent moderate level sensitisation to alpha-gal, even in the absence of clinical reactivity to meat, has been identified in similar studies from areas endemic to helminth infections in Kenya (17) and Zimbabwe (5).

A. lumbricoides sIgE was significantly associated with alpha-gal IgE with a moderate level correlation in cases and in controls. The higher concentration of *A. lumbricoides* antibodies in alpha-gal allergic cases than controls could thus be due to two mechanisms. Since we have shown alpha-gal epitopes in *A. lumbricoides*, subjects with primary alpha-gal sensitisation could also be positive on *A. lumbricoides* ImmunoCAP, similar to that seen with ImmunoCAPs to whole extracts of beef, pork and other mammalian meats. However, this explanation cannot be true as this would then be the case in all patients and cannot account for the patients with significantly raised IgE to alpha-gal and no detectable cross-binding to *A. lumbricoides* extract on the ImmunoCAP. Alternatively, this may reflect higher exposure to *A. lumbricoides* in those with alpha-gal allergy, implying that in addition to ticks, exposure to *A. lumbricoides* may be a causative factor in causing sensitisation in a subset of subjects.

Moreover, we present data showing that *A. lumbricoides* E/S antigens and rABA-1 antigen are capable of inducing an allergic cellular response in the presence of serum from patients with alpha-gal allergy. Neither *A. lumbricoides* E/S nor recombinant ABA-1 contain alpha-gal

moieties, as the E/S did not demonstrate any alpha-gal in blotting experiments (Figure 2D and 2E) and the recombinant allergen was produced in human HEK293-6E cells, which do not possess the enzymatic machinery to produce alpha-gal. This suggests that the activation seen in the RS-ATL8 luciferase reporter assay is indicative of true exposure to *A. lumbricoides* (i.e. past and/or current infection), rather than being caused by cross-reactivity with alpha-gal. This further strengthens the likelihood of a causal link between *A. lumbricoides* infection and sensitization to alpha-gal. Together, these findings present infection with *A. lumbricoides* as a new potential source of sensitization to alpha-gal.

Implication of ticks as the source of sensitization leading to the production of sIgE against red meat has been shown in various locations worldwide (17). However, alpha-gal IgE has been shown in places with no reported tick bites, and in places where there is no alpha-gal allergy. In this study, participants reported exposure to ticks, scabies, and helminths. The self-reported exposure to different parasites did not differ in individuals with alpha-gal allergy and the controls(22), whereas in European and North American cohorts a closer association with self-reported exposure to ticks is seen (4).

To investigate potential sources of sensitisation in this cohort we tested endemic parasites for the presence of alpha-gal epitopes. As expected, the presence of the alpha-gal epitope in both male and female *A. hebraeum* and *R. evertsi* was found but with higher concentrations in *A. hebraeum*, particularly the males. *A. hebraeum* (“bont tick”), is a three-host tick whose definitive host are large mammals such as cattle, goats and sheep (34). It is also commonly reported to feed on humans. *R. evertsi* (red-legged tick) is a two-host tick of domesticated animals placing it in proximity with humans. Endogenous production or exogenous sources of alpha-gal for these tick species are yet to be determined, as the specimens used had already fed on mammalian hosts that may be their source of alpha-gal. However, localization of the alpha-gal in salivary acini organized around a lumen into which the saliva is excreted (Figure

4D) suggests endogenous alpha-gal production. The staining also localized to specific cell types in an acinus. Staining of vesicles associated with large vacuole supports secretion of glycosylated granules during feeding. In *Ixodes ricinus*, tick saliva has been shown to contain IgE-binding alpha-gal carrying proteins, which by allergenomics were revealed to be vitellogenins (35). The expression of the alpha-gal transferase genes b4galt7, a4galt-1, and a4galt-2 by some tick species and the induction of the gene transcription during feeding (36) suggests that elevated corresponding protein levels during feeding results in a higher level of alpha-gal bearing proteins. Immunoblotting showed the presence of more than one positive protein band as having an alpha-gal epitope (Figure 2A), concentrated in salivary acini (Figure 4). This suggests the possibility of multiple glycosylated proteins induced by feeding in tick saliva.

The occurrence of alpha-gal as a terminal carbohydrate moiety has been described in *Parelaphostrongylus tenuis* (20), *Haemonchus contortus* (19), and *Echinococcus granulosus* (21), but has not been hitherto shown in *A. lumbricoides*. In this study, we identified the occurrence of high amounts of alpha-gal in *A. lumbricoides*, very low levels in *E. granulosus*, in agreement with existing findings (21) and no binding in *T. crassiceps* and *N. brasiliensis* (Supplementary Figure E1 A and B). In our study, worms were isolated from a non-alpha-gal-producing human host. The role of alpha-gal or its source in *A. lumbricoides* is unknown. The presence of alpha-gal in *A. lumbricoides* suggests the possibility of this agent to sensitise the human host to alpha-gal. *A. lumbricoides* is an orally introduced parasite in which antigen sampled from the lumen may be transported to the mesenteric lymph nodes promoting induction of T- regulatory cells (37). However, the larval stage of A. *lumbricoides* in which tissue penetration, lung invasion and recurrence of gut infestation occurs, may also contribute to sensitization.

Our data localised alpha-gal in adult *A. lumbricoides* intestinal lining, gonads, and eggs (Figure 3D). Eggs are released from the female covered by a light brown, mamillated, albuminous outer coat, which may not be accessible to immune cells and cause sensitization. *A.*

lumbricoides eggs may also be secreted in their decorticated form (38) which may make it possible for them to cause sensitization. The presence of alpha-gal in the intestinal lining could result from endogenous proteins or from the ingestion of alpha-gal moieties that may be present in the human host's diet.

We have only sought for the presence of alpha-gal containing glycoproteins (given the methodology used) rather than glycolipids. An *in vitro* model of alpha-gal transport across an electrically tight Caco-2 monolayer (as a model of the gut epithelium), showed only alpha-gal containing lipids, but not proteins carrying the same moiety, were able to cross the epithelial cell layer (39). Assuming these findings reflect the *in vivo* context, this may point to a role of alpha-gal containing lipids, rather than glycoproteins, in inducing sensitization to red meat, perhaps also explaining the delayed allergic reaction in patients with clinical manifestations. Most recently, Iweala and co-authors demonstrated the capacity of alpha-gal bearing lipids to activate human basophils in an IgE-dependent manner (40).

In both ticks and helminths, GSI-B4 lectin showed more staining (Figures 3D and 4A-D) and higher arbitrary values (Supplementary Figure E2) than the anti-alpha-gal chicken scFv antibody. This may be an observation of potential lectin promiscuity with the GSI-B4 lectin known to interact with a range of terminal galactose structures (Gal-α1,3-R, Gal-α1,2-R and Gal-α1,4-R) (41). However, the anti-alpha-gal chicken scFv antibody used for profiling has been demonstrated to have specificity for the Gal-α1,3-Gal structure (25). The structurally similar glycan, Gal(α1-3)GalNAc(β1-4)GlcNAc on glycosphingolipids of *Ascaris suum* adults (42) may cause cross-reactivity and be mistaken for alpha-gal using the GSI-B4 lectin but not the scFv antibody.

CONCLUSION

Our data show a significant positive correlation between anti-alpha-gal sIgE and *A. lumbricoides* sIgE in serum of subjects with alpha-gal allergy. There was detection of multiple alpha-gal containing glycoproteins in the gut of *A. lumbricoides* and the salivary acini of the ticks *A. hebraeum* and *R. evertsi*. RS-ATL8 IgE reporter cells primed with serum from allergic subjects resulted in significant activation of basophil luciferase signal when exposed to non-alpha gal containing *A. lumbricoides* antigens in comparison to non-allergic control serum, indicating a possible causal role of exposure to *A. lumbricoides* for alpha gal sensitisation and elicitation of clinical reactivity.

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Figure Legends

Figure 1: Correlations between Ascaris IgE and Alpha-gal IgE in cases and controls

Figure 2: (A)Detection of alpha-gal in lysates from endo- and ectoparasites. (B) Relative abundance of alpha-gal glycosylated proteins calculated through densiometric analysis of alpha-gal-stained bands normalised against synthetic alpha-gal HSA. (C) Detection of alpha-gal in lysates from *A. lumbricoides* and ticks by ELISA (D) SDS-PAGE comparison between adult A. lumbricoides somatic and E/S antigen (E) Immunoblotting comparison between adult *A. lumbricoides* somatic and E/S antigen. (F) Inhibition of patient IgE binding to *A. lumbricoides* somatic extract in the presence of 2 mM bovine thyroglobulin.

Figure 3: Immunolocalization of alpha-gal glycosylated moieties on longitudinal cut sections of an adult female *A. lumbricoides*. Staining of cut sections was performed by (A-B) hematoxylin and eosin, (C) an anti-alpha gal chicken scFv antibody, (D) GSI-B4 lectin and (E) secondary antibody only. Positive staining for alpha-gal resulted in a brown color as shown by a thick black arrow in C and D.

Figure 4: Immunolocalization of alpha-gal glycosylated moieties on longitudinal cut sections of (A) *Rhipicephalus evertsi* female (low resolution), (B) Rhipicephalus evertsi female (high resolution), (C) *Rhipicephalus evertsi* male (high resolution) and (D) *Amblyomma hebraeum* male (high resolution) ticks using anti-alpha-gal chicken scFv antibody and GSI-B4 lectin, and (E) Secondary antibody only.Positive staining was in the form of a purple color as indicated by a thick black arrow.

Figure 5: Luminescence from RS-ATL8 reporter cells after sensitization with sera from alpha-gal negative, alpha-gal objective and alpha-gal subjective patients and subsequent treatments with A) no stimulation as the negative control; stimulation with positive controls B) ConA; C) anti-IgE; D) native *A. lumbricoides* E/S or recombinant allergens E) rAsc l 1 and F) rAsc l 3.

653

654 **Table 1 Description of control vs alpha-gal allergy participants**

Category		Control n=26	Alpha-gal allergy n=84	P-value 655
Exposure, n (%)	Tick bite	1 (3.9)	9 (10.7)	0.29
	Scabies	14 (53.9)	39 (46.4)	0.65
	Worms	8 (30.8)	22 (26.2)	0.63
	Schistosomiasis	2 (7.7)	8 (9.5)	1.00
Alpha-gal IgE kU/L, median (IQR)		0.5 (0–1.2)	12.0 (4.2–33.4)	<0.01
Alpha-gal IgE: total IgE ratio, median (IQR)		0.1 (0-0.4)	4.2 (1.9–11.0)	<0.01
Ascaris IgE kU/L, median (IQR)		0.25 (0.03-1.13)	1.12 (0.47-2.59)	0.01
Ascaris IgE: total IgE ratio, median (IQR)		0.08 (0.05-0.10)	0.17 (0.09-0.31)	<0.001