

# Ascaris lumbricoides and ticks associated with sensitization to galactose $\alpha$ 1,3-galactose and elicitation of the alpha-gal syndrome

Murangi, Tatenda; Prakash, Prema; Moreira, Bernardo Pereira; Basera, Wisdom; Botha, Maresa; Cunningham, Stephen; Facey-Thomas, Heidi; Halajian, Ali; Joshi, Lokesh; Ramjith, Jordache; Falcone, Franco H; Horsnell, William; Levin, Michael E

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1 ***Ascaris lumbricoides* and ticks associated with sensitisation to Galactose  $\alpha$ 1,3-**  
2 **galactose and elicitation of the alpha-gal syndrome.**

3

4 **<sup>1</sup>Tatenda Murangi (Bsc (Hons)), <sup>6</sup>Prema Prakash (PhD), <sup>6</sup>Bernardo Pereira Moreira**  
5 **(PhD), <sup>2,3</sup>Wisdom Basera (MPH), <sup>4</sup>Maresa Botha (MD), <sup>5</sup>Stephen Cunningham (PhD), <sup>2</sup>**  
6 **Heidi Facey-Thomas (DipNursCert), <sup>7</sup>Ali Halajian (PhD), <sup>5</sup>Lokesh Joshi (PhD), <sup>8</sup>**  
7 **Jordache Ramjith (MSc), <sup>6</sup>Franco H. Falcone (PhD) \*, <sup>1,9</sup>William Horsnell (PhD) \*, <sup>4</sup>**  
8 **Michael E. Levin (PhD) \***

9

10 <sup>1</sup>Wellcome Centre for Infectious Diseases Research in Africa, Institute of Infectious Disease  
11 and Molecular Medicine, Department of Pathology, Division of immunology, University of  
12 Cape Town, Cape Town, South Africa.

13 <sup>2</sup>School of Public Health and Family Medicine, University of Cape Town, South Africa

14 <sup>3</sup>Burden of Disease Research Unit, South African Medical Research Council, South Africa

15 <sup>4</sup>Division of Paediatric Allergy, Department of Paediatrics, University of Cape Town, Cape  
16 Town, South Africa

17 <sup>5</sup>Glycoscience Group, Biomedical Sciences, National University of Ireland Galway, Ireland

18 <sup>6</sup>Institute of Parasitology, Biomedical Research Center Seltersberg (BFS), Justus Liebig  
19 University Giessen, Giessen, Germany.

20 <sup>7</sup>Research Administration and Development, University of Limpopo, Sovenga, 0727, South  
21 Africa

22 <sup>8</sup> Department for Health Evidence, Biostatistics Research Group, Radboud Institute for  
23 Health Sciences, Radboud University Medical Center, Nijmegen, Netherlands

24 <sup>9</sup>Institute of Microbiology and Infection, College of Medical and Dental Sciences, University of  
25 Birmingham, Birmingham, UK.

26

27 \* Joint senior authors

28

29 Corresponding author: Prof. Michael Levin

30 Room 516, ICH building, Red Cross Children's Hospital, Klipfontein Road

31 Cape Town, South Africa

32 Tel: +27 21 658 5111 | Mobile: +27 824680989

33 Email: [Michael.levin@uct.ac.za](mailto:Michael.levin@uct.ac.za)

34

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36 None

37

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49

50 **Abstract**

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

55 **Objectives**

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

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

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

65

66 **Results:** Alpha-gal IgE correlated with *A. lumbricoides*-specific IgE. Alpha-gal protein at 70-  
67 130kDa was detected in *A. lumbricoides* at concentrations higher than those found in  
68 *Rhipicephalus evertsi* and *Amblyomma hebraeum* ticks. IHC localised alpha-gal in tick salivary  
69 acini and the helminth gut. Non-alpha-gal containing *A lumbricoides* antigens activated RS-  
70 ATL8 basophils primed with serum from AGS subjects.

71

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

77

## 78 **Keywords**

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

82

## 83 **Abbreviations used**

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

85 IHC: Immunohistochemistry

86 E/S: Excretory-secretory

87 BSA: Bovine Serum Albumin

88 GSI-B4: Griffonia simplicifolia isolectin B4

89 BTG: Bovine Thyroglobulin

90 ConA: Concanavallin

91

## 92 **Capsule Summary**

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

96

## 97 **Clinical Implications**

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

## 100 INTRODUCTION

101 Galactose-alpha-1,3-galactose (alpha-gal), an oligosaccharide antigen found in non-primate  
102 mammalian cells (1,2), causes delayed anaphylaxis to mammalian meat (3,4). Despite wide  
103 variation in alpha-gal-IgE levels in patients with clinical reactivity to meat and the presence of  
104 sensitisation to alpha-gal without clinical meat allergy in some populations (5), clinical  
105 reactivity to meat is associated with higher levels of specific-IgE (sIgE) against alpha-gal and  
106 a higher anti-alpha-gal sIgE: total IgE ratio. The values above which there is a 95% probability  
107 of having meat allergy are alpha-gal sIgE of >5.5kU/L and alpha-gal sIgE: total IgE ratio of  
108 2.12% (6).

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

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

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

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

133

## 134 **METHODS**

### 135 **Participants**

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



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

157

### 158 **Antigen acquisition and preparation**

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

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

171 Somatic antigen was prepared from *A. lumbricoides* (adult worm), *Nippostrongylus brasiliensis*  
172 (third larval stage - L3), *Taenia crassiceps* (L3) *Echinococcus* sp., adult ticks, pork kidney, and  
173 boiled egg. Tissue was homogenized in RIPA buffer (Merck) and then centrifuged at 2000 x g  
174 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

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

203

#### 204 **Alpha-gal inhibition assay**

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

217

#### 218 **Immunolocalization of alpha-gal**

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

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

230

### 231 **Cell Culture**

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

237

### 238 **Serum Samples**

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

242

### 243 **Recombinant expression and purification of Ascaris allergens**

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

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

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

262

### 263 **Humanised Rat Basophilic Leukaemia Cell Line RS-ATL8 Assay**

264 *Cell Sensitization:* RS-ATL8 cells were resuspended in medium to a concentration of 1x10<sup>6</sup>  
265 cells/mL. The cells were sensitized with 5 min heat-inactivated serum at a 1:100 dilution. 50  
266 µL of the sera and the cell suspension were added to NUNC white-96 well plate (ThermoFisher  
267 Scientific) and incubated in a humidified incubator at 37°C and 5% CO<sub>2</sub> for 18-20 h.

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

273 test samples contained 10 µg/mL *A. lumbricoides* E/S protein or the recombinant allergens of  
274 Asc I 1 and Asc I 3 both at 1 µg/mL and incubated for 3.5 hours in a humidified incubator at  
275 37°C and 5% CO<sub>2</sub>. Using the ONE-Glo Luciferase Assay System (Promega), 50 µL of ONE-  
276 Glo Reagent was added to each well. The luminescence was then measured using a  
277 CLARIOstar plus multimode microplate reader.

278

## 279 **Statistical analysis**

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

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

295

## 296 **RESULTS**

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

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

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

312

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

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

324 Semi-quantitative estimation of alpha-gal concentration established by densitometry (Figure  
325 2B) and ELISA (Figure 2C) detected a higher concentration of alpha-gal in pork kidney and *A.*  
326 *lumbricoides* in comparison to tick extracts. Densitometric analysis identified a raised relative  
327 abundance of alpha-gal glycosylated protein in all sensitising organisms, and pork kidney (p-  
328 value=0.002) (Figure 2B). *A. lumbricoides* alpha-gal abundance was higher than that of both  
329 tick species ( $5.34\pm 0.61$  vs  $3.38\pm 2.19$ ;  $p = 0.10$ ). There was a marked higher concentration of  
330 alpha-gal in males than female *A. hebraeum* ticks ( $p=0.006$ ) and a smaller, non-significant  
331 difference in male vs female *R. evertsi* (Figure 2B and 2C).

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

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

343

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

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



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

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

357

### 358 **RS-ATL8 Assay**

359 To identify the potential for *A. lumbricoides* antigens to induce an allergic reaction we  
360 assessed the ability of both native *A. lumbricoides* E/S and recombinant *A. lumbricoides*  
361 allergens to induce luciferase activity in a humanized IgE basophil reporter system RS-ATL8  
362 (27) (28) primed with serum from patients with and without alpha gal-allergy.

363 As expected, there were no differences between the groups in the negative control (Figure  
364 5A) or the two positive controls (Figure 5B and C). However, luciferase responses were  
365 significantly higher to the rAsc I 1 antigen in the "alpha-gal objective" patient group compared  
366 to both the "alpha gal negative" patient group and the "alpha gal subjective" patient group  
367 (Figure 5E). With native *A. lumbricoides* E/S antigen and rAsc I 3 antigens, there was a  
368 significant higher response in the "alpha gal objective" group when compared to those without  
369 alpha gal allergy, but not between any other groups (Figure 5 D and F).

370

### 371 **DISCUSSION**

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

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

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

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

398 moieties, as the E/S did not demonstrate any alpha-gal in blotting experiments (Figure 2D and  
399 2E) and the recombinant allergen was produced in human HEK293-6E cells, which do not  
400 possess the enzymatic machinery to produce alpha-gal. This suggests that the activation seen  
401 in the RS-ATL8 luciferase reporter assay is indicative of true exposure to *A. lumbricoides* (i.e.  
402 past and/or current infection), rather than being caused by cross-reactivity with alpha-gal. This  
403 further strengthens the likelihood of a causal link between *A. lumbricoides* infection and  
404 sensitization to alpha-gal. Together, these findings present infection with *A. lumbricoides* as a  
405 new potential source of sensitization to alpha-gal.

406

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

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

424 4D) suggests endogenous alpha-gal production. The staining also localized to specific cell  
425 types in an acinus. Staining of vesicles associated with large vacuole supports secretion of  
426 glycosylated granules during feeding. In *Ixodes ricinus*, tick saliva has been shown to contain  
427 IgE-binding alpha-gal carrying proteins, which by allergenomics were revealed to be  
428 vitellogenins (35). The expression of the alpha-gal transferase genes b4galt7, a4galt-1, and  
429 a4galt-2 by some tick species and the induction of the gene transcription during feeding (36)  
430 suggests that elevated corresponding protein levels during feeding results in a higher level of  
431 alpha-gal bearing proteins. Immunoblotting showed the presence of more than one positive  
432 protein band as having an alpha-gal epitope (Figure 2A), concentrated in salivary acini (Figure  
433 4). This suggests the possibility of multiple glycosylated proteins induced by feeding in tick  
434 saliva.

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

448 Our data localised alpha-gal in adult *A. lumbricoides* intestinal lining, gonads, and eggs (Figure  
449 3D). Eggs are released from the female covered by a light brown, mamillated, albuminous  
450 outer coat, which may not be accessible to immune cells and cause sensitization. *A.*

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

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

464 In both ticks and helminths, GSI-B4 lectin showed more staining (Figures 3D and 4A-D) and  
465 higher arbitrary values (Supplementary Figure E2) than the anti-alpha-gal chicken scFv  
466 antibody. This may be an observation of potential lectin promiscuity with the GSI-B4 lectin  
467 known to interact with a range of terminal galactose structures (Gal- $\alpha$ 1,3-R, Gal- $\alpha$ 1,2-R and  
468 Gal- $\alpha$ 1,4-R) (41). However, the anti-alpha-gal chicken scFv antibody used for profiling has  
469 been demonstrated to have specificity for the Gal- $\alpha$ 1,3-Gal structure (25). The structurally  
470 similar glycan, Gal( $\alpha$ 1-3)GalNAc( $\beta$ 1-4)GlcNAc on glycosphingolipids of *Ascaris suum* adults  
471 (42) may cause cross-reactivity and be mistaken for alpha-gal using the GSI-B4 lectin but not  
472 the scFv antibody.

473

474 **CONCLUSION**

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

483

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486

487

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623

## 624 **Figure Legends**

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

626

627

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

635

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

641

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

648

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

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

Category		Control n=26	Alpha-gal allergy n=84	P-value	655
<b>Exposure, n (%)</b>	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	
<b>Alpha-gal IgE kU/L, median (IQR)</b>		0.5 (0–1.2)	12.0 (4.2–33.4)	<0.01	
<b>Alpha-gal IgE: total IgE ratio, median (IQR)</b>		0.1 (0-0.4)	4.2 (1.9–11.0)	<0.01	
<b>Ascaris IgE kU/L, median (IQR)</b>		0.25 (0.03-1.13)	1.12 (0.47-2.59)	0.01	
<b>Ascaris IgE: total IgE ratio, median (IQR)</b>		0.08 (0.05-0.10)	0.17 (0.09-0.31)	<0.001	