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Firdaus Hamid, M.D., PhD, Serge A. Versteeg, Bsc, Apriliano E. Wiria, M.D., PhD, Linda J. Wammes, M.D., PhD, Sitti Wahyuni, M.D., PhD, Taniawati Supali, PhD, Erliyani Sartono, PhD, Ronald van Ree, PhD, Maria Yazdanbakhsh, PhD

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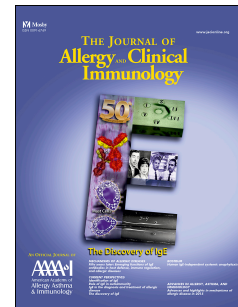
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**Molecular diagnostics and lack of clinical allergy in helminth-endemic areas in
Indonesia**

Firdaus Hamid, M.D., PhD^{a,b}, Serge A Versteeg, Bsc^c, Aprilianto E Wiria, M.D.,
PhD^{b,d}, Linda J Wammes, M.D., PhD^{b*}, Sitti Wahyuni, M.D., PhD^e, Taniawati Supali,
PhD^d, Erliyani Sartono, PhD^b, Ronald van Ree, PhD^{c,##}, Maria Yazdanbakhsh, PhD^{b#}

^aDepartment of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar,
Indonesia

^bDepartment of Parasitology, Leiden University Medical Center, Leiden, The
Netherlands

^cDepartment of Experimental Immunology, Academic Medical Center, University of
Amsterdam, Amsterdam, The Netherlands

^dDepartment of Parasitology, Faculty of Medicine, Universitas Indonesia, Jakarta,
Indonesia

^eDepartment of Parasitology, Faculty of Medicine, Hasanuddin University, Makassar,
Indonesia

^fDepartment of Experimental Immunology and Department of Otorhinolaryngology,
Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

*Currently at Department of Medical Microbiology & Infectious Diseases, Erasmus
Medical Center, Rotterdam, the Netherlands

[#]these authors contributed equally

Corresponding authors:

1. Maria Yazdanbakhsh, Dept. of Parasitology L4-Q, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands, Phone: +31715265067, Fax: +31715266907, Email: m.yazdanbakhsh@lumc.nl
2. Ronald van Ree, Departments of Experimental Immunology and Otorhinolaryngology, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands, Phone: +31205666076, Fax: +31205669756, Email: r.vanree@amc.uva.nl

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decision to publish or preparation of the manuscript. This study would not been possible without enthusiastic cooperation of children, their parents and teachers.

Capsule summary:

Allergen microarray characterization of sensitization to common allergen sources in a helminth-endemic area of Indonesia shows that helminth induced cross-reactivity to cross-reactive carbohydrate determinants (CCD), and not primary sensitization to their major allergens, is the dominant feature.

Key words

Specific IgE, IgE cross-reactivity, microarray, natural and recombinant allergens, helminth infection, cross-reactive carbohydrate determinants, skin prick test, Indonesia

Abbreviations

Th2, T helper 2; IgE, Immunoglobulin-E; SPT, skin prick test; CCDs, cross-reactive carbohydrate determinants; ISAAC, International Study of Asthma and Allergy in Childhood; HDM, house dust mite; ISU/L, ISAC standardized units/Liter; ELISA, Enzyme Linked Immunosorbent Assay; IU/ml, International Units/milliliter; SEA, soluble egg antigen; CI, confidence intervals.

To the editor:

Parasitic helminths and allergens are potent inducers of T helper 2 (Th2) responses that lead to high levels of immunoglobulin (Ig) E [1]. Despite the similar immunological profiles of helminth infections and allergic disorders, several cross-sectional and interventional studies have indicated a negative association between helminth infections and skin prick test (SPT) positivity to allergens [2]. In addition to the possible role of immune-regulatory networks induced by chronic helminth infections, cross-reactive IgE to helminthes and environmental/food allergens with low biological activity may prevent mast cell degranulation [3].

The allergen that results in the induction of the original allergic responses is the primary sensitizer, while others are considered cross-reactive allergens. IgE cross-reactivities can be due to homologous proteins, or to complex N-glycans on plant and invertebrate glycoproteins, known as cross-reactive carbohydrate determinants (CCDs) [4]. The characterization of IgE in helminth-endemic areas has largely been based on allergen extracts. IgE against CCD has a very broad spectrum of cross-reactivity across the plant kingdom, invertebrates such as insects (venoms), and even parasites. Importantly, oral challenge with a CCD-bearing allergen in patients with IgE to CCD proved that CCD-specific IgE is of poor biological activity and has limited, if any, clinical relevance [5]. To fully understand the association between helminths and allergies, it is important to characterize IgE reactivity to allergen components at the molecular level.

The ImmunoCAP-ISAC microarray has recently been developed with individual purified natural and recombinant inhalant and food allergens [6]. This allows testing for individual molecular IgE recognition profiles providing

comprehensive information about likely sources of primary sensitization and cross-reactivity and helps elucidate the process of sensitizations and identify risk and protective factors for clinical phenotypes.

To elucidate the molecular basis of the extremely high prevalence of sensitization to common allergen sources (65% - 85%) amongst Indonesian school children, living in a helminth endemic area, who have very low SPT reactivity and virtually absent clinical allergy, the IgE profiles to allergen components on ImmunoCAP-ISAC chips was studied. The study population selection from the ImmunoSPIN (www.immunospin.org) program [7], and all methods are given in the supplementary information in this article's online repository at www.jacionline.org. Of the study subjects, 93% were infected with soil transmitted helminths (hookworm, *Ascaris lumbricoides* and *Trichuris trichiura*) with high total IgE (Geometric mean 2816 IU/ml). Sensitization to common inhalant and food allergen sources by ImmunoCAP assay was high: grass pollen; *Phleum pratense* (90%), cockroach; *Blattella germanica* (87%), shrimp; *Penaeus aztecus* (79%), HDM; *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) (74%) and peanut; *Arachis hypogaea* (65%) (supplementary table E1 and figure 1). However, SPT reactivity was considerably lower than sensitization assessed by IgE: HDM (*D. pteronyssinus* and/or *D. farinae* (27%), followed by cockroach (24%), peanut (6%) and shrimp (5%) (table E1). Wheeze or eczema in the past 12 months was reported for only 5 children. None of the study subjects reported any symptoms of food allergy.

ImmunoCAP-ISAC microarray results indicated that IgE against commonly accepted major allergens of HDM (*D. pteronyssinus*), cockroach, grass pollen, shrimp and peanut was only observed in 0% to 5% of cases (figure 1, 2 and table E2). However,

many individuals recognized several natural purified glycoprotein allergens from pollen (nCyn d 1, nPhl p 4, nCry j 1, nCup a 1, nOle e 1, nPla a 2) and from food (nJug r 2), all carrying typical CCD glycans (figure 2), reflected in positivity of most plasma to MUXF3 representing a common CCD glycan. IgE responses to these natural glycoproteins and to MUXF3 were highly correlated ($0.60 < r < 0.77$), supporting CCD-based cross-reactivity (figure E1). To further confirm CCD-reactivity, a sub-group of 30 subjects, with sufficient plasma, recognizing natural glycoproteins, was tested for IgE against bromelain which is a glycoprotein carrying the MUXF3 glycan structure. All were positive to bromelain and these responses correlated (figure E2) with IgE to grass pollen and peanut ($r=0.87$ and $r=0.84$, respectively). Association with cockroach and shrimp were still significant ($r=0.53$ and $r=0.48$, respectively), which was not the case for HDM (*D. pteronyssinus*), implying that typical CCD structures can most likely not (fully) explain reactivity to these allergen sources. CCD-specific IgE, titrated ImmunoCAP inhibition assays confirmed CCD-based cross-reactivity was significant for peanut and grass pollen but was limited or absent for cockroach and HDM (*D. pteronyssinus*), respectively (figure E3a). A similar picture emerges upon inhibition with soluble egg antigen (SEA) of *Schistosoma haematobium* (figure E3b). On the other hand, *A. lumbricoides* antigen was a very poor inhibitor of IgE-binding to all four allergen sources (figure E3c). These data indicate that whereas schistosome eggs can be a source of cross-reactive glycans present on grass pollen (and less so on cockroach or HDM (*D. pteronyssinus*)), this is not the case for *A. lumbricoides* adult worms. In general, IgE reactivity to recombinant allergens not carrying CCD-type glycans was very rare ($\leq 5/150$ subjects per allergen). There were however four exceptions: rPla a 1, an

invertase inhibitor from plane tree, recognized by 19 subjects (12.7%) and three insect venom allergens, i.e. honey bee phospholipase A₂ (rApi g 1) recognized by 28 subjects (18.7%) and group 5 allergens from paper wasp (rPol d 5) and from common wasp (rVes v 5), recognized by 55 (36.7%) and 51 (34.0%) subjects, respectively. With the exception of significant increase in odds of IgE reactivity to rVes v 5 in *A. lumbricoides* infected subjects (OR, 3.42; 95% CI 1.22-9.59; $p=0.019$) no significant associations were found between helminth infections and IgE reactivity. Taken together these data indicate that the observed IgE antibodies are not the result of primary sensitization to allergens tested, even though exposure can be great, for example to peanut and shrimp, which are very common ingredients in the Indonesian diet. Using the allergen microarray, we found the high degree of sensitization against common inhalant and food allergens is unique in that there is virtually no recognition of established major allergens. Instead there is evidence of CCD-cross-reactivity, explaining most of the sensitization to pollen and peanut. For the high sensitization to HDM (*D. pteronyssinus*) and cockroach, an explanation is still missing. It is however very likely that other cross-reactive protein or carbohydrate structures in helminths, other parasites or insects are involved. These results are in line with observations from Ghana [8] and the Philippines [9] and can be used to design better microarrays for use in clinical diagnosis of allergic disorders not only in affluent but also in low to middle income countries.

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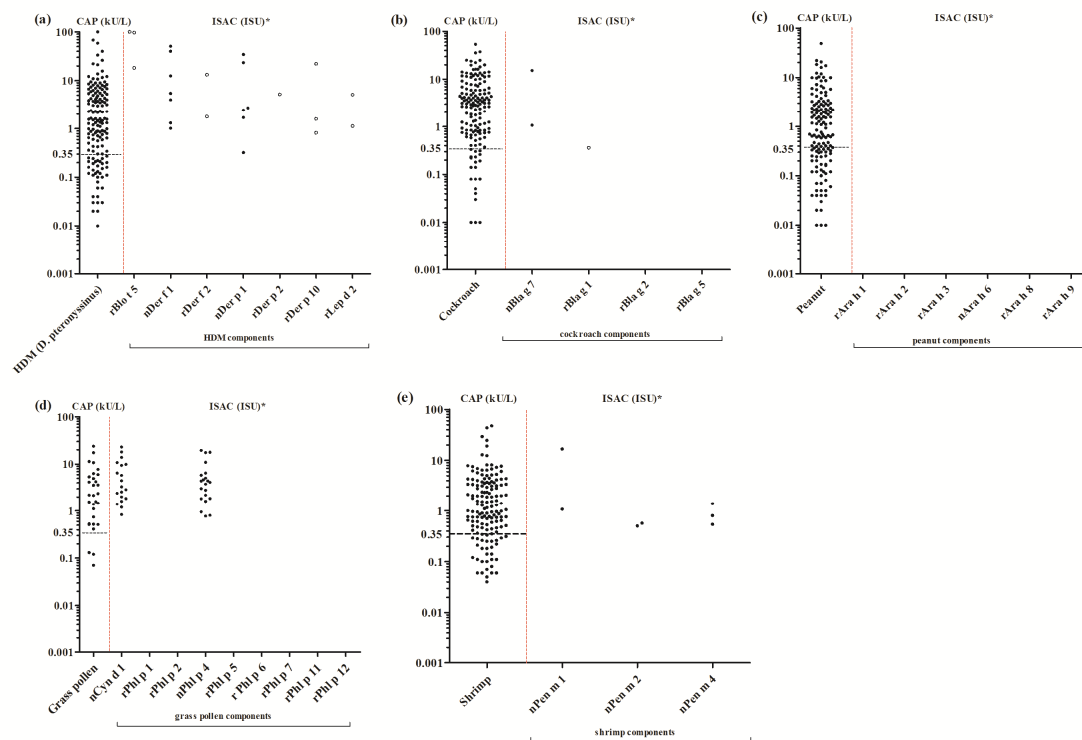
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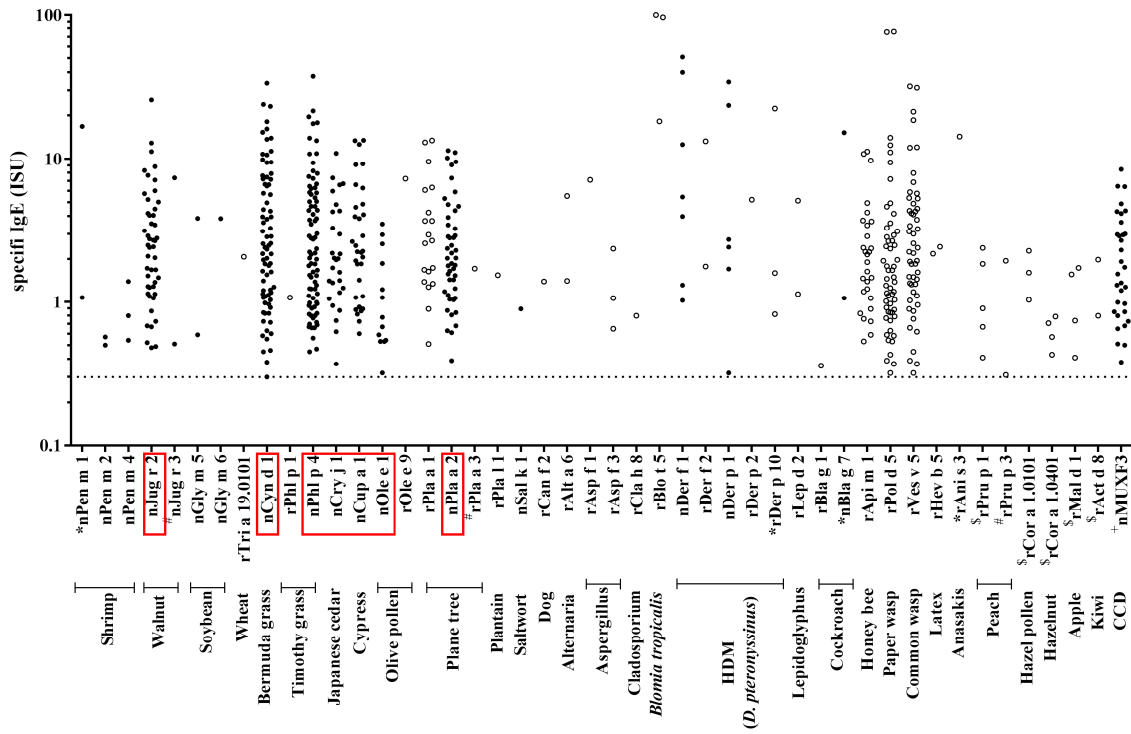
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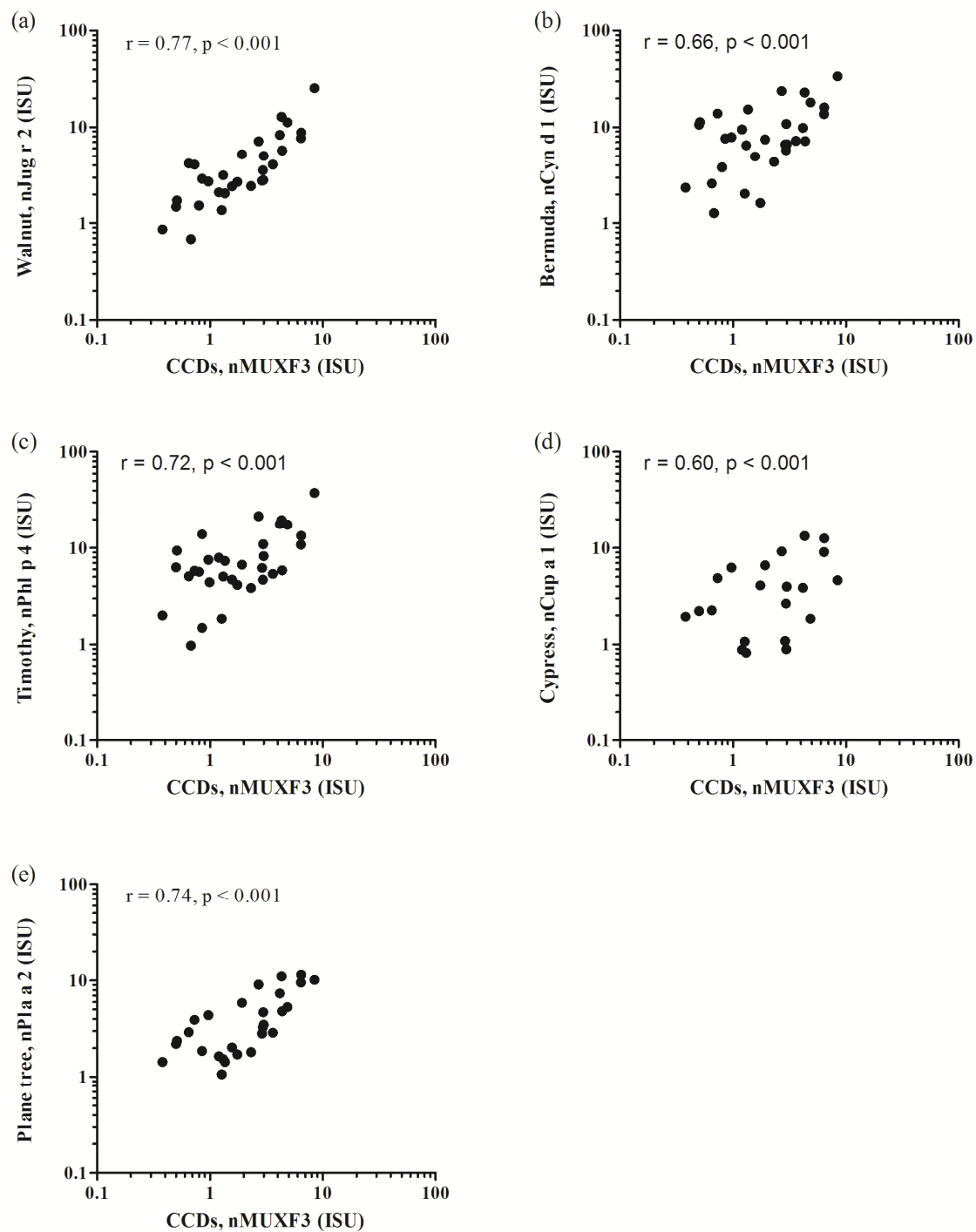
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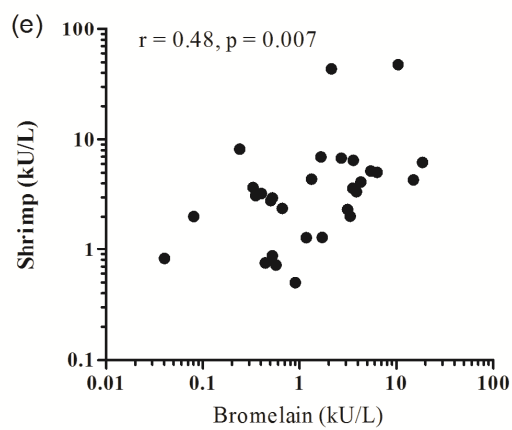
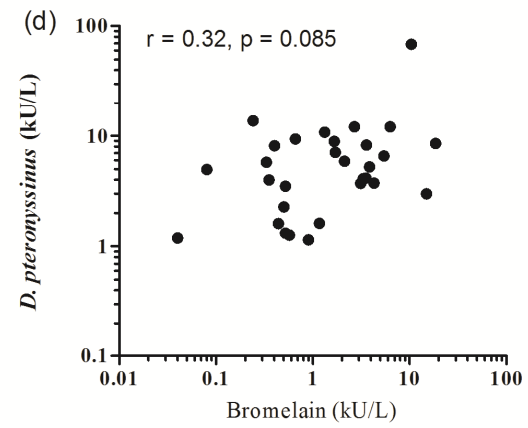
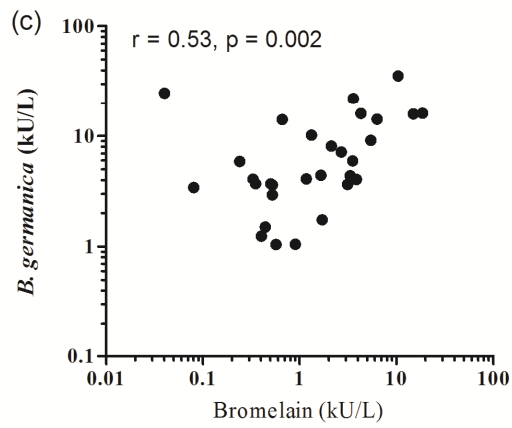
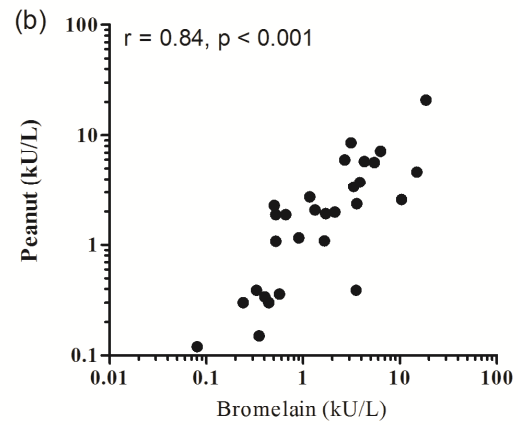
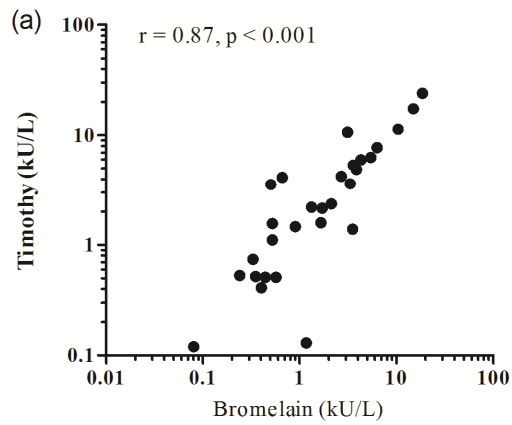
Figure 1. IgE measured by ImmunoCAP and ImmunoCAP-ISAC. IgE to; (a) HDM (*D. pteronyssinus*), (b) cockroach, (c) peanut, (d) grass pollen, and (e) shrimp measured by ImmunoCAP and ImmunoCAP-ISAC. The single allergens depicted for ISAC are related to the whole allergen extracts tested in CAP. Solid circles represent natural (n) component allergens, open circles represent recombinant (r) component allergens. Horizontal dotted lines represent the cut off value for ImmunoCAP positivity. IgE levels are given as KU/L for ImmunoCAP and as ISAC standardized units (ISU)/L for ImmunoCAP-ISAC. *For ImmunoCAP-ISAC assay, only positive results are shown.

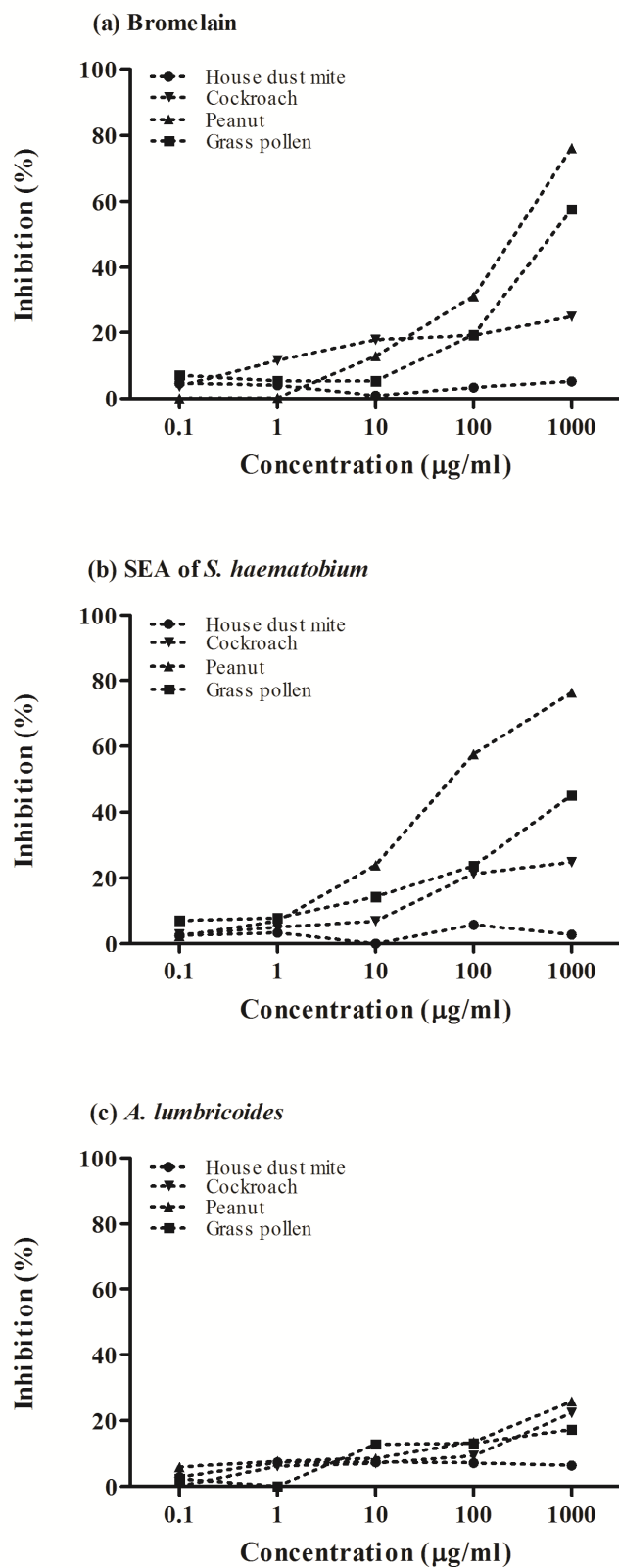
Figure 2. IgE to allergens. IgE to purified natural and recombinant allergens recognised on ImmunoCAP-ISAC (N=150). Solid circles represent natural (n) component allergens, open circles represent recombinant (r) component allergens. Cross reacting allergen to: *tropomyosin, #non-specific lipid transfer protein (nsLTP), \$the pathogenesis-related (PR)-10 protein, +Cross reactive carbohydrate determinants (CCDs). Each circle represents a single positive subject and the dotted line indicates the cut-off value for the ISAC assay (0.3 ISU). ISU, ISAC Standardized Unit. The allergen abbreviations are given in Table E2. Indicated in red squares are allergens carrying CCD.











Supplemental Figures

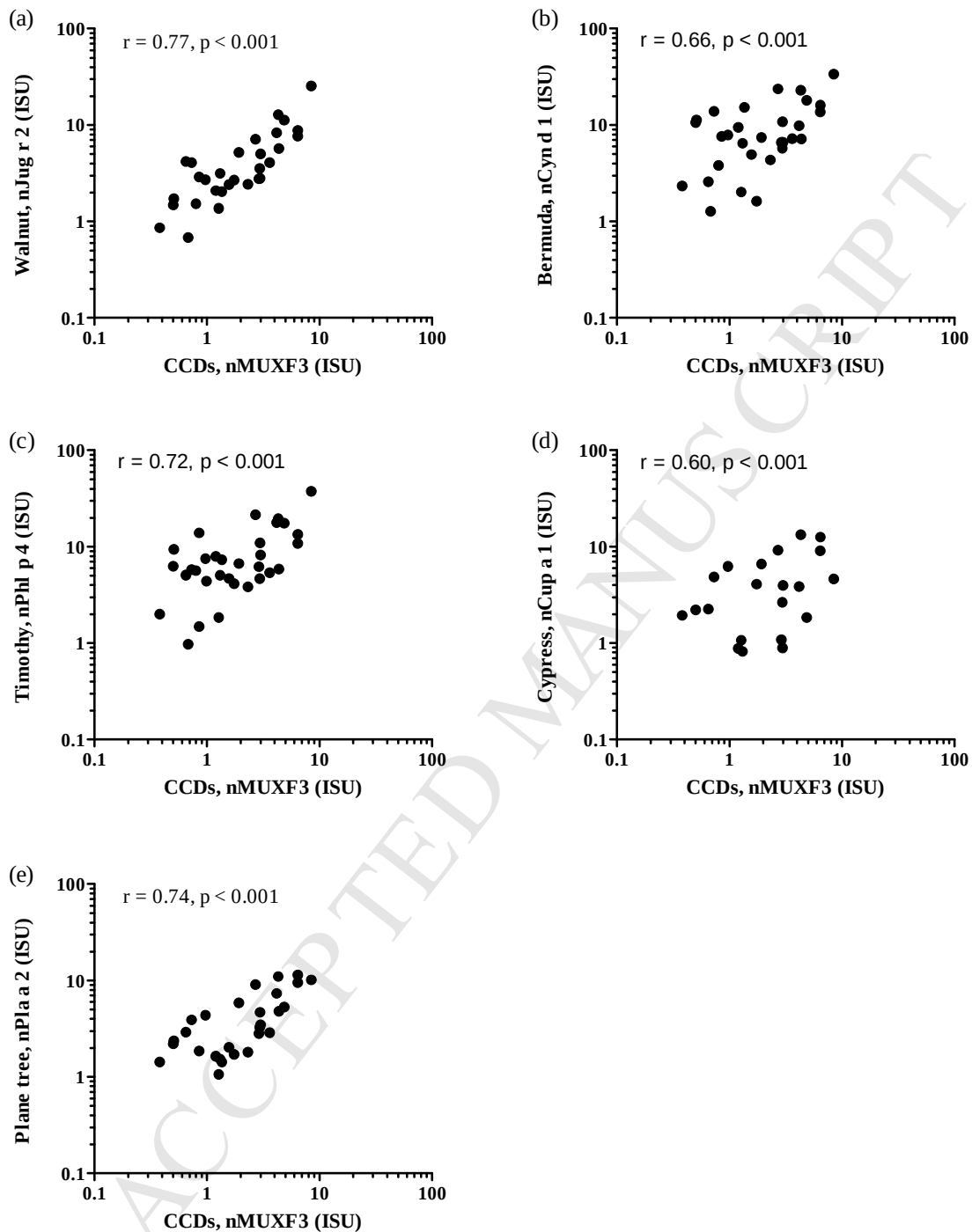


Figure E1. Correlation between IgE to CCD and natural allergens on ImmunoCAP-ISAC; (a) walnut, (b) bermuda, (c) timothy, (d) cypress, and (e) plane tree. nMUXF3 is a marker glycoprotein for CCD reactivity on ISAC assay. r = correlation coefficient. ISU, ISAC Standardized Unit.

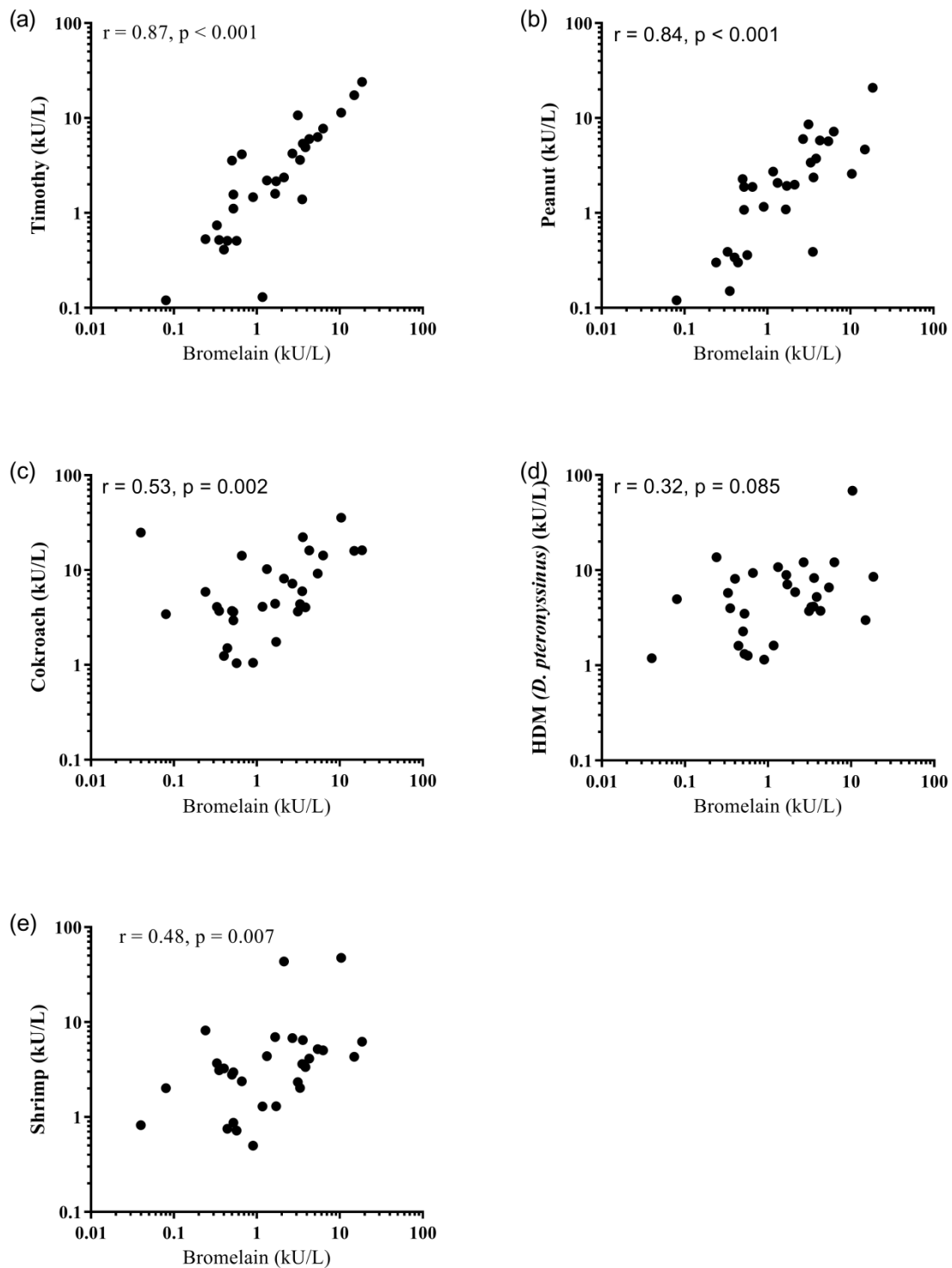


Figure E2. Correlation between IgE levels to bromelain and allergens on ImmunoCAP; (a) timothy, (b) peanut, (c) cockroach, (d) HDM (*D. pteronyssionus*), and (e) shrimp. Bromelain is a marker glycoprotein for CCD reactivity. P values and r (correlation coefficient) are given.

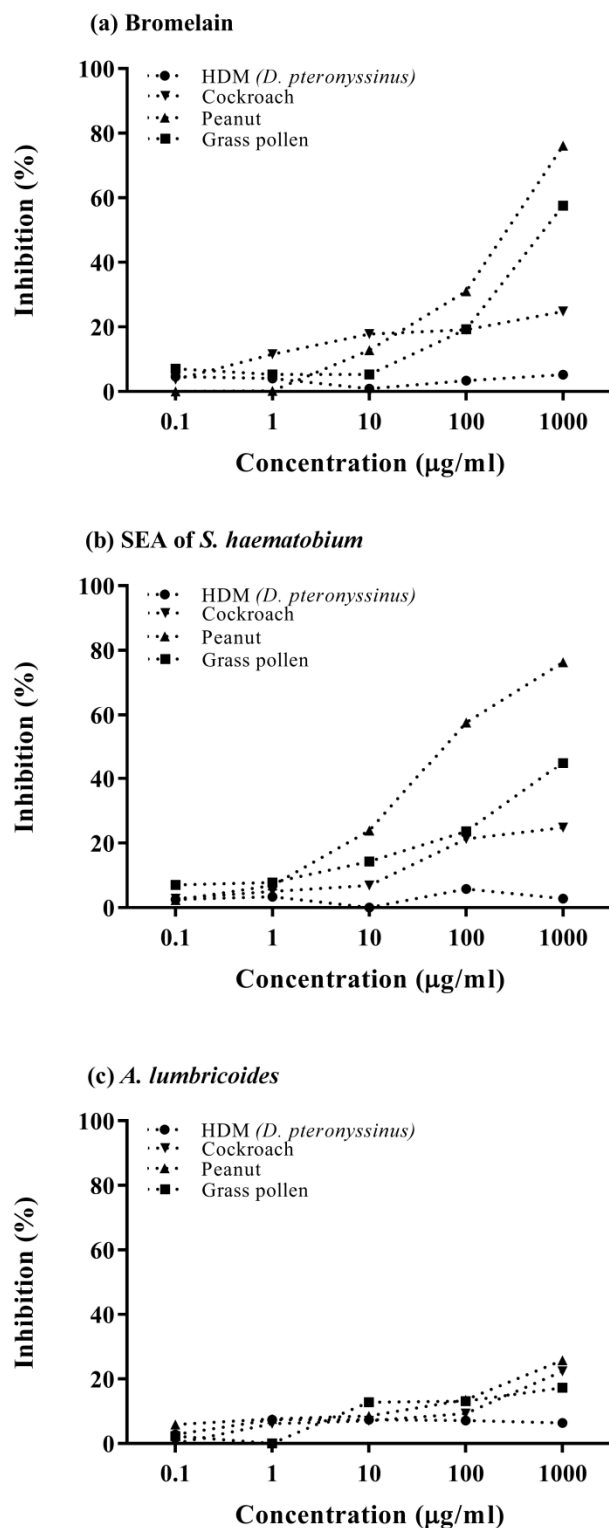


Figure E3. IgE inhibition assay. IgE cross-reactivity of HDM (*D. pteronyssinus*), cockroach, peanut and grass pollen extract tested by using an IgE ImmunoCAP inhibition assay. Bromelain, soluble egg antigens (SEA) of *S. haematobium* and *A. lumbricoides* were used as inhibitors and values were expressed as the percentage of IgE inhibition.

Supplementary Tables

Table E1. Characteristics of the study population.

	N	
Age (years; mean, $\pm$ SD)	150	10.6 ($\pm$ 2.9)
Total IgE IU/ml (geometric mean, 95% CI)	133	2816 (2257-3515)
		n (%)
Sex (female)	150	83 (55.3)
Specific IgE (cut off $\geq$ 0.35 kU/L)		
HDM (<i>D. pteronyssinus</i>)	150	111 (74.0)
Cockroach	150	130 (86.7)
Peanut	150	97 (64.7)
Shrimp	150	119 (79.3)
Grass pollen*	30	27 (90)
Bromelain*	30	26 (86.7)
Skin prick test positive		
HDM (<i>D. pteronyssinus</i> and/or <i>D. farinae</i>)**	150	40 (26.7)
Cockroach	150	36 (24.0)
Peanut	150	8 (5.3)
Shrimp	150	7 (4.7)
Helminth infection		
Any helminths	74	69 (93.2)
Hookworms ¹	76	58 (76.3)
<i>Ascaris lumbricoides</i> ¹	76	32 (42.1)
<i>Trichuris trichiura</i> ²	104	30 (28.8)

SD: standard deviation. CI: confidence intervals. Helminth infection was diagnosed by ¹PCR. ²microscopy. *Sufficient sera was available from 30 subjects only. ** Considered positive if reaction to either *D. pteronyssinus* and/or *D. farinae*.

Table E2. The prevalence of sensitization on ImmunoCAP-ISAC.

Component allergens	n (%)	Component allergens	n (%)
Food components		Cross-reactive	
Shrimp (nPen m 2)	2 (1.3)	Tropomyosin	
Shrimp (nPen m 4)	3 (2.0)	Anisakis (rAni s 3)	1 (0.7)
Walnut (nJug r 2)	48 (32.0)	Cockroach (nBla g 7)	2 (1.3)
Soybean (nGly m 5)	2 (1.3)	<i>D. pteronyssinus</i> (rDer p 10)	3 (2.0)
Soybean (nGly m 6)	1 (0.7)	Shrimp (nPen m 1)	2 (1.3)
Wheat (rTri a 19.0101)	1 (0.7)	nsLTP	
Grass pollen		Walnut (nJug r 3)	2 (1.3)
Bermuda grass (nCyn d 1)	76 (50.7)	Peach (rPru p 3)	2 (1.3)
Timothy grass (rPhl p 1)	1 (0.7)	Plane tree (rPla a 3)	1 (0.7)
Timothy grass (nPhl p 4)	80 (53.3)	PR-10 protein	
Tree pollen		Hazel pollen (rCor a 1.0101)	3 (2.0)
Japanese cedar (nCry j 1)	28 (18.7)	Hazelnut (rCor a 1.0401)	4 (2.7)
Cypress (nCup a 1)	32 (21.3)	Apple (rMal d 1)	4 (2.7)
Olive pollen (nOle e 1)	13 (8.7)	Peach (rPru p 1)	5 (3.3)
Olive pollen (rOle e 9)	1 (0.7)	Kiwi (rAct d 8)	2 (1.3)
Plane tree (rPla a 1)	19 (12.7)	CCD	
Plane tree (nPla a 2)	46 (30.7)	CCD (nMUXF3)	32 (21.3)
Weed pollen		Natural (n) and recombinant (r) allergen components.	
Plantain (rPla l 1)	1 (0.7)	The number of positives, n out of 150 subjects examined is given as (%).	
Saltwort (nSal k 1)	1 (0.7)		
Animal			
Dog (rCanf2)	1 (0.7)		
Mold			
Alternaria (rAlt a 6)	2 (1.3)		
Aspergillus (rAsp f 1)	1 (0.7)		
Aspergillus (rAsp f 3)	3 (2.0)		
Cladosporium (rCla h 8)	1 (0.7)		
HDM			
<i>Blomia tropicalis</i> (rBlo t 5)	3 (2.0)		
<i>D. farinae</i> (nDer f 1)	7 (4.7)		
<i>D. farinae</i> (rDer f 2)	2 (1.3)		
<i>D. pteronyssinus</i> (nDer p 1)	6 (4.0)		
<i>D. pteronyssinus</i> (rDer p 2)	1 (0.7)		
<i>Lepidoglyphus destructor</i> (rLep d 2)	2 (1.3)		
Cockroach			
Cockroach (rBla g 1)	1 (0.7)		
Venom			
Honey bee (rApi m 1)	28 (18.7)		
Paper wasp (rPol d 5)	55 (36.7)		
Common wasp (rVes v 5)	51 (34.0)		
Latex			
Latex (rHev b 5)	2 (1.3)		

1 *Letter to the Editor, JACI*

2 **Online Repository Materials**

3

4 **Molecular diagnostics and lack of clinical allergy in helminth-endemic areas in**
5 **Indonesia**

6

7 **Supplementary methods**

8 *Study population*

9 This study was part of a longitudinal study of the ImmunoSPIN programme
10 (www.immunospin.org) investigating the effect of anthelmintic treatment on malarial
11 parasitemia and allergy, described in detail elsewhere [1, 2]. In short, skin prick
12 testing was performed, blood was drawn for serological determinations, and a stool
13 sample was collected for diagnosis of soil-transmitted helminth infections. In addition,
14 parents/guardians were asked to fill out a questionnaire regarding allergy symptoms.
15 Written informed consent was obtained from parent or guardian of each child. This
16 study was approved by the Ethical Committee of the Medical Faculty, University of
17 Indonesia, Jakarta and filed by the ethics committee of the Leiden University Medical
18 Center, the Netherlands. The study was registered as clinical trial
19 (ISRCTN83830814).

20 A total of 1674 schoolchildren aged 5-15 years old were included in the study
21 of allergic outcomes. Total and specific IgE has been measured in 883 subjects, of
22 which 576 had sufficient plasma for ImmunoCAP-ISAC analysis. Due to budgetary
23 constraints, only 150 of these plasma samples could be analyzed in the current
24 study. To increase the chance of finding differences in IgE recognition profiles of SPT

25 positive versus SPT negative, we over-represented plasma samples from SPT
26 positives, i.e. 52 out of 150 (34% instead of 22% in original 576).

27 Questionnaire

28 Symptoms of asthma and atopic dermatitis in the past 12 months were recorded
29 using a modified version of the International Study of Asthma and Allergy in
30 Childhood (ISAAC) questionnaire as reported before [2].

31

32 Skin prick test

33 House dust mite (*Dermatophagoides pteronyssinus* and/or *D. farinae*), peanut,
34 shrimp (kindly provided by Paul van Rijn, HAL Allergy, Leiden, The Netherlands) and
35 cockroach (*Blattella germanica*; Lofarma, Milan, Italy) were used for SPT. A SPT was
36 considered positive when the wheal size (mean of longest diameter and diameter
37 perpendicular to that) was ≥ 3 mm as described previously [2].

38

39 IgE antibody measurement

40 ImmunoCAP (ThermoFisher Scientific, Uppsala, Sweden) was used to measure IgE
41 against house dust mite (*D. pteronyssinus*), cockroach, grass pollen, bromelain (as
42 a marker for CCD), peanut and shrimp, following to the manufacturer's instructions.
43 Levels of ≥ 0.35 kU/L were considered positive.

44 The ImmunoCAP-ISAC 112 with 112 allergen molecules (kindly provided by
45 Anita Kober, ThermoFisher Scientific) was used following manufacturer's instructions.
46 Briefly, arrays were incubated with 30 μ L of plasma for 2 hours. After washing and
47 drying, arrays were incubated for 30 minutes with fluorescently labeled anti-human
48 IgE. After further washing, arrays were read with a laser scanner. Results were

49 expressed as ISAC standardized units (ISU)/L. Levels of ≥ 0.3 ISU/L were
50 considered as positive.

51 Total IgE was measured by ELISA as described previously [1, 2]. The results
52 were expressed in International Units (IU/ml).

53

54 IgE inhibition assay

55 Four separate pools of plasma were prepared, a HDM (*D. pteronyssinus*)-, a
56 cockroach-, a grass pollen- and a peanut-positive pool which each contained equal
57 volumes (n=16, n=11, n=3, and n=18, respectively) of plasma samples that had ≥ 5
58 kU/L of specific IgE for the respective allergen sources. Inhibitors used were
59 bromelain (marker for CCD), SEA (soluble egg antigen of *Schistosoma*
60 *haematobium*, a source of helminth CCD) [3] and *Ascaris lumbricoides* antigen
61 (locally endemic parasite). Each 75 μ L of pooled plasma was mixed with equal
62 volume of inhibitor at different dilutions (titrated inhibition) and pre-incubated at room
63 temperature for 1 hour. Subsequently, samples were analyzed for HDM-, cockroach-,
64 grass pollen- and peanut-specific IgE, following the normal ImmunoCAP protocol.
65 Results were expressed as percentages of an uninhibited control (PBS) [3].

66

67 Parasitological examinations

68 Of the 150 studied subjects, 74 had provided stool samples for parasitological
69 testing. Characteristics of subjects providing stool samples and those who did not,
70 showed no differences in atopic sensitization (SPT and IgE), age or sex. *Trichuris*
71 *trichiura* was detected by microscopy and a multiplex real-time PCR was used for
72 detection of hookworms (*Ancylostoma duodenale*, *Necator americanus*), *A.*
73 *lumbricoides*, and *Strongyloides stercoralis* DNA as detailed previously.

74

75 Statistical analysis

76 Statistical analyses were performed using SPSS (IBM Corp., Armonk, New York,
77 USA) version 20. Data were expressed as means ($\pm$ standard deviations), frequency
78 (percentage of measured data) and geometric means [95% confidence intervals (CI)].
79 The correlations was examined using Spearman rank correlation coefficients. Logistic
80 regression was used to examine the association between helminth infection and IgE
81 reactivity to allergen component measured on ImmunoCAP-ISAC as well as the
82 association between helminth infection and IgE reactivity to whole allergen extract
83 tested on ImmunoCAP with adjustment for age, sex, and area. A *p-value* < 0.05 was
84 considered statistically significant.

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