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Allergenomics of the tick *Ixodes ricinus* reveal important α-Gal-carrying IgE-binding proteins in red meat allergy

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To the Editor

Red meat allergy known as mammalian meat allergy, caused by IgE antibodies against galactose-α-1,3-galacotse (α-Gal), is nowadays recognized worldwide and strong associations with tick bites have been identified for different tick species and geographic locations.[1] Time relationship between tick exposure and increased IgE levels to α-Gal has further supported the strong evidence that tick bites are the primary cause of the IgE antibodies.[2] All developmental stages of ticks can bite humans and in the US, high IgE levels to α-Gal following bites from larvae have been reported.[3] While we have previously demonstrated the presence of α-Gal in the gut of the European tick *Ixodes ricinus*[4], α-Gal-containing proteins in tick saliva from the South American, Japanese and European ticks, *A. sculptum*[5], *Haemaphysalis longicornis*[6] and *Hyalomma marginatum*[7], have recently been reported. However, the α-Gal-content of the *I. ricinus* proteome has not been investigated yet. Here we used allergenomics[8] and shotgun proteomics approaches to identify IgE-binding α-Gal carrying proteins in adult and larval stages as well as in saliva of *I. ricinus* ticks. Allergen-specific IgE antibody responses were assessed by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) among 32 Swedish and 18 US mammalian meat allergic patients to reveal differences in IgE reactivity between two geographical population groups. IgE- and α-Gal binding capacity, as well allergenicity of *I. ricinus* tick proteins were evaluated (details on methods and patients characteristics are presented in the Supporting information).

Our data revealed that nearly all Swedish and American meat allergic patients had IgE responses against both the larval and adult stages of *I. ricinus* and we noted that the four individuals IgE negative to *I. ricinus* had comparably low IgE levels to α-Gal (Table S1). Similarly to previous reports, moderate to high correlations between IgE to α-Gal and adult ticks were seen in the two patient groups (Figure S1).[4] Furthermore, the strong correlations between IgE reactivity to adult and larvae in both patient groups suggest that growth stages of *I. ricinus* ticks seems to be of less importance for the IgE recognition (Figure S1). In western-blot, IgE-reactive protein bands in the molecular weight range of 25–150 kDa in *I. ricinus* ticks were noted with comparable results for both tick stages using Swedish and American serum pools (Figure 1A and B respectively). To investigate whether the observed IgE reactivities were α-Gal related, the pools were pre-incubated with the α-Gal carrying glycoprotein bovine thyroglobulin prior to immunoblot analyses. The IgE binding to proteins from both adult and larval ticks was strongly diminished in Swedish as well as in US serum pools, revealing α-Gal as the main IgE target (Fig 1A and B lanes with α-Gal+) in both populations. Interestingly, proteins at the similar size were identified in *I. ricinus* saliva that bound red meat allergic patients' IgE and were recognized by the anti-α-Gal antibody (Figure 1C). Similar results have been reported for the analysis of saliva from the Japanese tick *H. longicornis*.[6] In addition, we investigated the IgE binding capacity with IgE inhibition ELISA where protein extract from *I. ricinus* was able to inhibit IgE binding to HSA-α-Gal by up to 77% (Fig S2). We also evaluated the allergenic potential of *I. ricinus* using blood from 14 mammalian meat allergic patients and noted that adult ticks induced basophil activation in 13 patients (Figure 1D and Figure S3). The allergenic activity towards ticks was higher compared with HSA-α-Gal, however HSA-α-Gal showed to be more sensitive. This shows that *I. ricinus* protein

epitopes also are of importance in basophil activation, which is in line with the IgE-binding capacity results (immunoblots and ELISA). When blood samples from four patients were stimulated with extract from *I. ricinus* larvae (Fig 1E), the allergenic activity was found to be similar to adult *I. ricinus* protein extract in three patients. None of the antigens activated basophils in two non-allergic individuals (Figure S3), indicating that the observed reactions were IgE-dependent. Basophil activation with adult *I. ricinus* protein extract was dose-dependent reaching 72.7% of CD63-positive cells (median, 34.4%; range, 7.5% to 72.7%,at concentration 50µg/ml) giving a sensitivity of 93%. Furthermore, a strong correlation between %CD63-positive basophils for adult *I. ricinus* protein extract and HSA-α-Gal (Fig S4) was noted, pointing out the dominant role of the α-Gal epitope in activating red meat allergic patients' basophils.

We used an allergenomics approach with 2D PAGE and 2D immunoblots together with mass spectrometry to identify α-Gal-carrying IgE binding proteins in adults and larvae ticks (Fig S5 and Fig S6, for details please see supporting information). Analysis of the obtained MS/MS spectra gave high identification scores to 43 protein accession numbers for adult and 37 for larvae from the *Ixodida* order (Table S2), grouped into six protein groups: vitellogenins, SERPIN, actin, α-2-macroglobulin, chitinase like-lectins and transport or channel forming proteins (Table 1). Comparing data from the 2D immunoblots with IgE binding (Fig S5A-C), and the anti-α-Gal antibody (Fig S5D) protein spots in the range of 75-100 kDa were shown to contain α-Gal carrying proteins. These proteins belonged to the vitellogenins and α-2-macroglobulin protein groups (Table 1). The other protein groups (actin, SERPIN, chitinase-like lectins and transport forming proteins) seem not to carry α-Gal. However, since these proteins possess IgE-binding properties and possibly allergenic activity, as shown in the basophil

activation test with the higher response to *I. ricinus* extract compared to HSA-α-Gal, point to the fact that they most likely play a role in immune responses against ticks. Vitellogenins (isoforms 1-4), which was the major α-Gal-carrying IgE binding protein group, are produced in mid gut cells, fat bodies and salivary glands of ticks and are present in glycosylated and non-glycosylated forms. These carbohydrate-binding proteins have major function in tick reproduction.[9] The recognition of similar proteins in the 1D immunoblotting of saliva and identification of vitellogenins by mass spectrometry with high score (Table S3) indicated that they are abundantly present in tick saliva as well. Recently, Cabezas-Cruz and colleagues reported the expression of galactosyltransferase in *Ixodes scapularis*, an enzyme needed for the production of α-Gal.[10] Thus, taken together these data support that ticks produce the α-Gal epitope. The α-Gal-containing vitellogenin and α-2-macroglobulin are probably delivered into the host's skin by tick bites and could presumably be involved in the induction of an anti-α-Gal immune response.

In conclusion, our results give new insight into mammalian meat allergy. Firstly, proteins from adults and larvae of the European tick *I. ricinus* are recognized by IgE from meat allergic patients and have allergenic activity, which are α-Gal and tick protein specific. In addition, larvae were not fed on the host which supports that α-Gal carrying proteins originate from ticks. Moreover, *I. ricinus* tick saliva contains IgE binding α-Gal carrying proteins which by allergenomics revealed to be vitellogenins. The results support the strong relationship with tick bites for the development of mammalian meat allergy. Thus, red meat allergic patients should be advised to avoid further tick bites.

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Author's contribution

DA participated in all stages of the project, performed the experiments and interpreted the data. JM and TCV were involved in the proteomics analysis. MS, SC, TPM provided the patient material. MW, MK and HS provided tick saliva material. DA, CH and MvH wrote the manuscript. All authors provided critical review of the manuscript.

Conflict of interest statements:

S.P. Commins has received support from Genentech as speaker's bureau and from UptoDate honorarium for topic author. The rest of the authors declare that they have no relevant conflicts of interest.

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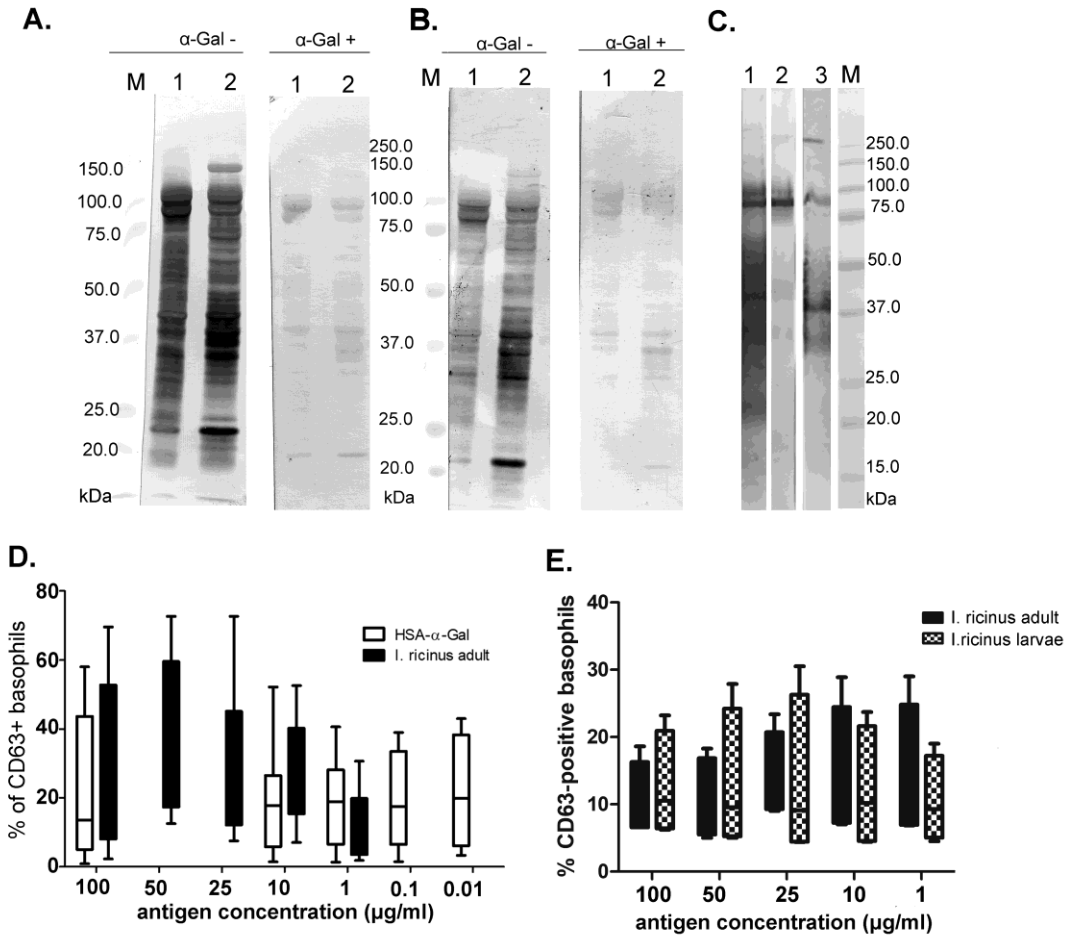
Figure legends

Fig. 1 IgE-binding activity and allergenic activity of *I. ricinus* proteins from adult and larvae in mammalian meat allergic patients. A) IgE immunoblot with the Swedish serum pool with (α-Gal+) and without inhibition with bovine thyroglobulin (α-Gal-) and B) IgE immunoblot with the US serum pool with (α-Gal+) and without inhibition with bovine thyroglobulin (α-Gal-); Lane 1- protein extract from adult *I. ricinus* ticks; Lane 2 - protein extract from larvae *I. ricinus* ticks; C) IgE binding to tick saliva proteins Lane i) with the Swedish serum pool Lane ii) and after inhibition with bovine thyroglobulin; Lane iii) with monoclonal anti-α-Gal binding; M-Molecular markers. D) Allergenic activity of 14 Swedish meat allergic patients on HSA-α-Gal and *I.ricinus* adult E) Allergenic activity of four Swedish meat allergic patient on *I. ricinus* adult and larvae

Tables

Table 1 - Identified IgE and α-Gal binding proteins in *Ixodes ricinus* ticks

Protein group	IgE and α-Gal binding on 2D PAGE			
	***I. ricinus* adults**	***I. ricinus* larvae**	**α-Gal**	**Spots from 2D**
Vitellogenins	+	+	+	1-4,6-7,9-15
α-2-macroglobulin	-	+	+	13
SERPIN	+	+	-	8,16
Actin	+	+	-	8,16
Transport or channel forming proteins	+	-	-	5,8
Chitinase-like lectins	+	-	-	1,2,7


A.
α-Gal -
α-Gal +
M 1 2 1 2
150.0
100.0
75.0
50.0
37.0
25.0
20.0
kDa
B.
α-Gal -
α-Gal +
M 1 2 1 2
250.0
150.0
100.0
75.0
50.0
37.0
25.0
20.0
kDa
C.
1 2 3 M
250.0
150.0
100.0
75.0
50.0
37.0
25.0
20.0
15.0
kDa
D.
% of CD63+ basophils
HSA-α-Gal
I. ricinus adult
100 50 25 10 1 0.1 0.01
antigen concentration (µg/ml)
E.
% CD63-positive basophils
I. ricinus adult
I.ricinus larvae
100 50 25 10 1
antigen concentration (µg/ml)

Online Supporting information`s

Methods

Table S1 - IgE levels of mammalian meat allergic patients

Table S2 - MS/MS analysis of spots from 2D PAGE

Fig. S1 Correlations between allergen-specific IgE responses in Swedish and US patients with mammalian meat allergy. A) IgE reactivity to α-Gal and protein extract from adult *I. ricinus*, B) IgE reactivity to α-Gal and protein extract from larvae *I. ricinus,* and C) IgE reactivity to protein extracts from adult and larvae *I. ricinus.*

Fig S2. IgE inhibition ELISA.

Fig. S3. Allergenic activity of *I. ricinus*. Allergenic activity of *I. ricinus* proteins from adult, larvae and anti-FcεRI (positive control) was determined by basophil activation in blood from 14 Swedish mammalian meat-allergic patients (S19-S32, Table S1), one non-allergic individual (H1) and one atopic individual (A1). Degranulation is presented as proportion (%) of CD63-positive out of CD203c-positive cells by flow cytometry (y-axes) in response to different allergen concentrations (x-axes).

Fig S4. Basophil activation correlations between HSA-α-Gal and protein extract from *Ixodes ricinus* (TE)

Fig S5. 2D immunoblot analysis of *I. ricinus*. 2D immunoblot of adult *I. ricinus* developed with A) the Swedish serum pool (S1-S18 Table S1) and B) the US serum pool (US1-US18 Table E1 in this article's Online Repository); C) 2D immunoblot of larvae developed with the Swedish serum pool (S1-S18 Table E1 in this article's Online Repository); and D) 2D immunoblot of adult *I. ricinus* developed with the anti-α-Gal antibody; M-Molecular weight markers.

Fig S6. Comparative 2D PAGE with spot picking A) adult *I. ricinus* protein extract; B) larvae *I. ricinus* protein extract. The protein spots were visualized by colloidal CBB staining.