



9. Weisser K, Stübler S, Matheis W, Huisinga W. Towards toxicokinetic modelling of aluminium exposure from adjuvants in medicinal products. *Regul Toxicol Pharmacol* 2017;88:310-321.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

DOI: 10.1111/all.13978

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

To the Editor

Red meat allergy known as mammalian meat allergy, caused by IgE antibodies against galactose- α -1,3-galactose (α -Gal), is nowadays recognized worldwide, and strong associations with tick bites have been identified for different tick species and geographical 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 Ticks in all developmental stages can bite humans, and in the United States, 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, *Amblyomma 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 allergenomics⁸ and shotgun proteomics approaches to identify IgE-binding α -Gal-carrying proteins in adult and larval stages and in saliva of *I ricinus* ticks. Allergen-specific IgE antibody responses were assessed by ImmunoCAP (Thermo Fisher Scientific) 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 capacities, as well as allergenicity of *I ricinus* tick proteins, were evaluated (details on Methods and Patients' characteristics are presented in the Appendix S1).

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). Similar 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 seem to be of less importance for the IgE recognition (Figure S1). In Western blot, IgE-reactive protein bands in the molecular 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,B, respectively). To investigate whether the observed IgE

reactivities were α -Gal-related, the pools were preincubated with the α -Gal-carrying glycoprotein bovine thyroglobulin prior to immunoblot analysis. The IgE-binding to proteins from both adult and larval ticks was strongly diminished in Swedish and in US serum pools, revealing α -Gal as the main IgE target (Figure 1A,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% (Figure S2). We also evaluated the allergenic potential of *I ricinus* using blood from 14 mammalian meat allergic patients and noted that protein extract from 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 (Figure 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 nonallergic 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 50 μ g/mL) giving a sensitivity of 93%. Furthermore, a strong correlation between %CD63-positive basophils for adult *I ricinus* protein extract and HSA- α -Gal (Figure 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 (Figure S5 and Figure S6; for details, please see Appendix S1). 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

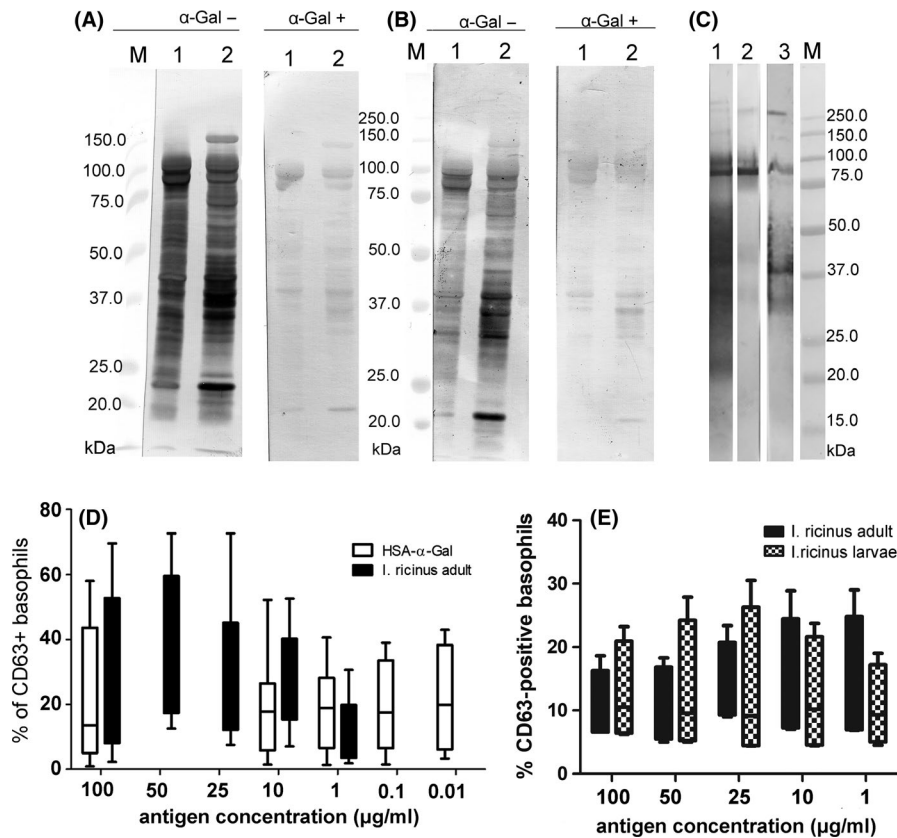


FIGURE 1 IgE-binding activity and allergenic activity of *Ixodes ricinus* proteins from adults and larvae in mammalian meat allergic patients. A, IgE immunoblot with the Swedish serum pool with (α-Gal+) and without inhibition with bovine thyroglobulin (α-Gal-). Lane 1 protein extract from adult *I. ricinus* ticks, and Lane 2 protein extract from larvae *I. ricinus* ticks. 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, and Lane 2 protein extract from larvae *I. ricinus* ticks. C, IgE- and α-Gal-binding to tick saliva proteins: Lane 1 with the Swedish serum pool; Lane 2 after inhibition with bovine thyroglobulin; and Lane 3 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

channel-forming proteins (Table 1). Comparing data from the 2D immunoblots with IgE-binding (Figure S5A-C) and the anti-α-Gal antibody (Figure 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 or channel-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, we 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 midgut cells, fat bodies and salivary glands of ticks and are present

in glycosylated and nonglycosylated 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

Protein group	IgE- and α-Gal binding on 2D PAGE			Spots from 2D PAGE
	<i>I. ricinus</i> adults	<i>I. ricinus</i> larvae	α-Gal	
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

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

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.

ACKNOWLEDGMENTS

We thank Neda Bigdeli for excellent technical assistance.

CONFLICT OF INTEREST

SP 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.

AUTHOR CONTRIBUTIONS

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 and 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.

FUNDING INFORMATION

This study was supported by the following funds: the Swedish Research Council, the Stockholm County Council (ALF project), the Swedish Asthma and Allergy Association's Research Foundation, the King Gustaf V 80th Birthday Foundation, the Swedish Heart-Lung Foundation, the Hesselman Foundation, the Konsul Th C Bergh Foundation, the Swedish Cancer and Allergy Foundation, the Magnus Bergvall Foundation, the Swedish Association for Allergology, the European Union's Horizon 2020 Research and Innovation Program—ID-LYME GA No. 720480, the European Union's Horizon 2020 FoodEnTwin project, GA No. 810752, and Ministry of Education Science and Technological Development Republic of Serbia GA No. 172024. The EC does not share responsibility for the content of the article.

Danijela Apostolovic¹ 

Jelena Mihailovic²

Scott P. Commins³

Michiel Wijnveld⁴

Maria Kazimirova⁵

Maria Starkhammar⁶

Hannes Stockinger⁴

Thomas A. E. Platts-Mills⁷

Tanja Cirkovic Velickovic^{2,8,9}

Carl Hamsten¹ 

Marianne van Hage¹

¹Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet and University Hospital, Stockholm, Sweden

²Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Center of Excellence for Molecular Food Sciences, Belgrade, Serbia

³Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, USA

⁴Institute for Hygiene and Applied Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

⁵Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia

⁶Department of Internal Medicine, Södersjukhuset, Stockholm, Sweden

⁷Asthma and Allergic Diseases Center, University of Virginia Health System, Charlottesville, VA, USA

⁸Ghent University Global Campus, Incheon, South Korea

⁹Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

Correspondence

Marianne van Hage, Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet, NKS Bioclinicum J7:17 Visiongatan 4, SE-171 64 Solna, Sweden.
Email: marianne.van.hage@ki.se

ORCID

Danijela Apostolovic  <https://orcid.org/0000-0001-8388-6916>

Carl Hamsten  <https://orcid.org/0000-0002-1830-9431>

REFERENCES

- Hilger C, Fischer J, Wolbing F, Biedermann T. Role and mechanism of galactose- α -1,3-galactose in the elicitation of delayed anaphylactic reactions to red meat. *Curr Allergy Asthm R*. 2019;23(1):3.
- Commins SP, James HR, Kelly LA, et al. The relevance of tick bites to the production of IgE antibodies to the mammalian oligosaccharide galactose- α -1,3-galactose. *J Allergy Clin Immun*. 2011;127(5):1286-U1310.
- Platts-Mills TA, Schuyler AJ, Tripathi A, Commins SP. Anaphylaxis to the carbohydrate side chain α -gal. *Immunol Allergy Clin North Am*. 2015;35(2):247-260.
- Hamsten C, Starkhammar M, Tran TA, et al. Identification of galactose- α -1,3-galactose in the gastrointestinal tract of the tick *Ixodes ricinus*; possible relationship with red meat allergy. *Allergy*. 2013;68(4):549-552.
- Araujo RN, Franco PF, Rodrigues H, et al. Amblyomma sculptum tick saliva: α -Gal identification, antibody response and possible association with red meat allergy in Brazil. *Int J Parasitol*. 2016;46(3):213-220.

6. Chinuki Y, Ishiwata K, Yamaji K, Takahashi H, Morita E. Haemaphysalis longicornis tick bites are a possible cause of red meat allergy in Japan. *Allergy*. 2016;71(3):421-425.
7. Mateos-Hernandez L, Villar M, Moral A, et al. Tick-host conflict: immunoglobulin E antibodies to tick proteins in patients with anaphylaxis to tick bite. *Oncotarget*. 2017;8(13):20630-20644.
8. Di Girolamo F, Muraca M, Mazzina O, Lante I, Dahdah L. Proteomic applications in food allergy: food allergenomics. *Curr Opin Allergy Clin Immunol*. 2015;15(3):259-266.
9. Donohue KV, Khalil S, Mitchell RD, Sonenshine DE, Roe RM. Molecular characterization of the major hemolipoglycoprotein in ixodid ticks. *Insect Mol Biol*. 2008;17(3):197-208.
10. Cabezas-Cruz A, Espinosa PJ, Alberdi P, et al. Tick galactosyltransferases are involved in alpha-Gal synthesis and play a role during Anaplasma phagocytophilum infection and Ixodes scapularis tick vector development. *Sci Rep*. 2018;8(1):14224.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

DOI: 10.1111/all.13970

Construction of multiple visual analog scale (UAD-9) for measuring severity of symptoms and quality of life of patients with respiratory allergy

To the Editor,

The concept of united airway disease (UAD) declares upper and lower airways as unified morphological and functional system.¹ Since 2008, according to ARIA and GINA, assessment of quality of life of the patients with respiratory allergy is also emphasized and might be considered with assessing clinical symptoms. For the use in UAD, four questionnaires (RHINASTHMA, RHINASTHMA-Adolescent, RAPP, and CARAT) were published.²⁻⁴ However, none of them is suitable for easy interpretation of the results: graphical visualization allowing information “at first glance” (snapshot) and using symptomatic visual analog scale for rating the answers.

The aim of the study was to develop and examine psychometric properties of the newly designed spatio-visual evaluating tool—multiple visual analog scale for united airway disease (UAD-9) for adolescent and adult patients. The questionnaire is suitable for daily use in clinical practice and research; it is simple to complete and to score (Figure 1). Intended group are all patients with allergic airways disease—allergic rhinitis/rhinosinusitis, cough equivalent asthma, allergic asthma with/without rhinitis.

UAD-9 has been constructed as a circle consisting of nine VAS lines forming umbrella arms, subsequently divided into three logical segments: upper airway (UAW), lower airway (LAW), and social life (SL). The study was realized on a cohort consisting of 170 adult patients (51.2% males). Data analysis evaluated basic psychometric properties of the scale and correlations between subscales, and confirmatory factor analyses were used to test the proposed factor structure and alternative model. Scale construction, characteristics of sample, and data analysis are attached in Data S1.

Internal consistency of the questionnaire subscales expressed as Cronbach's alpha was on the edge of acceptance in the case of upper ($\alpha = 0.685$, mean inter-item correlation 0.420) and lower airways ($\alpha = 0.626$, mean inter-item correlation 0.365) but acceptable

for social life subscale ($\alpha = 0.831$, mean inter-item correlation 0.622). Correspondence of UAD-9 upper and lower airways scores with medical evaluation achieved acceptable level (upper airways intraclass correlation (ICC) = 0.790 (95% CI 0.702-0.852) $P < 0.001$; lower airways ICC = 0.911 (95% CI 0.873-0.937) $P < 0.001$).

Results of confirmatory factor analyses showed relatively low but still acceptable goodness of fit to support three factorial compositions ($\chi^2 = 73.33$ $P < 0.001$; CMIN/DF = 2.790; GFI = 0.908, NFI = 0.841, TLI = 0.833, CFI = 0.888; RMSEA = 0.12 [0.09-0.15]). To achieve better goodness of fit, we modified the factorial model. We introduced second-order factor “all airways” that consisted of first-order factors of upper and lower airways. Such modified model achieved good goodness of fit and better reflected clinical reality ($\chi^2 = 36.51$ $P = 0.027$; CMIN/DF = 1.660; GFI = 0.941, NFI = 0.913, TLI = 0.938, CFI = 0.962; RMSEA = 0.07 [0.03-0.11]) (Figure 2).

Our study showed acceptable psychometrical properties of newly designed UAD-9 in the sense of reasonable significance of its convergent and discriminant properties and good goodness of fit. UAD-9 is the first published questionnaire for the use in daily clinical practice (1 patient) and for groups (research) that allows spatio-visual and numeric evaluation—by simple view over gap-opened umbrella (Figure 1). There is a lack of such an evaluating instrument not only among allergy specialists but even in the GP community where asthma is managed solely on reported symptoms and clinical examination.⁵

In spite of relatively low but acceptable internal consistency of the subscale, we still claim that our scale is reasonable for clinical use. According to Schmitt,⁶ even relatively low (eg, 0.50) levels of “reliability” do not seriously attenuate validity coefficients. It might be counter-productive for clinical purposes to seek high internal consistency as standard measurement theory would require.⁷

Confirmatory three-factor analysis showed relatively low consistency, but still acceptable goodness of fit to support three-segment

Supporting information

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

Danijela Apostolovic, PhD¹, Jelena Mihailovic, MSc², Scott P. Commins, MD, PhD³, Ing., Michiel Wijnveld⁴, Maria Kazimirova, PhD⁵, Maria Starkhammar, MD⁶, Hannes Stockinger, PhD⁴, Tomas A. E. Platts-Mills, MD PhD⁷, Tanja Cirkovic Velickovic PhD^{2,8,9}, Carl Hamsten, PhD^{1*}, Marianne van Hage, MD, PhD^{1*}

¹ Division of Immunology and Allergy, Department of Medicine Solna, Karolinska Institutet, and University Hospital, Stockholm, Sweden;

² University of Belgrade - Faculty of Chemistry, Department of Biochemistry, Center of Excellence for Molecular Food Sciences, Belgrade, Serbia;

³ Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, NC, USA;

⁴ Institute for Hygiene and Applied Immunology, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria;

⁵ Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia;

⁶ Department of Internal Medicine, Södersjukhuset, Stockholm, Sweden;

⁷ Asthma and Allergic Diseases Center, University of Virginia Health System, Charlottesville, VA, USA;

⁸ Ghent University Global Campus, Yeonsu-gu, Incheon, South Korea;

⁹ Ghent University, Faculty of Bioscience Engineering, Ghent, Belgium.

* Shared authorship

Correspondence to:

Marianne van Hage, MD, PhD

Karolinska Institutet
Department of Medicine Solna
Division of Immunology and Allergy
Karolinska University Hospital Solna L2:04
SE - 171 76 Stockholm, Sweden
Tel +46-8-5177 5942, Fax +46-8-33 57 24
E-mail: marianne.van.hage@ki.se

Methods

Patient sera

Sera from 32 Swedish and 18 US red meat-allergic patients reporting delayed allergic reactions following red meat consumption were used. Sera were either used individually or as pools (of either Swedish or US sera). In addition, 14 red meat-allergic patients were selected for basophil activation tests. Serum from one healthy and one atopic subject with history of tick bites lacking IgE to α -Gal and beef ($<0.1 \text{ kU}_A/\text{l}$) were used as negative controls. The study was approved by the local ethics committee at Karolinska Institutet (2014/847-32 and 2016/1447-32), University of Virginia (IRB#13298), and University of North Carolina (16-1533). Sample collection was done after written informed consent was obtained from each participant.

Preparations of protein extract and tick saliva

Protein extracts from pathogen-free *I. ricinus* adults and larvae (purchased from IS Insect Service GmbH, Berlin, Germany) were prepared as previously described.¹ Adult ticks were fed on rabbits. Larval ticks were obtained directly from hatched eggs. Tick saliva was collected from 12 fully engorged female *I. ricinus* ticks as previously described with slight modifications.²

Ixodes ricinus ticks from laboratory colony of the Institute of Zoology (Slovak Academy of Sciences, Bratislava, Slovakia) were fed on rabbits until fully engorged in the animal facility of the Biomedical Research Centre of the Slovak Academy of Sciences. The ticks were immobilised, dorsal side up, on a microscopy slide using nontoxic plasticine and tape. A glass capillary tube was placed over the hypostome and fixed in place using plasticine. Ticks were stimulated with 3 μl topically applied 5% pilocarpine hydrochloride (in 95% ethanol) solution to stimulate saliva production, taking care that no pilocarpine solution entered the capillary. Ticks were checked every 15-30 minutes over the salivation time of 6 hours. The stimulation with pilocarpine was repeated when salivation was reduced. Saliva was collected in a sterile microcentrifuge tube and stored on ice throughout the whole collection period and protease inhibitor cocktail (animal component free, Sigma Aldrich, Vienna, Austria) was added accordingly. The saliva was stored at -80°C until further use.

IgE reactivity measurements

IgE-reactivity to tick extracts was analysed by coupling 5 µg of biotinylated protein extract from adult and larvae ticks to Streptavidin ImmunoCAPs (Thermo Fisher Scientific) followed by serum analysis as described in Hamsten et al.¹ The cut off for allergen-specific IgE was ≥ 0.1 kU_A/l. Experiment was performed in duplicate. IgE reactivity to α -Gal was measured with ImmunoCAP (o215, bovine thyroglobulin, Thermo Fisher Scientific).

Basophil activation assay

Blood from 14 randomly chosen red-meat allergic patients was stimulated with ten- or 2-fold serial dilutions (from 100 - 0.01 µg/ml) of HSA- α -Gal, HSA, *I. ricinus* adult and larvae extracts. HSA- α -Gal was used as reference material based on its high specificity and sensitivity³. Allergen-specific basophil degranulation was analyzed by monitoring the basophil activation markers CD203c and CD63, as previously reported.⁴ Heparinized venous blood samples were analyzed with HSA- α -Gal, HSA, *I. ricinus* adult and larvae extracts. Different serial dilutions of antigen were added to blood and incubated for 30 minutes at 37 °C. Anti-Fc ϵ RI (Bühlmann Laboratories AG, Schönenbuch, Switzerland) was used as positive control. The samples were further incubated with FITC conjugated anti-CD63 and PE conjugated anti-CD203c monoclonal antibodies, lysed and analyzed by flow cytometry using a BD FACS Canto II (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo (Treestar, Ashland, OR). Basophils were identified by gating at least 300 CD203c-positive cells and the magnitude of allergen-activation was calculated and expressed as the percentage of CD63-positive cells among the gated basophils. A cut-off of 10% CD63+ basophils was used for ticks, and 5% (or Stimulation index SI ≥ 2) for α -Gal as recommended by manufacturer for insects and carbohydrate cross-reactive determinates or hapten, respectively (Bühlmann, Switzerland). Basophil reactivity and maximum %CD63+ basophils (highest value of activated basophils by one allergen); the ratio of %CD63+ basophils induced by the protein extract from adult *I. ricinus* ticks to %CD63+ basophils after stimulation with anti-Fc ϵ RI (%CD63 allergen+/anti-Fc ϵ RI) and correlation between %CD63+ basophils stimulated with *I. ricinus* and HSA- α -Gal were determined.

Electrophoretic, immunoblot analysis and protein identification

SDS-PAGE of tick protein extracts and tick saliva were carried out as previously described.⁵ Ten micrograms of protein per lane were separated by 10% PAGE. For 2D

PAGE analysis, extracts from *I. ricinus* adult and larvae were diluted to 150 and 300 µg/mL in rehydration buffer (8 M Urea, 2% CHAPS, 0.5% IPG buffer 3-10NL, 0.002% bromophenol blue and 50mM DTT). The samples were loaded on 7 and 13 cm immobilized pH gradient strips, pH 3-10NL (GE Healthcare, Sweden). First dimension was carried out on an Ettan IPGphor system (GE Healthcare) according to the manufacturer instructions. Second dimension separation was carried out on 10% acrylamide hand cast gels using a MiniProteon II BioRad system (BioRad, USA). Colloidal CBB staining was used for visualization.

Proteins were transferred to PVDF membranes (0.2 µm pore size) after SDS-PAGE or 2D PAGE. For IgE-binding analysis, 1% HSA in PBS-T (containing 0.05% Tween) was used as a blocking solution, while 1% BSA in PBS-T was used for α-Gal binding. Incubation was performed at room temperature (RT) for 1h. After blocking, the membrane was incubated overnight with serum pools from Swedish (34 kU_A/L to α-Gal) or US (39.6 kU_A/L to α-Gal) red meat allergic patients diluted 1:4. For the inhibition experiments pooled sera were pre-incubated with bovine thyroglobulin (Sigma) in a final concentration of 100 µg/ml. Bovine thyroglobulin was used as reference material in order to have the same material as in the ImmunoCAP analysis. For α-Gal detection, the membrane was incubated for 3h at room temperature (RT) with the monoclonal anti-α-Gal antibody diluted 1:3 (M86, Enzo Life Sciences, Farmingdale, NY, US) and detection was done as described earlier.⁵ For IgE detection, membranes were incubated with rabbit anti-human IgE (MIAB, Sweden) in dilution 1:2000 for 1 h in room temperature (RT). After five washes with PBS-T, membranes were further incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (Jackson). For α-Gal detection, after M86 antibody, membrane were incubated with goat anti-mouse IgM antibody conjugated with alkaline phosphatase (Southern Biotech, Birmingham, AL, USA), for 1h at RT diluted 1:3000. Immunoblots were developed using alkaline phosphatase detection kit (BioRad). Experiments were performed at least in duplicates. 2D PAGE protein spots of interest were excised and subjected to in-gel digestion with trypsin from porcine pancreas (Proteomic Grade, BioReagent, Dimethylated; Sigma) as previously described.⁶ The peptides obtained were analysed according to the method reported by Apostolovic et al.⁵ using LTQ Orbitrap XL mass spectrometer with an EASY- nano liquid chromatography (nLC) II system (Thermo Fisher Scientific Inc., Bremen, Germany), with change in the Orbitrap resolution from 30000 to 60000. MS/MS data analysis was performed in PEAKs version 8.5 (Peaks 8.5, Bioinformatics

Solutions Inc., Waterloo, Canada). Search was performed against Uniprot derived Ixodida (taxonomy ID 6935), *Oryctolagus cuniculus* (taxonomy ID 9986) and cRAP (common Repository of Adventitious Proteins, www.thegpm.org/crap/) database combined into one FASTA file, downloaded on 08/05/2018. Ixodida database (ID 6935) contains 190 997 entries, from which 83 297 represent putative proteins generated from gene sequencing. Peptide and MS/MS tolerance were set to 10 ppm and 0.5 Da, respectively. Carbamidomethylation of Cys was set as fixed while oxidation of Met, and deamidation of Asn and Gln, of N-terminus were set as variable modifications. False Discovery Rate was set to 0.01 for strict and 0.05 for relaxed modes of peptide discovery.

Shotgun proteomic analysis of *I. ricinus* saliva

Protein extract from adult *I. ricinus* tick saliva was subjected to trypsin digestion in solution. Obtained peptides were run on nLC-MS/MS as described in Smiljanic et al.⁷ Protein identification and comparison was done with PEAKs 8.5 as described above.

ELISA experiment

Half-area microtiter plates (Greiner bio-one, Frickenhausen, Germany) were coated for 6 h at RT with 0.1 µg of α-Gal-HSA (Dextra Laboratories, Reading, UK), followed by blocking (PBS containing 1% HSA and 0.5% Tween 20, pH 7.4) overnight at 4 °C. The Swedish serum pool was pre-incubated with protein extract from adult *I. ricinus* or HSA-α-Gal at a concentration of 7.8-1000 µg/ml for 1 h at RT prior to adding them onto plates. Detection was performed with anti-human IgE antibody conjugated with HRP (1 h, 1:2500; abcam, UK). The absorbance was measured at 450 nm. Inhibition of IgE binding was calculated as $[(OD_{no\ inhibitor} - OD_{inhibitor}) / OD_{no\ inhibitor}] \times 100$. Experiment was done in duplicate. A chemically conjugated HSA with α-Gal trisaccharide was used as reference material since it is more sensitive than bovine thyroglobulin (data not shown).

Statistical analysis

Spearman's correlation test was used where $p < 0.05$ was considered significant. Analyses were performed using Graphpad Prism 5 software (Graphpad Software Inc., San Diego, CA).

Results and Discussion

Mammalian meat-allergic patients have IgE reactivity to α -Gal as well as to the adult and larvae stages of *I. ricinus*

Swedish patients had similar IgE levels against α -Gal (median 24 kU_A/l; range 1.9 - >100 kU_A/l) as the US patients (median 20.5 kU_A/l; range 1.5 - >100 kU_A/l). Most of the patient showed to have more than 10% of α -Gal specific of total IgE antibodies. Thirty-one out of 32 Swedish mammalian meat-allergic patients had IgE antibodies against *I. ricinus* proteins (Table S1). No difference in median IgE value to adult ticks (0.81 kU_A/l; 0.11-40.0 kU_A/l) compared to larvae (0.73 kU_A/l, 0.13-20.1 kU_A/l) was noted.

Table S1. IgE levels of red meat allergic patients

ID	α -Gal (kU _A /l)	<i>Ixodes ricinus</i> (kU _A /l)		Total IgE (kU _A /l)
		Adults	Larvae	
S1	54	30	12.1	260
S2	23	40	20.1	280
S3	6.2	2.8	2.62	870
S4	24	10	7.94	1800
S5	25	8.1	4.23	130
S6	23.1	0.44	0.85	110
S7	>100	6.93	11.3	705
S8	2.84	0.64	1.05	817
S9	>100	3.98	6.31	510
S10	6.3	0.27	0.29	166
S11	1.9	<0.1	<0.1	110
S12	19	0.28	0.27	67
S13	17	0.27	0.43	530
S14	100	1.31	3.33	320
S15	37	0.15	0.44	75
S16	4.8	0.18	0.28	32
S17	3.6	0.11	0.13	370
S18	63	0.33	0.44	350
S19	80	3.4	1.2	310
S20	5.2	0.13	0.13	47
S21	42.2	0.45	0.19	207
S22	95	4.63	3.41	1576
S23	4.9	0.22	0.11	18
S24	94	1.0	0.42	270
S25	12	0.32	0.2	81
S26	>100	11	5.4	420
S27	>100	2.43	2.36	2000
S28	87	0.83	0.46	217
S29	24	1.3	0.6	86
S30	13	0.13	<0.1	44

S31	10	2.9	2.0	48
S32	59	0.79	1.7	440
Median and range	24 1.9 - >100	0.81 0.11 - 40	0.73 0.13 - 20.1	
US1	21	1.39	3.27	95
US2	4.1	<0.1	<0.1	189
US3	96.7	4.04	8.07	n.p.
US4	7.3	<0.1	0.22	59.2
US5	12.7	0.14	0.14	128
US6	33.4	3.45	5.83	85
US7	65	3.98	6.61	374
US8	1.82	0.1	0.12	83
US9	11.3	0.36	0.55	64
US10	266	8.02	12.9	562
US11	44	0.79	1.1	280
US12	21	2.63	4.32	300
US13	>100	1.08	2.86	n.p.
US14	20	0.45	0.33	120
US15	8.3	0.25	0.38	720
US16	>100	0.94	1.79	780
US17	4.5	0.11	0.18	150
US18	1.5	<0.1	<0.1	140
Median and range	20.5 1.5 - >100	0.62 0.1 - 8.02	0.83 0.12 - 12.9	

There was a moderate correlation between IgE levels to α -Gal and *I. ricinus* adults and larvae stages among Swedish mammalian meat allergic patients (Fig S1A, $\rho=0.61$; and Fig S1B, $\rho=0.59$, respectively), and the correlation between IgE levels to *I. ricinus* adult and larvae stages was high (Fig S1C, $\rho=0.93$).

Among US mammalian meat-allergic patients, 16 of 18 had IgE reactivity towards *I. ricinus*. A high correlation between the IgE levels to α -Gal and *I. ricinus* adults and larvae was observed (Fig S1A, $\rho=0.89$; Fig S1B, $\rho=0.87$) as well as between the IgE levels to *I. ricinus* adults and larvae (Fig S1C, $\rho=0.98$). Swedish patients displayed a lower correlation between IgE to adult and larvae compared to American patients. This suggests that Swedish patients also have an α -Gal-independent IgE reactivity against *I. ricinus* proteins, which we confirmed with IgE inhibition ELISA (Fig S2).

IgE binding capacity to HSA- α -Gal was inhibited up to 77% when the serum pool was pre-incubated with protein extract from *I. ricinus* in comparison with homologous inhibition which reached 100% (Fig S2).

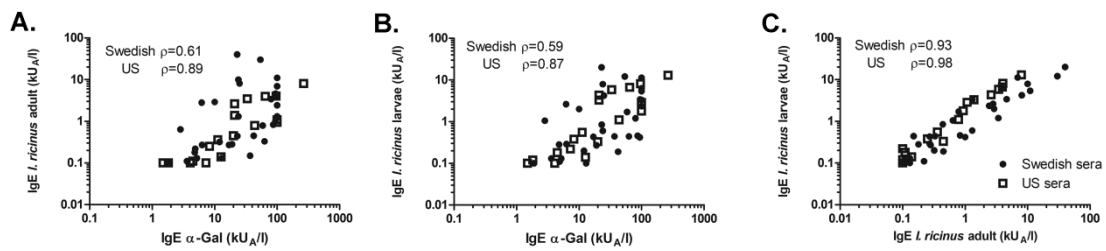


Fig. S1 Correlations between allergen-specific IgE responses in Swedish and US patients with red meat allergy. A) IgE reactivity to α-Gal and protein extract from adult *I. ricinus*, B) IgE reactivity to α-Gal and protein extract from larvae *I. ricinus*, and C) IgE reactivity to protein extracts from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

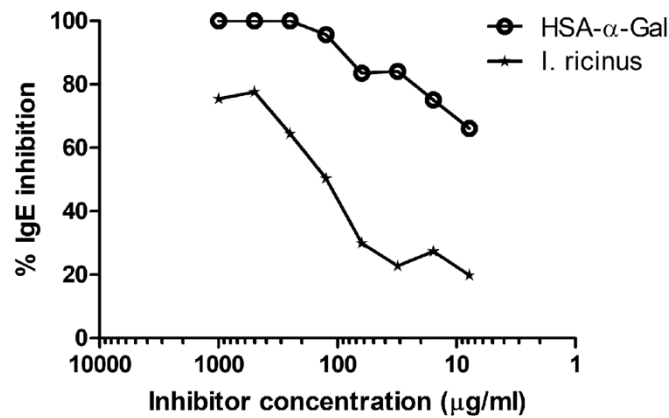


Fig S2. IgE inhibition ELISA.

Allergenic activity of tick proteins

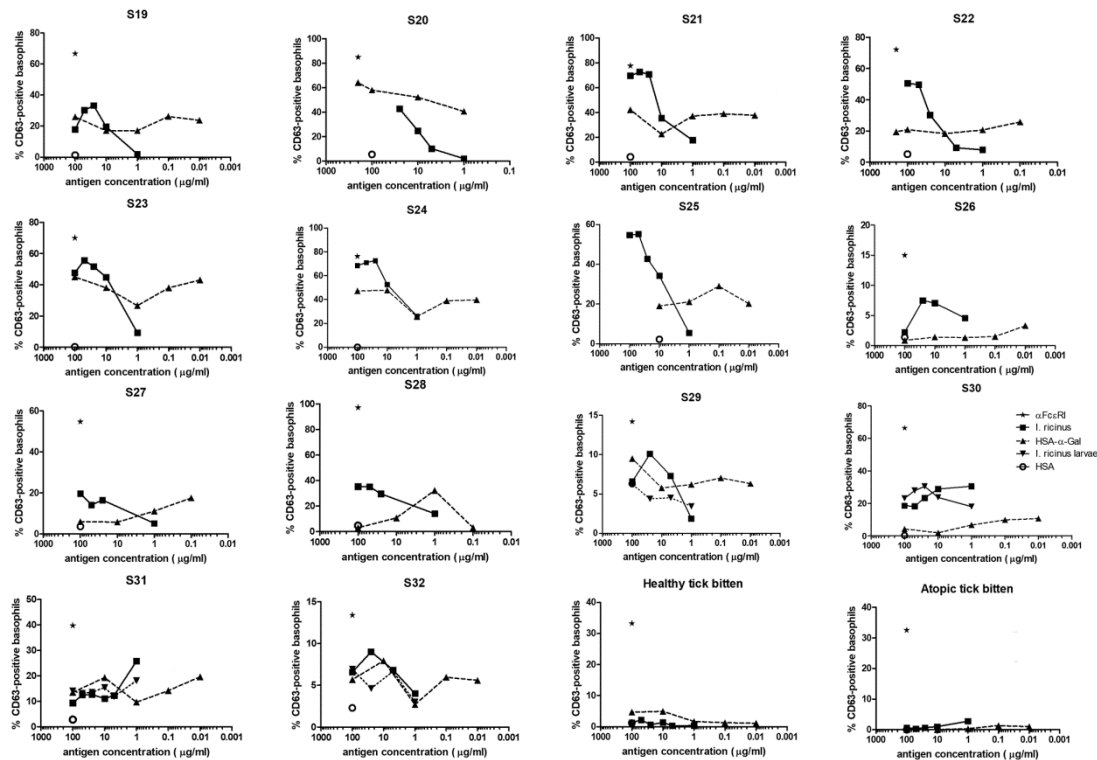


Fig. S3. Allergenic activity of *I. ricinus*. Allergenic activity of *I. ricinus* proteins from adult, larvae and anti-Fc ϵ RI (positive control) was determined by basophil activation in blood from 14 Swedish red meat-allergic patients (S19-S32, Table S1), one non-allergic individual (H1) and one atopic 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).

There was a strong correlation between %CD63-positive basophils for adult *I. ricinus* protein extract and HSA- α -Gal (Fig S4), pointing to the dominant role of the α -Gal epitope in activating red meat allergic patients' basophils.

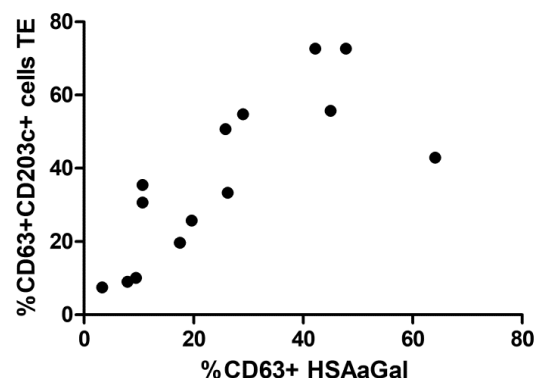


Fig S4. Basophil activation correlations between HSA- α -Gal and protein extract from *Ixodes ricinus* (TE)

Allergenomics of *I. ricinus* ticks reveal α -Gal carrying proteins

IgE binding profile of tick proteins by 2D immunoblot developed with the Swedish or American serum pools as well as with the monoclonal anti- α -Gal antibody (Fig S5). Mammalian meat-allergic patients' IgE antibodies from Swedish (Fig S5A) and US serum pools (Fig S5B) bound adult *I. ricinus* proteins at 37 kDa in the acidic pI region and 50-100 kDa in the acidic to basic pI region. The IgE-binding profile to larval *I. ricinus* proteins (Fig S5C) was similar as to adult. In 2D immunoblots developed with Swedish serum pool (Figure S5A and S5C) most of the spots were close one to another, but the dominant proteins were at approximately 100 kDa (Fig S5A and S5C). The monoclonal anti- α -Gal antibody recognized α -Gal carrying proteins (Fig S5D).

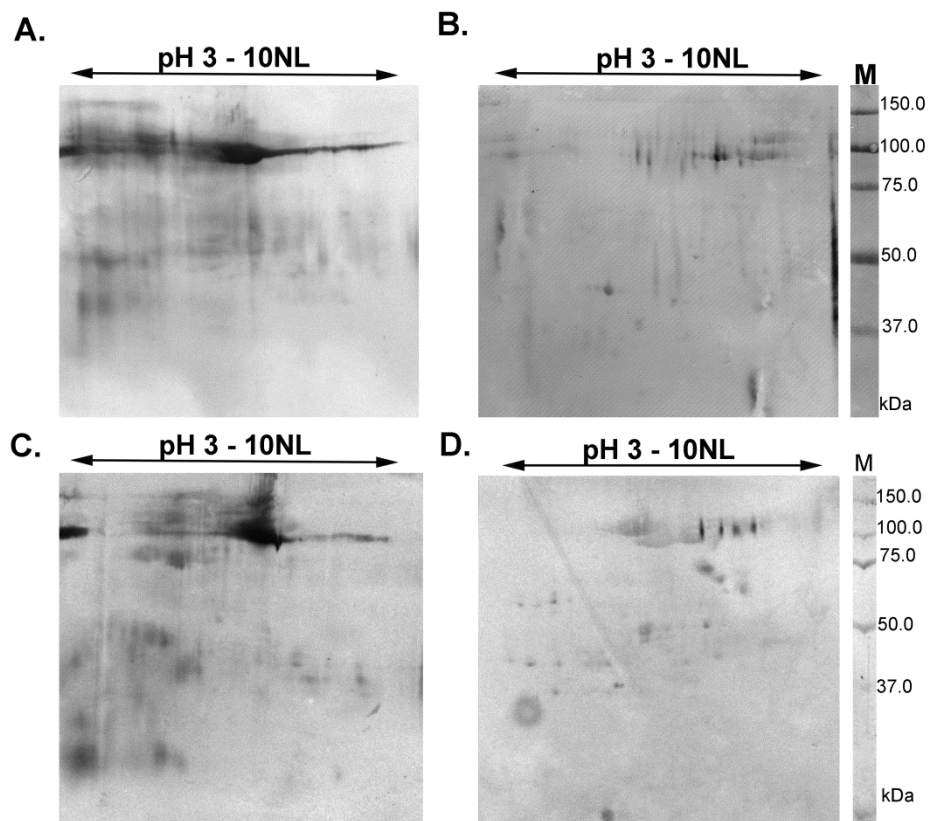


Fig S5. 2D immunoblot analysis of *I. ricinus*. 2D immunoblot of adult *I. ricinus* developed with A) the Swedish serum pool (S1-S18 Table S1) and B) the US serum pool (US1-US18 Table E1 in this article's Online Repository); C) 2D immunoblot of larvae developed with the Swedish serum pool (S1-S18 Table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

To identify the IgE binding α -Gal carrying proteins CBB visible spots or area of the gel (labeled 1-16, Fig S6), displaying IgE reactivity in mammalian meat-allergic patients, were excised from both gels (adult and larvae ticks), subjected to trypsin digestion and thereafter nLC-MS/MS analysis.

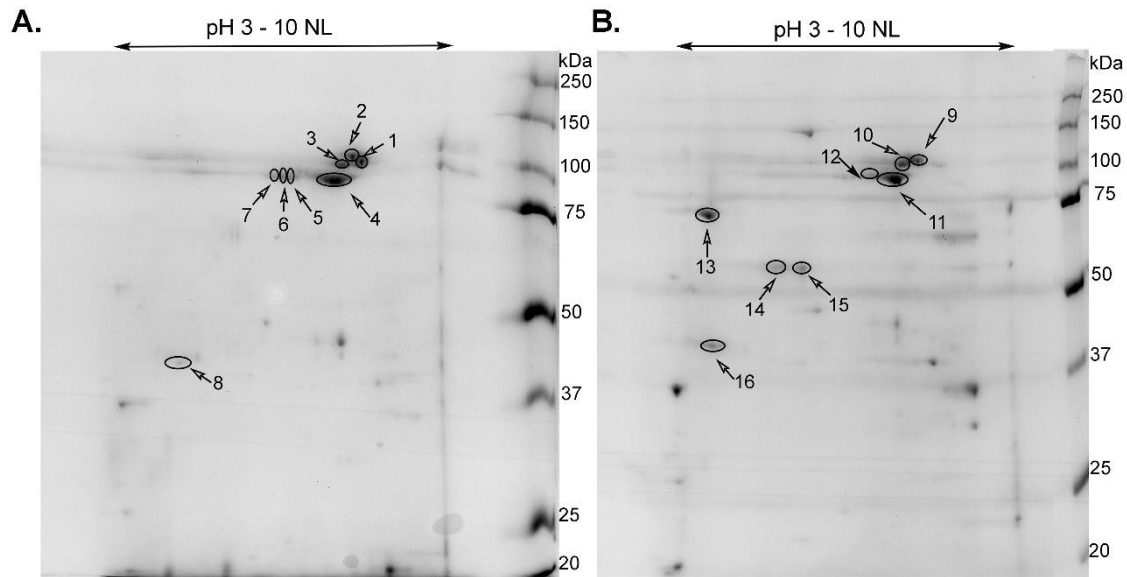


Fig S6. Comparative 2D PAGE with spot picking A) adult *I. ricinus* protein extract; B) larvae *I. ricinus* protein extract. The protein spots were visualized by colloidal CBB staining.

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. Five protein groups were found in adult *I. ricinus* and four in *I. ricinus* larvae. 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. Vitellogenin or hemelipoglycoprotein⁸ contains several cleavage sites that generate subunits in the molecular range from 50-150kDa^{9,10}, a result which we also noted. In addition, these proteins have been shown to have a higher expression rate after the ticks were fed⁸, and the recognition of similar proteins in the 1D immunoblotting of saliva indicated that they are also present in tick saliva (Fig 1C). That was confirmed by shotgun mass spectrometry analysis (Table S3). Moreover, the α -2-macroglobulin protein spot was only identified in the 2D PAGE of larvae proteins which is probably due to the lower resolution of the isoelectric focusing of the 2D PAGE. In addition, MS/MS spectra were screened against the database containing protein sequences from rabbit (*Ory c*) and against the cRAP database for background contaminants to

improve the quality of obtained data (data not shown). Some of the proteins showed to be human keratin. Other identified proteins were not assigned to originate from ticks and showed only 1 unique peptide with low protein score which makes them less likely to be a random match.

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

***Ixodes ricinus* adults (IRA)**

protein hits (first) in *Ixodes ricinus* adult body 2DE - LC-MS/MS
analysis identified by Peaks 8.5

Search algorithm: Peaks 8.5, Bioinformatics Solutions Inc.,
Waterloo, Canada

Database: Uniprot derived Ixodida(tax. Id 6935), Oryctolagus cuniculus (tax. Id 9986) and cRAP combined
into 1 fasta file, downloaded on 18/05/2018

Search filters: missed cleavages ≤ 2, PSM FDR: 1%, unique peptide ≥1, protein score ≥ 25, precursor mass
tolerance: 10 ppm, fragment mass tolerance: 0.5 Da

Protein groups

I

Vitellogenin

II

SERPIN

III

ACTIN

IV

chitinase like-
lectins
transport or
channel forming
proteins

V

Spot	Accession	Description	Organism	Avg. Mass	Protein score (-10lgP)	Coverage (%)	#Peptides	#Unique peptides	PTM
1	A0A131XR98	Putative sodium leak channel non-selective protein (Fragment)	<i>Ixodes ricinus</i>	195,788	30.83	1	1	1	Deamidation (NQ)
1	B7QGE3	Hemipoglyco-carrier protein (Fragment)	<i>Ixodes scapularis</i>	151,678	83.15	3	2	1	Carbamidomethylation
1	B7Q406	Hemipoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	89.57	2	4	2	Carbamidomethylation
1	V5GJN1	Putative vitellogenin-2	<i>Ixodes ricinus</i>	175,610	177.28	14	15	3	Carbamidomethylation; Oxidation (M)
1	A0A0D3RJ94	Hemipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	176.58	14	16	4	Carbamidomethylation; Oxidation (M)
1	V5H340	Putative vitellogenin-1	<i>Ixodes ricinus</i>	199,555	210.64	21	28	28	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
2	A0A0K8R491	Putative innexin (multimer)	<i>Ixodes ricinus</i>	15,218	29.63	14	1	1	Carbamidomethylation; Acetylation (N-term)
2	B7QGE3	Hemipoglyco-carrier protein (Fragment)	<i>Ixodes scapularis</i>	151,678	68.15	2	2	1	Deamidation (NQ)
2	A0A147BA93	Hemipoglycoprotein (Fragment)	<i>Carios mimon</i>	96,026	69.91	2	2	1	Carbamidomethylation

2	A0A0D3RJ94	Hemelpoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	121.06	4	5	2	
2	B7Q406	Hemelpoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	98.24	4	6	5	Carbamidomethylation
2	V5GJN1	Putative vitellogenin-2	<i>Ixodes ricinus</i>	175,610	128.42	6	6	3	
2	V5H340	Putative vitellogenin-1	<i>Ixodes ricinus</i>	199,555	265.01	32	49	45	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
3	B7QGE3	Hemelpoglyco-carrier protein (Fragment)	<i>Ixodes scapularis</i>	151,678	84.55	2	2	1	Deamidation (NQ)
3	V5GJN1	Putative vitellogenin-2	<i>Ixodes ricinus</i>	175,610	127.58	4	4	2	
3	B7Q406	Hemelpoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	105.83	4	5	4	
3	A0A0D3RJ94	Hemelpoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	177.62	4	6	4	Deamidation (NQ)
3	V5H340	Putative vitellogenin-1	<i>Ixodes ricinus</i>	199,555	278.5	22	28	28	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
4	B7QGE3	Hemelpoglyco-carrier protein (Fragment)	<i>Ixodes scapularis</i>	151,678	81.73	2	2	1	Deamidation (NQ)
4	B7Q406	Hemelpoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	101.29	4	6	3	Carbamidomethylation; Oxidation (M)
4	V5H7G7	Putative vitellogenin-2	<i>Ixodes ricinus</i>	161,482	158.06	8	8	1	Carbamidomethylation; Oxidation (M)
4	V5GJN1	Putative vitellogenin-2	<i>Ixodes ricinus</i>	175,610	177.83	9	10	1	Carbamidomethylation; Oxidation (M)
4	A0A0D3RJ94	Hemelpoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	195.79	9	12	3	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
5	A0A0K8RCB1	Putative enolase	<i>Ixodes ricinus</i>	47,145	61.66	3	1	1	
6	A0A0D3RJ94	Hemelpoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	281.02	13	13	4	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
6	V5GJN1	Putative vitellogenin-2	<i>Ixodes ricinus</i>	175,610	283.27	15	14	5	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
7	A0A293M6L4	Ribophorin II (Fragment)	<i>Ornithodoros erraticus</i>	63,852	28.94	2	1	1	
7	B7QGE3	Hemelpoglyco-carrier protein (Fragment)	<i>Ixodes scapularis</i>	151,678	88.21	2	3	1	Carbamidomethylation; Deamidation (NQ)
7	B7Q406	Hemelpoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	158.84	9	13	4	Deamidation (NQ); Oxidation (M)

7	B7Q406	Hemipoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	213.56	11	16	5	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
7	A0A0D3RJ94	Hemipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	196.02	14	19	2	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
7	V5H7G7	Putative vitellogenin-2	<i>Ixodes ricinus</i>	161,482	205.16	15	20	1	Deamidation (NQ); Oxidation (M)
7	A0A0D3RJ94	Hemipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	268.57	15	21	1	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
7	V5H7G7	Putative vitellogenin-2	<i>Ixodes ricinus</i>	161,482	256.73	17	22	1	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
7	A0A131Y9E6	Putative vitellogenin-2 (Fragment)	<i>Ixodes ricinus</i>	132,382	269.08	22	22	3	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
8	A0A131XRU7	Putative catalytically inactive chitinase-like lectin (Fragment)	<i>Hyalomma excavatum</i>	41,678	35.49	3	1	1	
8	B7PY91	Secreted protein putative	<i>Ixodes scapularis</i>	37,345	56.36	8	1	1	
8	B7Q5W3	Serpin-2 precursor putative (Fragment)	<i>Ixodes scapularis</i>	41,636	77.36	7	2	2	
8	A0A023GGD8	Putative actin (Fragment)	<i>Amblyomma triste</i>	25,804	97.7	30	3	3	Carbamidomethylation; Oxidation (M)
8	A0A068EU07	Blood meal-induced serine protease inhibitor	<i>Ixodes scapularis</i>	43,425	139.08	27	6	1	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
8	B7QL34	Secreted salivary gland peptide putative	<i>Ixodes scapularis</i>	43,546	150.43	28	7	1	Deamidation (NQ); Oxidation (M)
8	A0A0K8RCY5	Putative salivary serpin	<i>Ixodes ricinus</i>	43,570	197.39	38	9	3	Deamidation (NQ); Oxidation (M)
8	A0A147BWE2	Putative blood meal-induced serine protease inhibitor	<i>Ixodes ricinus</i>	43,541	187.3	41	10	5	Carbamidomethylation; Deamidation (NQ); Oxidation (M)

Ixodes ricinus larvae (IRL)

Ixodes ricinus larvae proteins resolved by 2DE PAGE and identified by LC- MS/MS through *Ixodida* database search

Search algorithm: Peaks 8.5, Bioinformatics Solutions Inc.,
Waterloo, Canada

Database: Uniprot derived *Ixodida*(tax. Id 6935), *Oryctolagus cuniculus* (tax. Id 9986) and cRAP
combined into 1 fasta file, downloaded on 08/05/2018

Search filters: missed cleavages ≤ 2, PSM FDR: 1%, unique peptide ≥1, protein score ≥ 25, precursor mass tolerance: 10 ppm,
fragment mass tolerance: 0.5 Da

BLAST was performed through Uniprot

Protein groups

I Vitellogenin

II SERPIN

III ACTIN

IV α2MG

Spot	Accession	Description	Organism	Avg. Mass	Protein score (-10lgP)	Coverage (%)	#Peptides	#Unique peptides	PTM
9	Q17174	GP80 (Fragment)	<i>Rhipicephalus microplus</i>	147,185	68.6	1	1	1	
9	B7Q406	Hemelipoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	93.7	3	3	3	Carbamidomethylation
9	B7QGE3	Hemelipoglyco-carrier protein (Fragment)	<i>Ixodes scapularis</i>	151,678	110.9	3	2	2	Deamidation (NQ)
9	A0A0D3RJ94	Hemelipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	165.5	6	6	2	Carbamidomethylation
9	V5H7G7	Putative vitellogenin-2	<i>Ixodes ricinus</i>	161,482	149.6	5	4	1	Carbamidomethylation
10	A0A0D3RJ94	Hemelipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	252.2	10	11	4	Carbamidomethylation; Oxidation (M)
10	V5GJN1	Putative vitellogenin-2	<i>Ixodes ricinus</i>	175,610	263.7	11	11	4	Carbamidomethylation; Oxidation (M)
11	B7Q407	Heme lipoprotein putative	<i>Ixodes scapularis</i>	152,642	254.5	15	14	1	Carbamidomethylation; Deamidation (NQ); Oxidation (M)

11	B7Q406	Hemelipoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	236.0	13	18	6	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
11	B7Q406	Hemelipoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	226.1	10	12	5	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
11	A0A0D3RJ94	Hemelipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	218.9	16	18	3	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
11	A0A0D3RJ94	Hemelipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	289.6	20	23	1	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
11	V5H7G7	Putative vitellogenin-2	<i>Ixodes ricinus</i>	161,482	205.9	15	17	1	Carbamidomethylation; Deamidation (NQ)
11	V5H7G7	Putative vitellogenin-2	<i>Ixodes ricinus</i>	161,482	285.5	24	25	1	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
12	Q17174	GP80 (Fragment)	<i>Rhipicephalus microplus</i>	147,185	55.4	1	1	1	
12	A0MVX0	Heme lipoprotein	<i>Amblyomma americanum</i>	177,038	89.5	2	3	1	
12	A0MVX0	Heme lipoprotein	<i>Amblyomma americanum</i>	177,038	88.5	2	3	1	
12	B7Q406	Hemelipoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	110.8	3	3	2	Carbamidomethylation
12	B7Q406	Hemelipoglyco-carrier protein	<i>Ixodes scapularis</i>	177,654	116.4	3	3	3	
12	B7QGE3	Hemelipoglyco-carrier protein	<i>Ixodes scapularis</i>	151,678	61.8	2	1	1	

		(Fragment)							
12	A0A0D3RJ94	Hemelipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	169.5	8	9	1	Deamidation (NQ); Oxidation (M)
12	A0A0D3RJ94	Hemelipoglyco-carrier protein CP3	<i>Ixodes ricinus</i>	175,839	185.9	9	10	1	Carbamidomethylation
12	V5GJN1	Putative vitellogenin-2	<i>Ixodes ricinus</i>	175,610	176.4	8	10	1	Deamidation (NQ); Oxidation (M)
12	V5GJN1	Putative vitellogenin-2	<i>Ixodes ricinus</i>	175,610	198.3	11	12	2	Carbamidomethylation
13	B7QGE3	Hemelipoglyco-carrier protein (Fragment)	<i>Ixodes scapularis</i>	151,678	68.0	2	1	1	
13	V5H7Z4	Putative alpha-macroglobulin	<i>Ixodes ricinus</i>	152,097	206.7	12	10	10	Carbamidomethylation
13	A0A147BKD0	Putative vitellogenin 2 (Fragment)	<i>Ixodes ricinus</i>	214,317	162.5	5	6	1	
13	B7Q7E5	Vitellogenin putative	<i>Ixodes scapularis</i>	184,771	160.2	7	7	2	
13	B7QJ67	Vitellogenin putative	<i>Ixodes scapularis</i>	218,884	80.8	1	2	2	Deamidation (NQ)
14	A0A147BKD0	Putative vitellogenin 2 (Fragment)	<i>Ixodes ricinus</i>	214,317	167.8	6	9	9	Carbamidomethylation; Deamidation (NQ); Oxidation (M)
15	A0A147BKD0	Putative vitellogenin 2 (Fragment)	<i>Ixodes ricinus</i>	214,317	168.3	6	9	9	Carbamidomethylation; Oxidation (M)
16	A0A293M4Q5	Actin (Fragment)	<i>Ornithodoros erraticus</i>	41,794	286.3	49	19	1	Carbamidomethylation; Deamidation (NQ); Oxidation (M); Acetylation (N-term)
16	A0A131XNB9	Putative actin-related protein (Fragment)	<i>Hyalomma excavatum</i>	40,482	288.8	58	20	2	Carbamidomethylation; Deamidation (NQ); Oxidation

									(M); Acetylation (N-term)
16	A0A147BWE2	Putative blood meal-induced serine protease inhibitor	Ixodes ricinus	43,541	222.1	48	12	11	Deamidation (NQ); Oxidation (M)
16	A0A0K8RCY5	Putative salivary serpin	Ixodes ricinus	43,570	221.8	37	9	3	Deamidation (NQ); Oxidation (M)
16	B7QL34	Secreted salivary gland peptide putative	Ixodes scapularis	43,546	188.8	26	7	1	Deamidation (NQ); Oxidation (M)

Table S3 - Shotgun analysis of protein extract from *Ixodes ricinus* saliva

Search algorithm: Peaks 8.5, Bioinformatics Solutions Inc., Waterloo, Canada									SERPIN
Database: Uniprot derived Ixodida(tax. Id 6935), Oryctolagus cuniculus (tax. Id 9986) and cRAP combined into 1 fasta file									microplusin
Search filters: missed cleavages ≤ 2, PSM FDR: 1%, unique peptide ≥1, protein score ≥ 25, precursor mass tolerance: 10 ppm, fragment mass tolerance: 0.5 Da									(chymo)trypsin inhibitor
Localization class was predicted through LocTree3 algorithm: LocTree3 prediction of localization Goldberg T, Hecht M, Hamp T, Karl T, Yachdav G, Ahmed N, Altermann U, Angerer P, Ansorge S, Balasz K, Bernhofer M, Betz A, Cizmadija L, Do KT, Gerke J, Greil R, Joerdens V, Hastreiter M, Hembach K, Herzog M, Kalemánov M, Kluge M, Meier A, Nasir H, Neumaier U, Prade V, Reeb J, Sorokoumov A, Troshani I, Vorberg S, Waldruff S, Zierer J, Nielsen H, Rost B. Nucleic Acids Research. 2014 Jul;42(Web Server issue):W350-5									metalloprotease
Nucleic Acids Research. 2014 Jul;42(Web Server issue):W350-5									Gly rich family
n/a - data not available									
									Rabbit protein
Accession	Description	Organism	Protein score (-10lgP)	#Peptides	#Unique peptides	Coverage (%)	Avg. protein mass (Da)	Localization	PTM
A0A0K8RHE3	Putative metalloprotease	Ixodes ricinus	101.1	1	1	5	27,889	secreted	
A0A0K8RKR5	Putative 24 kDa protein	Ixodes ricinus	106.4	2	2	14	24,996	secreted	Carbamidomethylation
A0A0K8RNV1	Putative til domain protein	Ixodes ricinus	109.0	2	2	20	11,485	secreted	Carbamidomethylation
A0A0K8RQG8	Putative metalloprotease (Fragment)	Ixodes ricinus	41.8	1	1	2	45,861	secreted	
A0A147BPH4	Putative serine proteinase inhibitor	Ixodes ricinus	46.3	1	1	9	43,456	secreted	Deamidation (NQ)
A0A147BVF3	Putative microplusin (Fragment)	Ixodes ricinus	149.6	3	3	25	10,106	n/a	Carbamidomethylation
B7Q2B8	Metalloprotease putative	Ixodes scapularis	77.0	1	1	3	53,772	secreted	Carbamidomethylation
B7Q406	Hemelipoglyco-carrier protein	Ixodes scapularis	79.3	2	1	1	177,654	secreted	Deamidation (NQ)
B7QKC1	Uncharacterized protein (Fragment)	Ixodes scapularis	82.1	1	1	20	6,755	secreted	Deamidation (NQ)
V5GJN1	Putative vitellogenin-2	Ixodes ricinus	237.7	10	9	11	175,610	secreted	Carbamidomethylation; Deamidation (NQ)
V5H5D1	Putative secreted protein	Ixodes ricinus	66.0	1	1	39	9,572	secreted	Carbamidomethylation
V5HLG8	Putative grp-2 471 glycine rich family (Fragment)	Ixodes ricinus	88.5	1	1	18	7,401	secreted	
V5IGT4	Uncharacterized protein	Ixodes ricinus	60.9	1	1	13	9,012	n/a	
A0A0A0MQP9	Protein S100	Oryctolagus cuniculus	89	1	1	12	14,568	secreted	

References

1. Hamsten C, Starkhammar M, Tran TA, et al. Identification of galactose-alpha-1,3-galactose in the gastrointestinal tract of the tick *Ixodes ricinus*; possible relationship with red meat allergy. *Allergy*. 2013;68(4):549-552.
2. Patton TG, Dietrich G, Brandt K, Dolan MC, Piesman J, Gilmore RD, Jr. Saliva, salivary gland, and hemolymph collection from *Ixodes scapularis* ticks. *J Vis Exp*. 2012(60).
3. Mehlich J, Fischer J, Hilger C, et al. The basophil activation test differentiates between patients with alpha-gal syndrome and asymptomatic alpha-gal sensitization. *J Allergy Clin Immun*. 2019;143(1):182-189.
4. Apostolovic D, Krstic M, Mihailovic J, et al. Peptidomics of an in vitro digested alpha-Gal carrying protein revealed IgE-reactive peptides. *Sci Rep*. 2017;7(1):5201.
5. Apostolovic D, Tran TA, Hamsten C, Starkhammar M, Cirkovic Velickovic T, van Hage M. Immunoproteomics of processed beef proteins reveal novel galactose-alpha-1,3-galactose-containing allergens. *Allergy*. 2014;69(10):1308-1315.
6. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature protocols*. 2006;1(6):2856-2860.
7. Smiljanic K, Apostolovic D, Trifunovic S, et al. Subpollen particles are rich carriers of major short ragweed allergens and NADH dehydrogenases: quantitative proteomic and allergomic study. *Clin Exp Allergy*. 2017;47(6):815-828.
8. Donohue KV, Khalil SM, Mitchell RD, Sonenshine DE, Roe RM. Molecular characterization of the major hemelipoglycoprotein in ixodid ticks. *Insect Mol Biol*. 2008;17(3):197-208.
9. Ramirez Rodriguez PB, Rosario Cruz R, Dominguez Garcia DI, et al. Identification of immunogenic proteins from ovarian tissue and recognized in larval extracts of *Rhipicephalus (Boophilus) microplus*, through an immunoproteomic approach. *Exp Parasitol*. 2016;170:227-235.
10. Boldbaatar D, Umemiya-Shirafuji R, Liao M, Tanaka T, Xuan X, Fujisaki K. Multiple vitellogenins from the *Haemaphysalis longicornis* tick are crucial for ovarian development. *J Insect Physiol*. 2010;56(11):1587-1598.