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 PhD²,³,⁸,⁹, Carl Hamsten, PhD¹*, Marianne van Hage, MD, PhD⁷*

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 kU/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. 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.

*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 analyzed 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 sensitivity\(^3\). 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-FcεRI (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-FcεRI (%CD63 allergen+/anti-FcεRI) 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 earliear. 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. The peptides obtained were analysed according to the method reported by Apostolovic et al. 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. 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 \[\left(\frac{\text{OD}_{\text{no inhibitor}} - \text{OD}_{\text{inhibitor}}}{\text{OD}_{\text{no inhibitor}}}\right) \times 100\]. 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).
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-FceRI (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.
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/l; range 1.9 - >100 kU/l) as the US patients (median 20.5 kU/l; range 1.5 - >100 kU/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/l; 0.11-40.0 kU/l) compared to larvae (0.73 kU/l, 0.13-20.1 kU/l) was noted.

Table S1. IgE levels of red meat allergic patients

| ID  | α-Gal (kU/l) | *Ixodes ricinus* (kU/l) | Total IgE (kU/l) |
|-----|--------------|------------------------|------------------|
|     | Adults       | Larvae                 |                  |
| S1  | 54           | 30                     | 12.1             |
| S2  | 23           | 40                     | 20.1             |
| S3  | 6.2          | 2.8                    | 2.62             |
| S4  | 24           | 10                     | 7.94             |
| S5  | 25           | 8.1                    | 15.8             |
| S6  | 23.1         | 0.44                   | 0.85             |
| S7  | >100         | 6.93                   | 18.0             |
| S8  | 2.84         | 0.64                   | 1.05             |
| S9  | >100         | 3.98                   | 6.31             |
| S10 | 6.3          | 0.27                   | 0.29             |
| S11 | 1.9          | <0.1                   | <0.1             |
| S12 | 19           | 0.28                   | 0.27             |
| S13 | 17           | 0.27                   | 0.43             |
| S14 | 100          | 1.31                   | 3.33             |
| S15 | 37           | 0.15                   | 0.44             |
| S16 | 4.8          | 0.18                   | 0.28             |
| S17 | 3.6          | 0.11                   | 0.13             |
| S18 | 63           | 0.33                   | 0.44             |
| S19 | 80           | 3.4                    | 3.7              |
| S20 | 5.2          | 0.13                   | 0.13             |
| S21 | 42.2         | 0.45                   | 0.19             |
| S22 | 95           | 4.63                   | 5.4              |
| S23 | 4.9          | 0.22                   | 0.11             |
| S24 | 94           | 1.0                    | 1.2              |
| S25 | 12           | 0.32                   | 0.2              |
| S26 | >100         | 11                     | 5.4              |
| S27 | >100         | 2.43                   | 2.36             |
| S28 | 87           | 0.83                   | 0.46             |
| S29 | 24           | 1.3                    | 1.6              |
| S30 | 13           | 0.13                   | <0.1             |

Table S1. IgE levels of red meat allergic patients
|   |   |   |   |   |
|---|---|---|---|---|
| S31 | 10 | 2.9 | 2.0 | 48 |
| S32 | 59 | 0.79 | 1.7 | 440 |
| Median and range | 24 | 0.81 | 0.73 | 60 |
|   | 1.9 - >100 | 0.11 - 40 | 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 | 0.62 | 0.83 |   |
|   | 1.5 - >100 | 0.1 - 8.02 | 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-FceRI (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 hemelipoglycoprotein contains several cleavage sites that generate subunits in the molecular range from 50-150kDa, a result which we also noted. In addition, these proteins have been shown to have a higher expression rate after the ticks were fed, 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

| Spot | Accession | Description | Organism | Avg. Mass | Protein score (-10lgP) | Coverage (%) | #Peptides | #Unique peptides | PTM |
|------|-----------|-------------|----------|-----------|------------------------|-------------|-----------|-----------------|-----|
| 1    | A0A131XR98| Putative sodium leak channel non-selective protein (Fragment) | *Ixodes ricinus* | 195,788   | 30.83                  | 1           | 1         | 1               | Deamidation (NQ) |
| 1    | B7QGE3    | Hemelipoglyco-carrier protein (Fragment) | *Ixodes scapularis* | 151,678   | 83.15                  | 3           | 2         | 1               | Carboxymethylation |
| 1    | B7Q406    | Hemelipoglyco-carrier protein | *Ixodes scapularis* | 177,654   | 89.57                  | 2           | 4         | 2               | Carboxymethylation |
| 1    | V5GJN1    | Putative vitellogenin-2 | *Ixodes ricinus* | 175,610   | 177.28                 | 14          | 15        | 3               | Carboxymethylation; Oxidation (M) |
| 1    | A0A0D3RJ94| Hemelipoglyco-carrier protein CP3 | *Ixodes ricinus* | 175,839   | 176.58                 | 14          | 16        | 4               | Carboxymethylation; Oxidation (M) |
| 1    | V5H340    | Putative vitellogenin-1 | *Ixodes ricinus* | 199,555   | 210.64                 | 21          | 28        | 28              | Carboxymethylation; Deamidation (NQ); Oxidation (M) |
| 2    | A0A0K8R491| Putative innexin (multimer) | *Ixodes ricinus* | 15,218    | 29.63                  | 14          | 1         | 1               | Carboxymethylation; Acetylation (N-term) |
| 2    | B7QGE3    | Hemelipoglyco-carrier protein (Fragment) | *Ixodes scapularis* | 151,678   | 68.15                  | 2           | 2         | 1               | Deamidation (NQ) |
| 2    | A0A147BA93| Hemelipoglycoprotein (Fragment) | Carios mimon | 96,026    | 69.91                  | 2           | 2         | 1               | Carboxymethylation |
|   | Accession     | Description                                      | Species       | MssM   | MssS   | MssE   | MssF   | MssG   | MssH   | Modification                                      |
|---|---------------|--------------------------------------------------|---------------|--------|--------|--------|--------|--------|--------|--------------------------------------------------|
| 2 | A0A0D3RJ94    | Hemelipoglyco-carrier protein CP3                | *Ixodes ricinus* | 175,839| 121.06 | 4      | 5      | 2      | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 2 | B7Q406        | Hemelipoglyco-carrier protein                    | *Ixodes scapularis* | 177,654| 98.24  | 4      | 6      | 5      | Carbamidomethylation                              |
| 2 | V5GJN1        | Putative vitellogenin-2                          | *Ixodes ricinus* | 175,610| 128.42 | 6      | 6      | 3      |                                                   |
| 2 | V5H340        | Putative vitellogenin-1                          | *Ixodes ricinus* | 199,555| 265.01 | 32     | 49     | 45     | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 3 | B7QGE3        | Hemelipoglyco-carrier protein (Fragment)        | *Ixodes scapularis* | 151,678| 84.55  | 2      | 2      | 1      | Deamidation (NQ)                                  |
| 3 | V5GJN1        | Putative vitellogenin-2                          | *Ixodes ricinus* | 175,610| 127.58 | 4      | 4      | 2      |                                                   |
| 3 | B7Q406        | Hemelipoglyco-carrier protein                    | *Ixodes ricinus* | 177,654| 105.83 | 4      | 5      | 4      |                                                   |
| 3 | A0A0D3RJ94    | Hemelipoglyco-carrier protein CP3                | *Ixodes ricinus* | 175,839| 177.62 | 4      | 6      | 4      | Deamidation (NQ)                                  |
| 3 | V5H340        | Putative vitellogenin-1                          | *Ixodes ricinus* | 199,555| 278.5  | 22     | 28     | 28     | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 4 | B7QGE3        | Hemelipoglyco-carrier protein (Fragment)        | *Ixodes scapularis* | 151,678| 81.73  | 2      | 2      | 1      | Deamidation (NQ)                                  |
| 4 | B7Q406        | Hemelipoglyco-carrier protein                    | *Ixodes scapularis* | 177,654| 101.29 | 4      | 6      | 3      | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 4 | V5H7G7        | Putative vitellogenin-2                          | *Ixodes ricinus* | 161,482| 158.06 | 8      | 8      | 1      | Carbamidomethylation; Oxidation (M)               |
| 4 | V5GJN1        | Putative vitellogenin-2                          | *Ixodes ricinus* | 175,610| 177.83 | 9      | 10     | 1      | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 4 | A0A0D3RJ94    | Hemelipoglyco-carrier protein CP3                | *Ixodes ricinus* | 175,839| 195.79 | 9      | 12     | 3      | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 5 | A0A0K8RCB1    | Putative enolase                                  | *Ixodes ricinus* | 47,145 | 61.66  | 3      | 1      | 1      |                                                   |
| 6 | A0A0D3RJ94    | Hemelipoglyco-carrier protein CP3                | *Ixodes ricinus* | 175,839| 281.02 | 13     | 13     | 4      | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 6 | V5GJN1        | Putative vitellogenin-2                          | *Ixodes ricinus* | 175,610| 283.27 | 15     | 14     | 5      | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 7 | A0A293M6L4    | Ribophorin II (Fragment)                         | *Ornithodoros erraticus* | 63,852 | 28.94  | 2      | 1      | 1      |                                                   |
| 7 | B7QGE3        | Hemelipoglyco-carrier protein (Fragment)        | *Ixodes scapularis* | 151,678| 88.21  | 2      | 3      | 1      | Deamidation (NQ)                                  |
| 7 | B7Q406        | Hemelipoglyco-carrier protein                    | *Ixodes scapularis* | 177,654| 158.84 | 9      | 13     | 4      | Deamidation (NQ); Oxidation (M)                  |
| ID   | Accession   | Description                                      | Species         | Mw (Da) | pI     | MImo | Mim | MId |
|------|-------------|--------------------------------------------------|-----------------|---------|--------|------|-----|-----|
| 7    | B7Q406      | Hemelipoglyco-carrier protein                    | *Ixodes scapularis* | 177,654 | 213.56 | 11   | 16  | 5   |
|      |             |                                                  |                 |         |         |      |     |     |
| 7    | A0A0D3RJ94  | Hemelipoglyco-carrier protein CP3                | *Ixodes ricinus* | 175,839 | 196.02 | 14   | 19  | 2   |
|      |             |                                                  |                 |         |         |      |     |     |
| 7    | V5H7G7      | Putative vitellogenin-2                          | *Ixodes ricinus* | 161,482 | 205.16 | 15   | 20  | 1   |
|      |             |                                                  |                 |         |         |      |     |     |
| 7    | A0A0D3RJ94  | Hemelipoglyco-carrier protein CP3                | *Ixodes ricinus* | 175,839 | 268.57 | 15   | 21  | 1   |
| 7    | V5H7G7      | Putative vitellogenin-2                          | *Ixodes ricinus* | 161,482 | 256.73 | 17   | 22  | 1   |
| 7    | A0A131Y9E6  | Putative vitellogenin-2 (Fragment)               | *Ixodes ricinus* | 132,382 | 269.08 | 22   | 22  | 3   |
| 8    | A0A131XRU7  | Putative catalytically inactive chitinase-like lectin (Fragment) | *Hyalomma excavatum* | 41,678 | 35.49  | 3    | 1   | 1   |
| 8    | B7PY91      | Secreted protein putative                        | *Ixodes scapularis* | 37,345 | 56.36  | 8    | 1   | 1   |
| 8    | B7Q5W3      | Serpin-2 precursor putative (Fragment)           | *Ixodes scapularis* | 41,636 | 77.36  | 7    | 2   | 2   |
| 8    | A0A023GGD8  | Putative actin (Fragment)                        | *Amblyomma triste* | 25,804 | 97.7   | 30   | 3   | 3   |
| 8    | A0A068EU07  | Blood meal-induced serine protease inhibitor     | *Ixodes scapularis* | 43,425 | 139.08 | 27   | 6   | 1   |
| 8    | B7QL34      | Secreted salivary gland peptide putative         | *Ixodes scapularis* | 43,546 | 150.43 | 28   | 7   | 1   |
| 8    | A0A0K8RCY5  | Putative salivary serpin                         | *Ixodes ricinus* | 43,570 | 197.39 | 38   | 9   | 3   |
| 8    | A0A147BWE2  | Putative blood meal-induced serine protease inhibitor | *Ixodes ricinus* | 43,541 | 187.3  | 41   | 10  | 5   |
**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

| Spot | Accession | Description | Organism | Avg. Mass | Protein score (-10lgP) | Coverag e (%) | #Peptides | #Unique peptides | PTM |
|------|-----------|-------------|----------|-----------|------------------------|---------------|------------|------------------|-----|
| 9    | Q17174    | GP80 (Fragment) | *Rhipicephalus microplus* | 147,185  | 68.6                    | 1             | 1          | 1                |     |
| 9    | B7Q406    | Hemelipoglyco-carrier protein | *Ixodes scapularis* | 177,654  | 93.7                    | 3             | 3          | 3                | Carbamidomethylation |
| 9    | B7QGE3    | Hemelipoglyco-carrier protein (Fragment) | *Ixodes scapularis* | 151,678  | 110.9                   | 3             | 2          | 2                | Deamidation (NQ) |
| 9    | A0A0D3RJ94 | Hemelipoglyco-carrier protein CP3 | *Ixodes ricinus* | 175,839  | 165.5                   | 6             | 6          | 2                | Carbamidomethylation |
| 9    | V5H7G7    | Putative vitellogenin-2 | *Ixodes ricinus* | 161,482  | 149.6                   | 5             | 4          | 1                | Carbamidomethylation |
| 10   | A0A0D3RJ94 | Hemelipoglyco-carrier protein CP3 | *Ixodes ricinus* | 175,839  | 252.2                   | 10            | 11         | 4                | Carbamidomethylation; Oxidation (M) |
| 10   | V5GJN1    | Putative vitellogenin-2 | *Ixodes ricinus* | 175,610  | 263.7                   | 11            | 11         | 4                | Carbamidomethylation; Oxidation (M) |
| 11   | B7Q407    | Heme lipoprotein putative | *Ixodes scapularis* | 152,642  | 254.5                   | 15            | 14         | 1                | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
|   | Accession | Protein Name                  | Species                  | MW [kDa] | Molar Extinction Coefficient | Charge | Molar Mass [Da] | Modification(s)                                      |
|---|-----------|-------------------------------|--------------------------|----------|-------------------------------|--------|-----------------|-----------------------------------------------------|
| 11| B7Q406    | Hemelipoglyco-carrier protein | *Ixodes scapularis*      | 177,654  | 236.0                         | 13     | 18              | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 11| B7Q406    | Hemelipoglyco-carrier protein | *Ixodes scapularis*      | 177,654  | 226.1                         | 10     | 12              | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 11| A0A0D3RJ94| Hemelipoglyco-carrier protein CP3 | *Ixodes ricinus*       | 175,839  | 218.9                         | 16     | 18              | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 11| A0A0D3RJ94| Hemelipoglyco-carrier protein CP3 | *Ixodes ricinus*       | 175,839  | 289.6                         | 20     | 23              | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 11| V5H7G7    | Putative vitellogenin-2       | *Ixodes ricinus*        | 161,482  | 205.9                         | 15     | 17              | Carbamidomethylation; Deamidation (NQ)               |
| 11| V5H7G7    | Putative vitellogenin-2       | *Ixodes ricinus*        | 161,482  | 285.5                         | 24     | 25              | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 12| Q17174    | GP80 (Fragment)              | *Rhipicephalus microplus* | 147,185  | 55.4                          | 1      | 1               |                                                     |
| 12| A0MVX0    | Heme lipoprotein              | *Amblyomma americanum*  | 177,038  | 89.5                          | 2      | 3               |                                                     |
| 12| A0MVX0    | Heme lipoprotein              | *Amblyomma americanum*  | 177,038  | 88.5                          | 2      | 3               |                                                     |
| 12| B7Q406    | Hemelipoglyco-carrier protein | *Ixodes scapularis*      | 177,654  | 110.8                         | 3      | 3               |                                                     |
| 12| B7Q406    | Hemelipoglyco-carrier protein | *Ixodes scapularis*      | 177,654  | 116.4                         | 3      | 3               |                                                     |
| 12| B7QGE3    | Hemelipoglyco-carrier protein | *Ixodes scapularis*      | 151,678  | 61.8                          | 2      | 1               |                                                     |
|   | Accession     | Description                        | Species     | Mass (kDa) | PSM | Ions | Modification                  |
|---|---------------|------------------------------------|-------------|-----------|-----|------|------------------------------|
| 12| A0A0D3RJ94    | Hemelipoglyco-carrier protein CP3  | *Ixodes ricinus* | 175,839   | 8   | 9    | Deamidation (NQ); Oxidation (M) |
| 12| A0A0D3RJ94    | Hemelipoglyco-carrier protein CP3  | *Ixodes ricinus* | 175,839   | 9   | 10   | Carbamidomethylation         |
| 12| V5GJN1        | Putative vitellogenin-2            | *Ixodes ricinus* | 175,610   | 10  | 1    | Deamidation (NQ); Oxidation (M) |
| 12| V5GJN1        | Putative vitellogenin-2            | *Ixodes ricinus* | 175,610   | 12  | 2    | Carbamidomethylation         |
| 13| B7QGE3        | Hemelipoglyco-carrier protein      | *Ixodes scapularis* (Fragment) | 151,678   | 1  | 1    | Carbamidomethylation         |
| 13| V5H724        | Putative alpha-macroglobulin       | *Ixodes ricinus* | 152,097   | 12  | 10   | Carbamidomethylation         |
| 13| A0A147BKD0    | Putative vitellogenin 2 (Fragment) | *Ixodes ricinus* | 214,317   | 6   | 9    | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 13| B7Q7E5        | Vitellogenin putative              | *Ixodes scapularis* | 184,771   | 7   | 7    | Carbamidomethylation         |
| 13| B7QJ67        | Vitellogenin putative              | *Ixodes scapularis* | 218,884   | 1   | 2    | Deamidation (NQ)             |
| 14| A0A147BKD0    | Putative vitellogenin 2 (Fragment) | *Ixodes ricinus* | 214,317   | 6   | 9    | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
| 15| A0A147BKD0    | Putative vitellogenin 2 (Fragment) | *Ixodes ricinus* | 214,317   | 6   | 9    | Carbamidomethylation; Oxidation (M) |
| 16| A0A293M4QS    | Actin (Fragment)                  | *Ornithodoros erraticus* | 41,794    | 49  | 19   | Carbamidomethylation; Deamidation (NQ); Oxidation (M); Acetylation (N-term) |
| 16| A0A131XN89    | Putative actin-related protein     | *Hyalomma excavatum* | 40,482    | 58  | 20   | Carbamidomethylation; Deamidation (NQ); Oxidation (M) |
|   | Accession     | Description                             | Species       | MW  | pI  | Score | Mods                      |
|---|---------------|-----------------------------------------|---------------|-----|-----|-------|---------------------------|
|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  
Database: Uniprot derived ixoida (tax. ID 6935), Oryctolagus cuniculus (tax. ID 9986) and cRAP combined into 1 fasta file  
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  
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, Anzorge S, Balasz K, Bernhofer M, Betz A, Cizmadija L, Do KT, Gerke J, Greil R, Joerdens V, Hastreiter M, Hembach K, Herzog M, Kalemanov M, Kluge M, Meier A, Nasir H, Neumaier U, Prade V, Reeb J, Sorokoumov A, Troshani I, Vorberg S, Waldraff S, Zierer J, Nielsen H, Rost B. Nucleic Acids Researc. 2014 Jul;42(Web Server issue):W350

| 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     | Carboxymethylation      |
| A0A0K8RRK5  | Putative 24 kDa protein                 | Ixodes ricinus    | 106.4                  | 2          | 2                | 14           | 24,996                 | secreted     | Carboxymethylation      |
| A0A0K8RNV1  | Putative til domain protein             | Ixodes ricinus    | 109.0                  | 2          | 2                | 20           | 11,485                 | secreted     | Carboxymethylation      |
| A0A0K8RQG8  | Putative metalloprotease (Fragment)     | Ixodes ricinus    | 41.8                   | 1          | 1                | 2            | 45,861                 | secreted     | Deamidation (NQ)        |
| A0A1478PH4  | 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          | Deamidation (NQ)        |
| B7Q2B     | Metalloprotease putative                | Ixodes scapularis | 77.0                   | 1          | 1                | 3            | 53,772                 | secreted     | Carboxymethylation      |
| B7Q406     | Hemelipoglyco-carrier protein           | Ixodes scapularis | 79.3                   | 2          | 1                | 1            | 177,654                | secreted     | Deamidation (NQ)        |
| B7OKC1     | 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     | Carboxymethylation; Deamidation (NQ) |
| V5HSD1     | Putative secreted protein               | Ixodes ricinus    | 66.0                   | 1          | 1                | 39           | 9,572                  | secreted     | Carboxymethylation      |
| V5HLG8     | Putative grp-2 471 glycine rich family (Fragment) | Ixodes ricinus | 88.5                   | 1          | 1                | 18           | 7,401                  | secreted     | Carboxymethylation      |
| 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.