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Immunogenomic analysis of insect bite hypersensitivity in a model horse population

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Abstract

Equine Insect bite hypersensitivity (IBH) is a seasonal IgE-mediated dermatosis caused by bites of insects of the genus *Culicoides*. A familial predisposition for the disease has been shown but, except for the MHC, the genes involved have not been identified so far. An immunogenomic analysis of IBH was performed in a model population of Old Kladrubby horses, all living in the same environment. Clinical signs of IBH were used as phenotypic manifestation of IBH. Furthermore, total serum IgE levels were determined in the sera of these horses and used as an independent phenotypic marker for the immunogenetic analysis. Single nucleotide polymorphisms (SNPs) in candidate immunity-related genes were used for association analyses. Genotypes composed of two to five genes encoding interferon gamma - *IFNG*, transforming growth factor beta 1 - *TGFB1*, Janus kinase 2 - *JAK2*, thymic stromal lymphopoietin - *TSLP*, and involucrin - *IVL* were associated with IBH, indicating a role of the genes in the pathogenesis of IBH. These findings were supported by analysis of gene expression in skin biopsies of 15 affected and 15 unaffected horses. Two markers associated with IBH, *IFNG* and *TGFB1*, showed differences in mRNA expression in skin biopsies from IBH-affected and non-affected horses ($p < 0.05$). Expression of the gene coding for the CD14 receptor molecule - *CD14* was different in skin biopsies at $p < 0.06$. When total IgE levels were treated as binary traits, genotypes of *IGHE*, *ELA-DRA*, and *IL10/b* were associated with this trait. When treated as a continuous trait, total IgE level were associated with genes *IGHE*, *FCER1A*, *IL4*, *IL4R*, *IL10*, *IL1RA*, and *JAK2*. This first report on non-MHC genes associated with IBH in horses is thus supported by differences in expression of genes known to play a role in allergy and immunity.

Keywords: insect bite hypersensitivity; horse; single nucleotide polymorphism; immunity related genes

1 Introduction

2 Insect bite hypersensitivity (IBH) is an important disease of the horse (Broström et al
3 1987; Björnsdóttir et al 2006; Cunningham and Dunkel, 2008; Marti et al., 2008; Schaffartzik
4 et al., 2012). It is mainly caused by IgE-mediated reactions to allergens present in the saliva
5 of biting insects (Hellberg et al., 2006). The mechanisms resulting to susceptibility or
6 resistance to IBH have not been completely clarified yet. IBH is a complex disease with
7 interplay of environmental and genetic factors. Midges (genus *Culicoides*) and possibly black
8 flies (family *Simuliidae*) are the most important groups of insects involved, although reactions
9 to antigens from black flies might be due to cross-reactivity (Schaffartzik et al., 2012).
10 Clinical manifestation of IBH is due to an allergic reaction sharing some similarities with
11 human atopic dermatitis (AD). IBH is also called summer eczema or summer dermatitis.
12 These names reflect clinical aspects as well as seasonality of the disease related to the
13 occurrence of the biting insects.

14 Genetic studies showed that horses from some families and breeds are more
15 susceptible than others (Lazary et al., 1994). The heritability for severity of summer eczema
16 was estimated at 0.30 in Icelandic horses (Eriksson et al. 2008) indicating that specific genes
17 can contribute to disease susceptibility. In humans, chromosome regions and specific genes
18 were found to be associated with allergies in general as well as with atopic dermatitis
19 (Hoffjan and Epplen, 2005).

20 Several approaches may be used for detecting genes contributing to
21 resistance/susceptibility to diseases. Genome-wide association studies (GWAS) using large
22 numbers of anonymous SNPs distributed over the genome are often used for this purpose. As
23 they do not always allow identification of specific genes, candidate gene studies can be a
24 useful tool. As compared to humans, domestic animal populations bred for a long time are
25 more homogenous and more informative, especially for clinically oriented association studies

with limited numbers of cases available. Well characterized small isolated model populations can even be valuable models for studies on allergic diseases (Laitinen, 2002). The Old Kladruby horse breed is a more than 400 year old population, with well-documented breeding records. This population is small, isolated and partially inbred. Its molecular genetic diversity in various types of markers has been characterized previously (Horin et al., 1998). All these horses have always been kept at the same place and always exposed to the same environmental factors, including biting insects. The occurrence of insect bite hypersensitivity in this population allowed using the population as an informative model.

Studies in humans have shown that the total serum IgE levels are influenced by genetic factors (Meyers et al. 1991; Weidinger et al. 2010). Some of these genes were found to be independent of the specific IgE response or of the susceptibility to atopy in humans as well as in animals (Delgado et al. 2010; Dizier et al. 1995; Meyers et al. 1991; Weidinger et al. 2010). Various studies have identified genes associated with total serum IgE levels (reviewed in Weidinger et al. 2011). In horses the genetic basis of total IgE levels has not been investigated so far, but a study in Lipizzan horses had estimated the heritability of mould specific IgE at 0.30 (Eder et al. 2001).

Based on the hypothesis that total IgE levels might be influenced by genetic factors in horses independently of the IBH status and that some of the candidate genes for atopy and the IgE response in general might be the same genes, the aim of this work was to investigate associations of selected candidate gene SNPs with total serum IgE levels and insect bite hypersensitivity and their expression in the population of Old Kladruby horses.

Materials and methods

The population and the disease

The nucleus population of the grey variety of the Old Kladruby horse kept always in a single location under the same conditions allowed a long-term follow-up, including clinical diagnostics, skin biopsies and analysis of gene expression. Sixty-one breeding mares, 4 to 22 years old, were available for the entire period of the study (10 years). Clinical manifestation of IBH - summer dermatitis - and total IgE levels were selected as phenotypic traits. Occurrence of clinical manifestations of IBH was recorded by the local veterinarian during at least 4 and maximum 10 seasons for individual horses. The intensity of clinical symptoms and the year of their onset varied among individual horses. The mean age of onset was between two and three years (2.7 yrs). The regions affected were, in order of appearance, mane, tail, *linea alba*, udder, head, chest and hind legs. In these regions, small itching areas and crusts extending progressively were observed. Due to extensive scratching, the areas affected were mutilated followed by appearance of bleeding wounds. Recurrence of the clinical signs in summer rather than quantitative expression was considered to be the attribute of susceptibility to IBH. Horses considered as resistant never showed even minor clinical signs of IBH during their life.

Entomological analysis showed occurrence of a variety of parasitic insect species with *Culicoides obsoletus* and *Odagmia ornata* representing species likely to cause IBH in this population (Sarkova, 2005). Altogether, twenty two mares were identified as susceptible to IBH and thirty-nine mares were considered to be resistant to IBH, as they had never had clinical signs of the disease during their lifespan. 19 stallions were used as sires of one to nine offspring during the period analysed.

Immunoblots on salivary gland extracts of Culicoides nubeculosus

Immunoblots on salivary gland extracts (SGE) from *Culicoides nubeculosus* were performed as described in Hellberg et al. (2006) with sera of 14 randomly selected IBH horses, because

of the limited availability of salivary gland extract. Briefly: SDS-PAGE was performed using the discontinuous system according to Laemmli (1970) on preparative 10-20% Tris HCl gels (BioRad, www.bio-rad.com) under denaturing reducing conditions using standard protocols. After electrophoresis, the proteins were electro-transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were dried and cut into strips. Horse sera were diluted 1:6 in washing buffer (20mM Tris, 0.25M NaCl, 0.125% Tween-20, pH7.5) and the strips incubated at 4°C overnight on a shaker. The following day the strips were washed and incubated with an anti-equine IgE mAb (0.5 µg/ml, 3H10, Wilson et al. 2006) for 3 h at RT on a shaker. After an additional washing step, the strips were incubated for 2h with horseradish peroxidase (HRP) conjugated goat anti-mouse-IgG ab (Sigma-Aldrich, www.sigmaaldrich.com) diluted 1:10,000 in washing buffer. Upon a final washing, the strips were developed with ECL Western blotting detection reagents (GE Healthcare Life Sciences, <http://www.gelifesciences.com>) according to the manufacturer's protocol.

Determination of total IgE levels in horse sera

Total serum IgE levels were determined by ELISA (enzyme linked immunoassay) as described in Wilson et al. (2006) and Scharrenberg et al. (2010). Briefly: Immunolon 2HB 96 well plates (Thermo Fisher Scientific, www.thermofisher.com) were coated with monoclonal anti-IgE 1C12 in 0.05 M carbonate buffer pH 9.6. Following blocking with PBS tween 0.5% BSA, sera (diluted 1:40 in blocking buffer) and standard samples were added to the plate, twofold serially diluted and incubated for 1.5 hours (h) at RT. A biotinylated anti-equine IgE mAb was added and incubated for 2 h, followed by incubation with extra-avidin alkaline phosphatase (Sigma-Aldrich; <http://www.sigmaaldrich.com>). The ELISA was developed with p-nitrophenyl phosphate. Plates were read when the OD 450 nm of the highest standard reached 1.00. As standard, duplicate samples of six doubling dilutions of a horse serum with a

known IgE concentration ranging from 128 - 4 ng/ml were used. The OD of the test samples were interpolated from the standard curve to give their IgE concentration in ng/ml. The concentration of the original test samples was calculated as the average of the values from those dilutions where the concentration fell within the standard curve.

Single nucleotide polymorphisms within candidate genes

SNP markers

46 SNP markers located in 29 candidate genes coding for CD14 receptor (*CD14*), Collagen, type VI, alpha 5 (*COL6A5*), MHC class II DQA (*ELA DQA*), MHC class II DRA (*ELA DRA*), Fc fragment of the IgE receptor, alpha chain (*FcER1A*), Interferon-induced guanylate-binding protein 1-like (*GBP1*), Histamine receptor H4 (*HRH4*), Interferon gamma (*IFNG*), Immunoglobulin heavy chain epsilon-like (*IGHE*), Interleukin 1 beta (*IL1B*), Interleukin 1 receptor antagonist (*IL1RN*), Interleukin 4 (*IL4*), Interleukin 4 receptor (*IL4R*), Interleukin 10 (*IL10*), Interleukin 12A (*IL12A*), Interleukin 12B (*IL12B*), Interleukin-12 receptor subunit beta-2 (*IL12RB2*), Interleukin-13 (*IL-13*), Interleukin-23 subunit p19 (*IL23A*), Involucrin (*IVL*), Janus kinase 1 (*JAK1*), Tyrosine-protein kinase JAK2-like (*JAK2*), Transforming growth factor beta 1 (*TGFB1*), Transforming growth factor beta 3 (*TGFB3*), Transforming growth factor beta receptor I (*TGFB1R*), Transforming growth factor, beta receptor II (*TGFB2R*), Tumor necrosis factor alpha (*TNFA*), Toll-like receptor 4 (*TLR4*), Thymic stromal lymphopoietin (*TSLP*) were used for analyzing the Old Kladruher population (Supplementary Table 1). Markers were identified by searching the Broad institute SNP database (<http://www.broad.mit.edu/mammals/horse/>) or newly developed by resequencing of pooled DNA samples from horses of the population under study (usually from five to ten horses). Horse-specific primers were designed using Primer3 software (<http://fokker.wi.mit.edu/primer3/>) on annotated genes from the NCBI horse genome

sequence assembly EcuCab2.0 (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9796) or genes predicted by BLAST search of horse genome for defined human/animal counterparts. Sequences with double or multiple peaks were aligned and analyzed by the BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and putative restriction enzyme cleavage sites were assigned by NEBcutter V2.0 (<http://tools.neb.com/NEBcutter2/index.php>).

Genotyping

In general, the total PCR reaction volume was 12.5 μ L with 1.25 μ L Hot Start Taq 10x buffer, 2.5 mM dNTPs, 5 pmol primers, 0.5 U Hot Star Taq polymerase (QIAGEN, Hilden, Germany), 100 ng DNA. The PCR protocol consisted usually of an initial denaturation at 95°C for 15min followed by 30 - 40 cycles of 30 sec at 94°C, 30 sec at annealing temperature (mostly 60°C), and extension at 72°C for 1min per kilobase, followed by a final extension at 68 – 72°C for 10 to 60 min. The primer sequences and SNP characteristics are given in Supplementary table 1. The majority of SNP markers were genotyped by PCR-RFLP. The fragments were run for 90 min at 300 V in 6% PAGE and silver stained. Enzymes (New England Biolabs, Ipswich, MA, USA) used for PCR-RFLP markers are in Supplementary table 2. If necessary, primers amplifying a PCR product of 200 to 400 bp were designed and SNPs were analyzed by PCR-SSCP.

MHC genes

Eqca-DRA exon 2 alleles. A RFLP-based genotyping system of *Eqca-DRA* alleles was developed based on the GenBank sequences available. Amplification of the 307 bp long product was carried out with standard primers Be3 and Be4 (Albright-Fraser et al., 1996). Aliquots of PCR products digested separately with restriction enzymes Cac8I, Hpy166II, BsaJI and produced specific fragments allowing identification of alleles *Eqca-DRA*0201*

(M60100), *Eqca-DRA*0301* (L47172), *Eqca-DRA*0401* (AJ575295) and *Eqca-DRA*0501* (FJ716134). The remaining allele *Eqca-DRA*0101* (L47174) could be identified by manual editing, subtracting sequences of the other alleles previously identified by RFLP. The nomenclature suggested by us previously (Janova et al., 2009) was used for designating *Eqca* alleles.

Eqca-DQA exon 2 alleles. *Eqca-DQA* genotypes were determined by PCR-SSCP according to the original method described by Fraser and Bailey (1998).

Data analysis

Clinical disease. 61 mares were analysed. The analysis was performed in two steps. After an initial scan allowing identification of individual markers potentially associated with IBH, complex genotypes integrating effects observed in previous analyses were identified and tested. A standard two-sided Fisher's exact test and Fisher's exact test based on Monte Carlo estimates (100 000 samples) of exact significance were used to assess associations between individual SNP alleles and/or genotypes and manifestation of the disease. Bonferroni corrections for multiple comparisons (numbers of markers analysed) were made in all tests. Odds ratios with 95% confidence intervals were computed on the basis of logistic regression analyses.

Total IgE levels. 58 mares were available for this analysis. Based on the sample distribution pattern (one-sample t-test), horses with total IgE levels higher than 32 µg/ml were identified as outliers ($p < 0.01$) and excluded. This selected group (N=50) was then analysed similarly to the complete group including outliers. Total IgE levels were treated in two ways. When the values of total IgE levels were treated as a continuous trait, effects of different alleles/genotypes on total IgE levels were tested by the standard non-parametric Mann

Whitney U test. For analysing them as a binary trait, cut-off values, which would allow binary coding of total IgE values were sought by an *a priori* performed association analysis, when significantly associated ($p \leq 0.1$) individual markers were selected in univariate logistic regression and used as input variables in the multivariate logistic regression model. Statistically conclusive associations were detected within the range 17-22 $\mu\text{g/ml}$. IBM SPSS For association analyses; IBM SPSS Advanced Statistics 19 for Windows (Release 19.0.1, © IBM Corporation 2010) was used.

Analysis of gene expression for selected markers

Genes associated with IBH at the population level (*IFNG*, *TGFB1*, *IVL*, *TSLP*, *CD14*) were analyzed for expression in peripheral blood leukocytes (PBL) and skin biopsies by real-time qPCR in 30 Kladruher horses.

Skin biopsies

Skin biopsies were collected by standard procedures from areas heavily affected with dermatitis in IBH-affected horses. Peripheral areas with signs of active dermatitis process but not damaged by scratching were biopsied. In non-affected horses, biopsies of intact skin were collected from the same areas. All biopsies were taken in summer. The samples were placed into RNALater solution (Qiagen, Carlsbad, CA) and stored at 4°C for 24 hours in order to allow the RNALater solution to penetrate into the tissue. After removing hair, the biopsies were stored at -21°C.

Stimulation of equine peripheral blood leukocytes

Due to low levels of expression of some genes in skin samples, leukocytes stimulated by phorbol and ionomycin were used for assessment of amplification efficacy. Peripheral blood

leukocytes were separated by hemolysis. Leukocyte suspension in the RPMI medium with 10% of fetal calf serum and antibiotics was incubated with 0.004 mg ionomycin and 0.0002 mg PMA at 37°C for 4 hours. After centrifugation, leukocytes were re-suspended in 1 ml TRI-RT solution (MRC, Cincinnati, OH, USA).

RNA extraction

Peripheral blood leukocytes. Total RNA from peripheral blood leukocytes (PBL) was isolated using TRIReagent-RT (MRC, UK), which eliminated all genomic DNA from RNA extracted, and subsequently purified on RNEasy Mini Kit columns (Qiagen, Carlsbad, CA) to eliminate phenol and other inhibitors of reverse transcription. Although this procedure itself avoided the possibility of genomic DNA being co-amplified, intron-spanning primers were designed, if possible. Only *CD14* and *Involucrin* gene sequences did not allow designing functional intron-spanning RT-PCR primers. RNA was then eluted to 20 µl and reverse transcribed by using 10 µl RNA oligoT a M-MLV (Invitrogen) in the final 20 µl volume. Following incubation at 37 °C for 1h 30, cDNA was diluted to the final volume 40 µl.

Skin biopsies. Biopsies stored in RNALater were dried and 10 mg of skin tissue were placed into 1 ml TRIReagent-RT (MRC), homogenized with 10 Zirconia/Silica Cat. No. 11079125z 2.3mm (BioSpec Products, Inc, USA) beads by using the MagnaLyzer (Roche, Switzerland). 100 µl bromanisol were added, and the samples were then treated as described above for PBL.

Real-time PCR

15 horses affected with IBH and 15 horses with no history or clinical signs of IBH at the time of sampling were analyzed. Gene expression was measured in triplicates. QuantiTect SYBR Green PCR Kit (Qiagen, USA) with 3 pmol of each primer in the final volume 3µl was used for real-time PCR on the LightCycler480 instrument (Roche, Switzerland). The PCR protocol was as follows: 15 min initial denaturation at 95 °C, 45 cycles at 95 °C 15 sec, 58 °C 30 sec

and 72 °C 30 sec, followed by PCR product melting temperature analysis (using continuous heating from 60 °C to 95 °C). cDNA obtained from stimulated leukocytes as described above was diluted 10x, 100x and 1000x, and used for measuring amplification efficacy for all primer pairs, the first three genes serving as reference genes. The primers for reference genes used for normalization were:

EqACTBF GGACCTGACGGACTACCTC

EqACTBR CACGCACGATTTCCTCTC

EqSDHAF GAGGAATGGTCTGGAATACTG

EqSDHAR GCCTCTGCTCCATAAATCG

EqHPRTe6F GCTTGCTGGTGAAAAGGACCCCTCG

EqHPRTe9R CACTAATGACACAAACGTGATTCAAATCCCTG

Analysis of variation in expression of candidate reference genes *SDHA*, *ACTb* a *HPRT* in skin and PBL samples was done by the geNORM software (Vandesompele et al., 2002). The least variation in expression was observed for the gene *HPRT*, which was therefore used as a reference gene for analysis. Real-time PCR was run as described above. The primers used for real-time PCR were:

EqIFNge3F GCCCAAAGCTAACCTGAGGAAGCGG

EqIFNge4R ATTGCAACGCTCTCCGGCCTCG

EqTGFb1e6F TCCACGAGCCCAAGGGCTACCAC

EqTGFb1e6e7R GGGCCAGGACCTTGCTGTACTGC

EqCD14F TTGCGCAAGCTCACTCACTCGC

EqCD14R CGCTCGCCCAGTCCAGGATTGTC

EqTSLPe2e3F2 AGACCGGCCGAGTTGTCTCACCG

EqTSLPe3R2 GGCAATGGCTGTTGAGCGTAGCG)

EqINVOFV3 GAAGGAAGGGTCTGCAGAGCAGC

EqINVORV3 CTGGCCGGGAGCTGGGACAAATG

EqJAK2IF GTGGCCTCAGATGTTTGGAGCTTTGG

EqJAK2IR TGCCAATCATACGCATAAATTCCGCTGG

The results obtained were expressed as ratio between expression of the gene of interest (goi) and expression of the reference gene (ref, here *HPRT*) weighed by amplification efficacy ($(1/\text{POWER}(E_{\text{goi}};Ct_{\text{goi}}))/(1/\text{POWER}(E_{\text{ref}};Ct_{\text{ref}}))$), (Zelnickova et al., 2008). Differences in gene expression between IBH-affected and non-affected groups or groups of different genotypes were examined by the Mann Whitney test.

Results

Clinical disease: summer dermatitis. After all corrections, significant associations of composed genotypes with IBH were found (Table 1). The genes whose SNPs were associated with summer dermatitis were interferon gamma (*IFNG*), transforming growth factor beta 1 (*TGFB1*), involucrin (*IVL*), Janus kinase 2 (*JAK2*) and thymic stromal lymphopoietin (*TSLP*) coding genes.

Total IgE levels. No difference in total IgE mean/median values between IBH-affected and non-affected horses was observed (19 361/21 309 vs. 17 430/12 510, respectively, $p=0.633$ in the Mann-Whitney U test). Genes associated with total IgE levels in the multivariate regression analysis are shown in Table 2. Associations with total IgE levels treated as a continuous trait are described in Table 3. Markers within *IGE*H and *IL10* genes were detected as associated by both methods used.

Immunoblots on salivary gland extracts of Culicoides nubeculosus

The immunoblots showed that overall, the sera of the Kladruby horses with IBH displayed IgE-binding to seven different protein bands of the salivary gland extract of similar molecular weights as those described previously (Hellberg et al., 2006), suggesting that they were

1 sensitized to similar *Culicoides* salivary glands allergens as the Icelandic horses living in
2 Switzerland.

3 *RNA expression.* Two markers associated with IBH, *IFNG* and *TGFB1* showed significant
4 differences in mRNA expression in skin biopsies from affected and non-affected horses
5 (Table 4) Non-significant ($p=0.065$) but yet 1.6 increase in *CD14* mRNA expression was
6 observed in IBH biopsies. No differences in mRNA expression of *JAK2* and *IVL* were found
7 in skin biopsies from affected and non-affected horses. Gene expression in peripheral blood
8 leukocytes was similar between IBH-affected and non-affected horses in all genes analyzed.

10 Discussion

11 In association analyses, phenotyping of the trait analysed is one of the crucial points.
12 Here, horses were classified as resistant or susceptible to clinical IBH based on presence or
13 absence of clinical manifestation of IBH over 4 to 10 summer seasons. The presence and
14 quantitative expression of IBH can be influenced by many non-genetic factors, such as the
15 degree of exposure to insect bites, seasonal variations, climatic variations between years etc.
16 Therefore, recurrence over seasons was selected as the main qualitative criterion of
17 susceptibility/resistance to IBH.

18 Total IgE levels were determined as an additional phenotypic marker. In humans, total
19 IgE levels are highly heritable (h^2 up to 0.8), less influenced by environmental factors and
20 correlate with clinical atopic dermatitis (Potaczek and Kabesch, 2012). It was shown
21 previously that total IgE levels are most probably strongly influenced by the degree of
22 infection with endoparasites in horses (Hamza et al.2010). Nonetheless, surprisingly, in one of
23 previous studies in horses of the Icelandic breed, horses affected with IBH had significantly
24 higher total serum IgE levels than non-affected control horses (Wilson et al. 2006). This may
25 be due to the fact that IBH-affected Icelandic horses often have very strong Th2-type, IgE-

mediated reactions (reviewed in Schaffartzik et al. 2012). We therefore investigated in this study whether total serum IgE levels could be associated with IBH in horses of another breed. The Kladruby horses were used for such a study because they live in the same stud, i.e. under similar environmental conditions. No differences in total IgE levels between IBH-affected and non-affected horses were observed. The association study showed that the IgE response and atopy behave as two different phenotypes, underlain by two different gene sets in the Kladruber horses.

All candidate genes were selected based on their role in immune responses and allergic reactions. Small, isolated and genetically defined populations were used successfully for analysis of human allergic disease. Major advantages of using such specific populations are a lower genetic variation, a higher predictability and more information on disease phenotype and environmental factors available (Laitinen, 2002). Candidate gene studies of canine atopic dermatitis revealed a need of breed and location specific analyses to increase the likelihood of finding associations with the disease (Wood et al. 2010). The Old Kladruber population is a small but definite and genetically homogenous group (Horin et al., 1998). Its small size and weak effects of individual genes did not allow a robust statistical identification of single marker associations. After Bonferroni corrections, no individual marker was associated with IBH at $p < 0.05$ (data not shown). However, combinations of genes associated with resistance/susceptibility to IBH in composed genotypes led to increased significance and strength of associations between composed genotypes and the disease, which may be interpreted in terms of a synergic (additive) action of the genes involved. Complex genotypes thus allowed identification of genes with weak individual effects. Sometimes, different SNPs located within the same genes were involved in composed genotypes associated with IBH. This probably reflects variation in numbers of corresponding genotypes present in the population, low numbers compromising statistical significance.

1 The Kladruby population is stratified due to inbreeding. In all cases, only results
 2 withstanding conservative Bonferroni corrections reflecting the numbers of markers studied
 3 were considered to minimize the risk of false positive results. No effect of age was observed.
 4 Similar results with lower p values due probably to smaller numbers were obtained by
 5 analyzing age-matched groups (data not shown). The data obtained are supported by similar
 6 observations made by us in a genetically distinct population of Icelandic horses where
 7 composed genotypes involving the same *TSLP* and *CD14* SNPs were associated with IBH
 8 (Vychodilova et al., submitted). Moreover, markers biologically related to IBH were
 9 identified, while different, but again biologically plausible markers showed associations with
 10 other phenotypes in the same group of mares with the same marker panel - total IgE levels
 11 (this study), and melanoma (Futas et al., 2012). Results of mRNA expression analysis and
 12 data from human and mouse studies are in agreement with the assumption of biological
 13 plausibility of the associations observed. Immunity-related genes associated with IBH in this
 14 study were reported to be associated with atopic dermatitis in humans (Arkwright et al., 2001;
 15 Nomura et al., 2003; Litonjua et al., 2005). Similarly to interferon gamma, JAK kinases are
 16 also expressed in keratinocytes. TSLP was shown to have a wide range of effects on cells of
 17 the immune system and on allergic inflammation (Roan et al., 2012). Associations between
 18 the TSLP-receptor encoding gene and atopic dermatitis were identified in several dog breeds
 19 (Wood et al. 2010). Associations of atopic disease with *CD14* polymorphisms (Litonjua et al.,
 20 2005), supported by functional studies (Sümegei et al. 2007) were found in humans. Besides
 21 IR markers, genes related to skin structure and function were also associated with human AD
 22 (Boguniewicz and Leung, 2011). Involucrin (*IVL*) is a specialized cytoplasmatic protein of the
 23 keratinocytes, major cells responsible for the structure of epidermis (Eckert and Green, 1986).
 24 RNA expression analysis showed down-regulation of interferon gamma production and up-
 25 regulation of *TGFB1*, *IVL* and *CD14* gene activity in the affected skin, similarly to human

atopic dermatitis. The differences observed for *IVL* and *CD14* were not significant, but the *p* values ranged between 0.05-0.10. No significant differences in TSLP expression were found in biopsies from affected horses compared to controls, while it was shown to be up-regulated in AD lesion of human patients. Although not significant ($p < 0.1$), expression of TSLP was lower in skin biopsies from IBH-affected compared to unaffected horses. The reason for this difference verified in two repeated tests is not clear – perhaps it could result from heterogeneity in TSLP expression in different parts of atopic skin regions or may be explained by the stage of the disease when biopsies were taken: biopsies were possibly taken too long after insect bites had occurred and TSLP was no longer increased. The timing of biopsy collection is probably a major limitation of this kind of studies. However, significant differences in gene expression between IBH skin lesion and skin from control horses were identified in a similar study in horses (Heimann et al. 2011). Transcription of *TGFB1* and *TGFB3* was shown to be enhanced in the regenerating epidermis and dermis in humans and pigs (Cox, 1995). Decreased expression of IL-10 and TGF- β 1 molecules was found to be involved in IBH in horses (Hamza et al., 2008). Here, we found no direct evidence for involvement of *TGFB3* in IBH. However, a strong association of IBH with the microsatellite marker *AHT004* closely linked to *TGFB3* on the horse chromosome 24 was observed in this group (data not shown). Moreover, *TGFB3* was associated with IBH in our study of Icelandic horses (Vychodilova et al., submitted).

Other genes were associated with total IgE levels, reflecting complex relationships between total IgE levels and clinical disease (Raap et al., 2012). In theory, genes associated with IgE levels may affect total antibody levels, total IgE levels as well as levels of specific IgE. Associations of IgE heavy chain (*IGH*) SNP markers with total IgE levels were identified by both statistical approaches used. SNPs in this gene seem to influence IgE levels, but with no association with clinical IBH. A variety of other immunity-related genes may

1 affect IgE antibody responses with or without associations with clinical disease (Choi et al.
2 2012). Associations of total IgE levels with the *FcER1A* gene correspond to recent findings in
3 humans (Potaczek and Kabesch, 2012). Curik et al. (2003) found associations between *ELA*
4 and levels of IgE antibodies against mould antigens.

5 Although they cannot reveal novel genes involved, studies of individual candidate loci
6 can be useful when genome-wide studies produce non-informative results or for a more
7 precise definition of mechanisms of the disease studied (Akey, 2009). This was also
8 confirmed for equine IBH by Andersson et al. (2012). They observed associations between
9 MHC class II region markers and IBH. Here, we found associations only with total IgE levels
10 due probably to limited polymorphism of the single *DRA* marker we used.

11 Taken together, our results identified genes associated with the equine IBH and IgE
12 levels similar to those reported in human atopic dermatitis. We showed differences in
13 expression of some of the IBH-associated genes in the affected and non-affected skin which
14 suggests potential functional importance of the statistical associations observed.

15 In general, the results of statistical analyses confirmed by gene expression data and by
16 similar observations in a genetically distinct population suggest that the role of the genes
17 involved merit to be further investigated. In specific terms, the results valid for this definite
18 endangered population can be used for designing a conservation programme aiming to reduce
19 the incidence of IBH and to maintain at the same time its diversity and specificity.

20 **Conflict of interest statement**

21 We disclose any financial and personal relationships with other people or organizations that
22 could inappropriately influence their work.

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Table 1 Composed genotypes associated with clinical summer dermatitis (R resistant, S susceptible)

Genotype associated	Horses tested (N)	Population frequency	Frequency in R	Frequency in S	p-value	p-value corrected	Odds ratio (CI 95%)
<i>IFNG/a (36 CG)/TGFB1(139 AA)</i>	61	0.246	0.359 (14/39)	0.045 (1/22)	0.006	0.042	0.085 (0.010 – 0.701)
<i>IFNG/a (36 CC or GG)/IVL/a (261 TC)</i>	58	0.172	0.053 (2/38)	0.400 (8/20)	0.002	0.013	12.000 (2.233 – 64.489)
<i>IFNG/a(36 CG)/JAK2/b(463 CT or TT)</i>	61	0.525	0.667 (26/39)	0.273 (6/22)	0.004	0.027	0.188 (0.059 – 0.592)
<i>IFNG/a(36 CG)/TSLP/b (130 GC or GG)</i>	61	0.295	0.436 (17/39)	0.045 (1/22)	0.001	0.008	0.062 (0.008 – 0.505)
<i>TGFB1(139 AA)/IVL/c (873/873 or 840/840)</i>	57	0.298	0.421 (16/38)	0.053 (1/19)	0.005	0.034	0.076 (0.009 – 0.633)
<i>TGFB1 (139 CC or CA)/TSLP/a (217 CC or CG)</i>	60	0.550	0.395 (15/38)	0.818 (18/22)	0.003	0.019	6.900 (1.950 – 24.415)
<i>TGFB1 (139 CC or CA)/TSLP/b (130 CC)</i>	61	0.328	0.179 (7/39)	0.591 (13/22)	0.002	0.012	6.603 (2.030 – 21.478)
<i>IVL/a (261 CC or TT)/JAK2/b(463 CT or TT)</i>	58	0.517	0.658 (25/38)	0.250 (5/20)	0.005	0.037	0.173 (0.051 – 0.584)
<i>IVL/c (873/873 or 840/840)/JAK2/b(463 CT or TT)</i>	57	0.632	0.789 (30/38)	0.316 (6/19)	0.001	0.007	0.123 (0.036 – 0.426)

Table 2 IgE level in association analyses – multivariate logistic regression model

Marker	IgE cutoff (μ g/ml)	Without outliers (N _{total} = 50)		With outliers (N _{total} = 58)	
		p-value	OR (95% CI)	p-value	OR (95% CI)
<i>IGHE/b</i>	17.0	0.043	26.955 (1.111; 654.046)		
<i>IGHE/a</i>	18.0	0.037	15.793 (1.180; 211.309)	0.030	13.626 (1.283; 144.715)
<i>FCER1A/b</i>	21.5	0.044	4.385 (1.041; 18.465)		
	22.0	0.014	10.766 (1.609; 72.012)		
<i>IL4</i>	18.0	0.056	0.290 (0.081; 1.031)		
<i>IL4R*</i>	18.0			0.033	0.157 (0.029; 0.861)
	19.0			0.025	0.139 (0.025; 0.778)
	20.0			0.045	0.190 (0.038; 0.963)
<i>IL4R</i>	20.0	0.008	0.239 (0.082; 0.693)		
	20.5	0.044	0.378 (0.147; 0.972)		
<i>IL10/b</i>	22.0			0.043	0.232 (0.056; 0.958)
<i>IL1RA</i>	17.0			0.037	0.221 (0.054; 0.910)
	19.0	0.075	0.230 (0.046; 1.163)	0.039	0.265 (0.075; 0.933)
<i>IL1RA</i>	20.5	0.064	0.263 (0.064; 1.082)	0.047	0.278 (0.079; 0.982)
<i>JAK2/b</i>	17.0	0.040	5.240 (1.077; 25.497)	0.105	2.450 (0.829; 7.242)
	18.0	0.048	3.098 (1.011; 9.489)	0.011	4.454 (1.417; 14.002)
<i>JAK2/b</i>	19.0	0.071	7.607 (0.841; 68.816)	0.023	9.814 (1.366; 70.530)

* Multiple values for the same marker refer to different alleles/genotypes associated

Table 3 Markers and genotypes associated with total IgE levels¹

Marker	Genotype	N	Total IgE level (ng/ml) Median	p-value
<i>IGHE/b</i>	allele A	11	24 428	0.020
	allele G	105	12 510	
<i>IGHE/a</i>	TT	51	11 757	0.018
	CT	7	24 498	
	allele C	7	24 498	0.021
	allele T	109	13 116	
<i>ELA-DRA</i>	<i>DRA*0201/-</i>	55	16 859	0.013
	others	3	4 513	
<i>IL10/b</i>	het CG	25	10 400	0.035
	CC+GG	33	19 763	
<i>COL29a1</i>	het CT	33	19 763	0.045
	CC+TT	25	10 400	

¹ Mann Whitney test; het heterozygote

Table 4 mRNA expression of selected genes in skin biopsies (S) and peripheral blood leukocytes (B); significant p-values in **bold**

gene	p-value*	fold Increase mean	IBH	mean	median	SE	SD	variance	tissue
<i>TGFB1</i>	0.0121	1.2736	+	0.3985	0.3855	0.0283	0.1097	0.0120	S
			-	0.3129	0.3007	0.0153	0.0595	0.0035	S
	0.4186		+	0.3985	1,6195	0.0283	0.1097	0.0120	B
<i>CD14</i>	0.0591	1.582	-	1.8290	1,3777	0.4090	1.5839	2.5089	B
			+	1.1432	1.0059	0.1570	0.6081	0.3698	S
	0.4186		-	0.7227	0.7121	0.0622	0.2410	0.0580	S
<i>IFNG</i>	0.0020	0.0589	+	1.6038	1,6650	0.1837	0.7117	0.5065	B
			-	1.4381	1,1989	0.1926	0.7459	0.5564	B
	0.8846		+	0.0582	0.0561	0.0047	0.0183	0.0003	S
<i>JAK2</i>	0.5476	1.0441	-	0.0988	0.0785	0.0115	0.0446	0.0020	S
			+	0.0690	0.0545	0.0093	0.0361	0.0013	B
	0.3952		-	0.0688	0.0632	0.0091	0.0352	0.0012	B
<i>IVL**</i>	0.0929	2.4470	+	0.8820	0.9049	0.0502	0.1945	0.0378	S
			-	0.8447	0.8090	0.0529	0.2049	0.0420	S
			+	0.6963	0.5875	0.1033	0.4002	0.1602	B
<i>TSLP</i>	0.093	0.841	-	0.6100	0.4379	0.1148	0.4444	0.1975	B
			+	1.5032	0.8825	0.4962	1.9216	3.6927	S
	NT	NT	-	0.6143	0.6000	0.0568	0.2200	0.0484	S
			+	0.6726	0.5374	0.0703	0.2724	0.0742	S
			-	0.7996	0.7708	0.0593	0.2300	0.0528	S
			+	0.2646	0.2348	0.0424	0.1641	0.0269	B
			-	0.2577	0.2090	0.0330	0.1277	0.0163	B