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Case Report

Interleukin 31 and targeted vaccination in a case series of six horses with chronic pruritus

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Summary

Chronic pruritus is defined as prolonged itching symptoms associated with a variety of skin conditions. These pruritic conditions clinically manifest in a dermatitis phenotype and commonly are of allergic origin with hypersensitivities towards environmental allergens. Interleukin-31 (IL-31) is a common player in allergic pruritus across species. The objective of the study was evaluation of the clinical efficacy of a therapeutic vaccine targeting IL-31 in horses with chronic pruritus of unknown origin (CPUO) and that could not be explained by insect bite hypersensitivity (IBH). This consecutive case series pilot study included client-owned horses with a long history of CPUO. Four horses affected by year-round CPUO were vaccinated with a vaccine consisting of equine IL-31 (eIL-31) covalently coupled to a virus-like particle (VLP) derived from cucumber mosaic virus containing a tetanus toxoid universal T cell epitope ($CuMV_{TT}$). Clinical signs and pruritic behaviour were documented by photography and owner questionnaire pre and post vaccination. In addition, in three CPUO horses, levels of IL-31, thymic stromal lymphopoietin (TSLP) and monocyte chemoattractant protein 1 (MCP-1) were quantified from skin punch biopsies. IL-31, TSLP and MCP-1 levels were upregulated in pruritic, alopecic skin lesions compared to healthy skin of the same horse. Clinical signs and pruritic behaviour improved in all four horses upon vaccination with eIL-31-$CuMV_{TT}$ vaccine. The vaccine was well tolerated without safety concerns throughout the study. The main limitations of this study are the absence control treated horses and allergy diagnostics. It was concluded that Anti-IL-31 therapy might be applied as an allergen-independent treatment option for horses with CPUO overcoming the challenges of identifying the allergic trigger.

Introduction

Chronic pruritus is defined by clinical signs of itching lasting longer than 6 weeks (Ikoma *et al*. 2006; Stander *et al*. 2007). In horses, chronic pruritus causes self-inflicted trauma, alopecia and secondary skin lesions. These lesions have a considerable impact on the quality of life of those affected individuals and often render horses unsound for work. The treatment of chronic pruritus is a serious challenge and depends on its cause. Numerous different causes such as hypersensitivity reactions to insect bites, environmental, food or contact allergens may lead to the development of chronic allergic pruritus (Fadok 1995; Fadok 2013; White 2015). Often the causative allergen is not known, making chronic pruritus of unknown origin (CPUO) difficult to treat. However, knowing the allergen is a prerequisite for allergen-specific immunotherapy (ASIT), the current treatment approach of CPUO relies on symptomatic treatment using a combination of topical and systemic therapies including antihistamines, essential fatty acids, pentoxifylline and glucocorticoids (Fadok 1995; Nowak and Yeung 2017). In addition, the therapy of horses with food hypersensitivity requires a cumbersome identification of the causative allergen(s) followed by the avoidance of the respective allergen(s).

Several molecular mechanisms are involved in the development of acute and chronic pruritus. The histamine-dependent (histaminergic) pathway is the most well-known mediator for acute pruritus in the context of allergies (White 1990). The pathophysiology of the histamine-independent (nonhistaminergic) and chronic pruritus is very complex and still not completely understood, particularly in animals. Pruritus originates in the epidermis and dermal–epidermal junction and is peripherally transmitted by two types of itch-selective C nerve fibres. Some of these fibres are sensitive to histamine, but the majority are not. The latter histamine-independent C fibres are also called cowhage-sensitive fibres (reviewed in Besner Morin and Misery 2019). A complex interplay between cowhage fibres and T cells, mast cells, neutrophils, eosinophils and keratinocytes, along with the release of cytokines, proteases and neuropeptides lead to exacerbations of pruritus (Yosipovitch and Bernhard 2013). This histamine-independent pruritus involves, amongst other mediators, the inflammatory cytokine IL-31 (Scott *et al*. 2004; Wilson *et al*. 2013; Yamada *et al*. 2018; Besner Morin and Misery 2019).

Thymic stromal lymphopoietin (TSLP) triggers scratching behaviour in mice by acting on sensory neurons and is produced by inflammatory cells leading to an allergen-dependent activation of Th2 cells followed by subsequent IL-31 secretion (Potenzieri and Undem 2012; Wilson *et al*. 2013). The IL-31 receptor is found on subsets of nociceptive neurons in the dorsal root ganglia (DRG) (Cevikbas *et al*. 2014). Therefore, IL-31 may directly mediate itch signals from the immune system into peripheral nerves supporting the neuro-immune crosstalk (Sonkoly *et al*. 2006; Mizuno *et al*. 2009). In addition, the IL-31 receptor is expressed on other cell types, including keratinocytes, macrophages and eosinophils (Zhang *et al*. 2008; Kasraie *et al*. 2010, 2011). Numerous

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studies in humans found that IL-31 is highly upregulated in the skin of patients with pruritic skin diseases such as atopic dermatitis. Also, in mice IL-31 levels were increased in a model of atopic dermatitis and transgenic overexpression of IL-31 resulted in the development of severe pruritus and skin lesions (reviewed in Dillon *et al*. 2004; Potenzieri and Undem 2012). Blocking IL-31 via anti-IL-31 antibodies decreased scratching behaviour in the murine atopic dermatitis model (Grimstad *et al*. 2009; Potenzieri and Undem 2012). Recently, we showed that there is an exclusive expression of IL-31 in pruritic skin lesions of horses with insect bite hypersensitivity (IBH) in contrast to healthy equine skin samples (Olomski *et al*. 2019). Furthermore, when targeting IL-31 with our eIL-31-CuMV$_{TT}$ vaccine, induction of auto-antibodies led to a reduction of clinical signs in horses with IBH compared to placebo treatment and previous IBH seasons without treatment (Olomski *et al*. 2019).

Here we show that IL-31 is upregulated in skin biopsies of three horses with chronic pruritus, while being absent in healthy skin of the same horses. Also, we present a pilot study consisting of a consecutive case series of four horses with chronic pruritus, which could not be explained by IBH. These horses differed in breed and age and were vaccinated with our eIL-31-CuMV$_{TT}$ vaccine. Clinical signs and pruritic behaviour were assessed prior and post vaccination. A positive response was documented upon treatment with the eIL-31-CuMV$_{TT}$ vaccine in this pilot study, thus providing support for conducting a prospective controlled clinical trial in horses with chronic pruritus independent of the allergen.

Materials and methods

Horses and clinical study

Prior to studies in client-owned horses, the vaccine was tested extensively in *in vitro* and in murine safety and efficacy studies using the respective murine analogue vaccine. Moreover, target animal safety (TAS) studies in horses were performed. Such testings follow strict regulatory rules in order to ensure safety, quality and efficacy. Subsequent to preclinical testing and TAS studies, these clinical studies were conducted during 2018 and 2019 and had been approved by the respective cantonal veterinary authorities. All vaccinated horses were monitored according to approved score sheets. Of note, no adverse reactions were found. All horse owners signed informed consent.

Biopsies

Three horses with chronic allergic or hypersensitivity induced pruritus and without clinical signs compatible with IBH (i.e. pruritus and alopecia, papulocrusting and scaling dermatitis and excoriations affecting the base of the mane, tail, ears, intermandibular space, axillae, ventral midline and groyne with a seasonality at least during initial years reflecting the insect season) were included for biopsy collection during their symptomatic period, which was autumn to winter. Biopsies of affected lesional skin (fresh lesion) and healthy nonlesional skin were collected at the same timepoint for each horse: Horse A with chronic pruritus due to exposure to bird mites from co-housed chicken (confirmed by exclusion procedure), biopsy collected in December 2019, pruritus since February 2017; Horse B with suspected drug induced (penicillin, in August 2018) pruritus, which persisted over 6 weeks after drug withdrawal, biopsy collected in October 2018; Horse C, also presented as case report horse, Horse number 3, biopsy collected in December 2018, pruritus since autumn 2013 and severe pruritus from winter 2014/2015. Only Horse C was included in the clinical case series.

Clinical case series

Four horses; Horse 1 (pruritus since 2015), 2 (pruritus since 2014), 3 (see Horse C), 4 (pruritus since April 2017); with CPUO, persistent during the whole year and without clinical signs compatible with IBH, were included in the study. Clinical signs were recorded by photography of pruritic alopecic skin lesions before and at several time points post vaccination. In addition, the owners were frequently questioned on the pruritic behaviour of the horses and filled out a survey including a visual analogue scale after the third vaccination (https://de.surveymonkey.com/r/258372L).

Punch biopsies

Two mm skin punch biopsies of Horses A, B and C were taken from fresh lesional skin and from nonlesional skin from each horse and were collected into RNAlater™ Stabilisation Solution[1] for RNA extraction and quantitative PCR (qPCR).

RNA extraction and qPCR

Total RNA was extracted from punch biopsies using RNAqueous-Micro Kit[1]. Extraction was performed according to the manufacturer's protocol including DNase I treatment and inactivation. RNA was transcribed into cDNA using Reverse Transcription System[2]. Equine IL-31, monocyte chemoattractant protein 1 (MCP-1), TSLP and housekeeping gene βactin mRNA levels were quantified by qPCR. All qPCR experiments were performed using FastStart Universal SYBR Green Master[3] with duplicate samples on a Viia7 Real-Time PCR System[1], including negative (water) and positive control (IL-31 plasmid) runs. Gene expression levels were normalised by βactin expression. Primers are listed in **Supplementary item 7**. The IL-31 primer was designed by us, βactin (Bogaert *et al*. 2006), MCP-1 (Benarafa *et al*. 2000) and TSLP (Klukowska-Rotzler *et al*. 2012) were previously published.

Cloning, expression and purification of recombinant eIL-31 in E. coli

Described by Olomski *et al*. (2019).

Cloning, expression and purification of recombinant eIL-31 in eukaryotic HEK cells

Eukaryotic eIL-31-C-Strep was designed in silico by codon optimised mature eIL-31-coding DNA (mature Interleukin-31, equus caballus; UniProt F7AHG9, amino acid 27-152) which was C-terminally linked to a Strep-tag-coding sequence followed by a stop codon. The resulting fragment was termed eIL-31-C-Strep, synthesised[4] and integrated into the pCB-14 expression vector by isothermal cloning with HiFi Master Mix[5]. HEK 293T cells were grown to 80% confluency. Transfections were performed with Polyethylenimine[6] at a Polyethylenimine:DNA ratio of 2.5. Supernatant was harvested 5 and 13 days after transfection and purified via the Strep-tag by Strep-Tactin[7].

Circular dichroism (CD) spectroscopy of purified eIL-31-C-His & eIL-31-C-Strep

The far-UV CD spectrum of purified eIL-31-C-His and eIL-31-C-Strep (in phosphate buffered saline [PBS]) was measured on a J-710 spectropolarimeter[8] at 25°C using a 1 mm cuvette. After

correction for the buffer spectrum, ellipticity was converted to mean residue ellipticity as described (Milburn *et al*. 1993).

Coupling of eIL-31 to CuMV$_{TT}$

Described by Olomski *et al*. (2019).

Vaccine analysis by SDS-PAGE and Coomassie staining, Western Blot and endotoxin testing

Described by Olomski *et al*. (2019). Coupling efficiency was evaluated from the chromatogram of the last polishing step when comparing Area Under the Curve (AUC) values of vaccine peak vs. free, uncoupled eIL-31-C-His peaks compared to the amount of eIL-31-C-His added to the coupling reaction (**Supplementary item 1**). Vaccines were tested for endotoxin content by PyroGene™ recombinant Factor C Assay[9] and showed values lower than 200 endotoxin unit (EU)/mg.

Blood withdrawal from horses

Blood was collected from the jugular vein at the intersection of the proximal to middle third of the neck. Blood was taken on the day of the 1st vaccination (Day 0), and 2–4 weeks post the second and third vaccinations. Blood for serum was collected into serum tubes provided by IDEXX Diavet (Bäch, Switzerland) and centrifuged at 1200 ***g*** for 10 min, serum was transferred into fresh tubes.

Vaccine administration, immunisation regimen

To generate vaccine-induced self-reactive antibodies to equine IL-31, horses were injected subcutaneously with 300 μg of eIL31-C-His-CuMV$_{TT}$ VLP in 1000 μL of 20 mM NaP/ 2 mM EDTA, pH 7.5 without additional adjuvants, stored at 4°C. Horses received a prime-boost vaccination in Weeks 0, 4 and a booster in Week 15 ± 1.

Anti-CuMV$_{TT}$ and anti-IL-31 antibody titre determination

Maxisorp 96 well ELISA plates (Nunc[10]) were coated over night with purified eIL-31-C-His or purified CuMV$_{TT}$ (5 mg/L). Plates were washed 3 times with PBST (PBS + 0.1% Tween) and blocked with Superblock[10] for 2 h at room temperature. Plates were then washed 3 times with PBST and threefold dilutions of 1:10 diluted horse sera (in Superblock) were added and incubated at room temperature for 2 h. The plates were subsequently washed 3 times with PBST and incubated with anti-equine IgG (1:2000) conjugated with horseradish peroxidase (HRP)[11] at room temperature for 30 min. The plates were again washed 4 times with PBST and developing solution (TMB[10]) was added. After approximately 2 min of reaction at room temperature the ELISA was stopped with 5% H_2SO_4. Absorbance was measured at 450 nm (OD450) on a Tecan M200 spectrophotometer[12]. The antibody titres were calculated as OD_{50} (serum dilution on a logarithmic scale where OD450 was half maximal). All antibody titres are calculated with naïve serum subtracted on logarithmic scales, and presented as delta OD_{50} (ΔOD_{50}). Titres below 10 (and including 10) were considered background.

Results

IL-31 expression in skin biopsies of pruritic and healthy skin

Skin punch biopsies were collected from three horses affected with chronic allergic pruritus, including one horse with pruritus associated with birds' mites exposure (Horse A), one horse with drug induced, likely penicillin, hypersensitivity reaction (Horse B) and one horse presented in case report no. 3 (Horse C). Pruritic, alopecic skin lesions and autologous healthy punch biopsies were collected and mRNA of equine cytokines IL-31 (Horse A, B, C), TSLP (Horse B, C) and MCP-1 (Horse B, C) were quantified by qPCR in relation to the house keeping gene βactin. Levels of IL-31, TSLP and MCP-1 were upregulated in pruritic, alopecic skin lesions when compared to healthy skin of the same horse (**Fig 1a**). Moreover, neither IL-31 nor TSLP could be measured in healthy skin samples.

IL-31 and vaccine production

Recombinant eIL-31 with a C-terminal linker containing a free cysteine residue and a tag was generated by gene synthesis for protein production in *E. coli* (eIL-31-C-His) and for protein production in eukaryotic HEK cells (eIL-31-C-Strep). Prokaryotic protein expression, inclusion body preparation and purification were done according to the previously described method for eIL-5 (Fettelschoss-Gabriel *et al*. 2018, 2019). Prokaryotic eIL-31-C-His was sequentially dialysed for refolding into final sodium phosphate buffer. Refolded protein was concentrated and purified by size exclusion chromatography. Purification steps are shown in **Supplementary item 2**. Eukaryotic eIL-31-C-Strep was produced in HEK cells and purified by affinity chromatography (**Supplementary item 1**, lane 8). Folding of purified eIL-31-C-His was confirmed by comparing far-UV circular dichroism (CD) spectroscopies of eukaryotic HEK expressed eIL-31-C-Strep and prokaryotic *E. coli* expressed eIL-31-C-His. Both showed comparable pattern with expected and indicated minima at 208 and 222 nm, representing mostly α-helical secondary structure (**Supplementary item 2**).

Comparably to our previously published eIL-5-CuMV$_{TT}$ vaccine (Fettelschoss-Gabriel *et al*. 2018, 2019; Olomski *et al*. 2019), eIL-31 proteins were chemically coupled to the derivatised CuMV$_{TT}$ VLPs using a heterobifunctional cross-linker. A typical 'VLP-ladder' upon derivatisation before (**Supplementary item 3**, lane 3) and after coupling of CuMV$_{TT}$ subunits with eIL-31-C-His molecules (**Supplementary item 3**, lane 2) was shown on a reducing SDS-PAGE gel. The additional coupling bands correspond to the molecular mass of monomeric or monomeric/dimeric eIL-31 plus monomeric or multimeric CuMV$_{TT}$ subunits (**Supplementary item 3**, lane 4). Successful covalent attachment of eIL-31-C-His to CuMV$_{TT}$ was confirmed by Coomassie staining (**Supplementary item 3**) and Western blot using an anti-His antibody, only staining His-tagged eIL-31 (**Supplementary item 4**). The vaccine was further polished by size exclusion chromatography in order to remove free uncoupled eIL-31 (**Supplementary items 3** and **4**, lane 6) from the vaccine (**Supplementary items 3** and **4**, lane 5). Coupling efficiency for all batches was approximately 70% (**Supplementary item 5**).

Anti-IL-31 and anti-VLP antibody titres in horses

Antibody titres in serum of horses were measured before and at several time points after vaccination. Horses received a subcutaneous basic vaccination consisting of two initial immunisations in a 4 weeks interval and a third vaccination was administered in week 15 ± 1. One vaccine dose contained 300 μg of eIL-31-CuMV$_{TT}$ formulated in sodium phosphate buffer without adjuvants and was injected subcutaneously. Anti-CuMV$_{TT}$ antibody titres of immunised

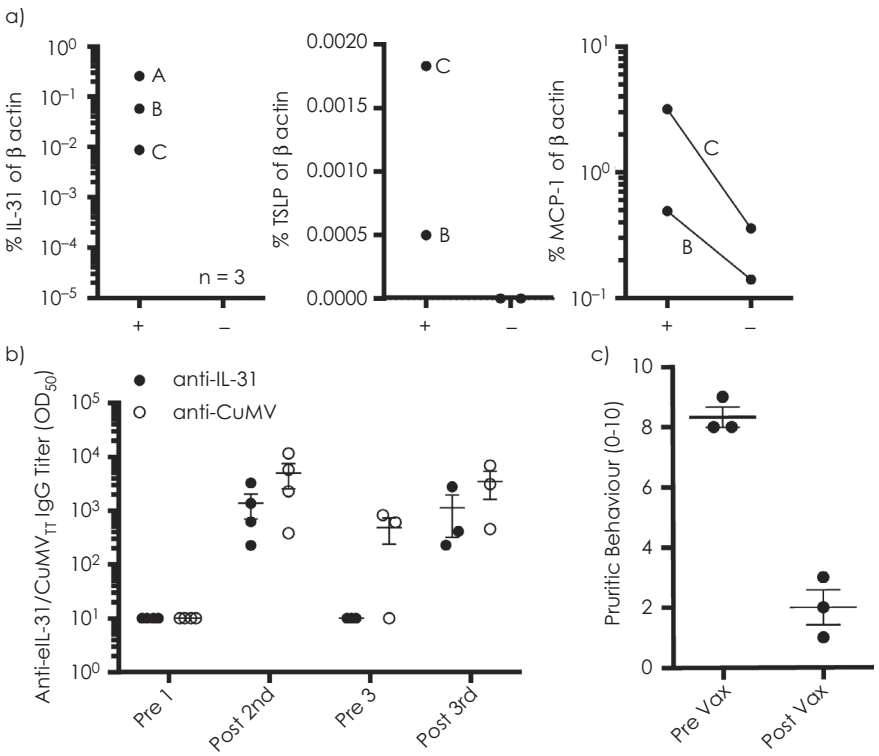

Fig 1: IL-31 expression in pruritic skin, induction of anti-IL-31 antibody titres, and evaluation of pruritus before and after vaccination by horse owners. a) Levels of eIL-31, eTSLP, and eMCP-1 expression levels relative to eβactin in skin punch biopsies from pruritic skin lesions (+) and matched healthy skin (–) of the same horse. Line connects matched samples. b) Antibody titre of anti-CuMV$_{TT}$ IgG and anti-eIL-31 IgG for horse 1–4 at time point of 1st vaccination (Pre 1), post second vaccination (post 2nd), at time point of third injection (Pre 3) and post third vaccination (post 3rd). All antibody titres are calculated with naïve serum subtracted on logarithmic scales, limit of detection are titres ≤ 10. c) Pruritic behaviour before vaccination (Pre Vax) and improvement of pruritic behaviour after the third vaccination (Post Vax) according to visual analogue scale filled out by horse owners. https://de.surveymonkey.com/r/258372L.

horses were used as a surrogate marker for successful vaccination. Upon second and third vaccination all horses developed antibodies against both IL-31 and CuMV$_{TT}$ (**Fig 1b**).

Reduced pruritic behaviour upon eIL-31-CuMV$_{TT}$ vaccination

An owners' evaluation in a survey (online questionnaire) on the pruritic behaviour of their horses, before vaccination and after the third vaccination (**Fig 1c**), showed that pruritic behaviour was reduced by vaccination against IL-31 (mean improvement by 76.4%). In addition, pruritus improved in all horses.

Vaccination using eIL-31-CuMV$_{TT}$ vaccine mitigates pruritus and clinical signs of skin lesions

Case study 1, included in February 2018

Thoroughbred gelding (* 2006) with chronic pruritus and alopecia since 2015 (or earlier) had been examined by several veterinarians, but treatment attempts yielded limited success, thus pruritus persisted during all seasons. In 2015, the horse had episodes of focal inflammatory alopecia of the head and neck but initially without pruritus, and was treated with the topical disinfectant Dexavetaderm. In early summer 2016 the horse developed lesions of the head and neck with marked pruritus. Alopecia was multifocal, rather annular with little crusts mainly at forehead, mandible, neck, below mane (here also with seborrhoea sicca) and the hairs were easily epilated. Fur and skin was otherwise without abnormalities. The horse received two injections of dermatophyte vaccine (Insol® Dermatophyton, Boehringer Ingelheim), but the pruritus progressed. Due to severe pruritus and impairment of general attitude, the horse was administered corticosteroids (unknown concentration and duration, Dexamedium, MSD) and a mild improvement was noticed short term, whereafter deterioration to severe pruritus affecting the head and neck region was again recorded. Skin biopsies from head and neck were taken and were compatible with allergic dermatitis with mainly perivascular infiltration of lymphocytes and eosinophils. In addition, perifollicular fibrosis was noted, possibly due to an earlier dermatophytosis. The horse was regularly dewormed. Although annular lesions that were initially nonpruritic and later pruritic suggested bacterial folliculitis, fungal elements or onchocerca infection, these were excluded upon histological examination. Other co-housed horses were not affected. The horse was considered allergic based on exclusion of ectoparasites and cutaneous infections. Causative allergic agents could not be identified by the previous examinations and exclusion procedures. Upon two vaccinations against IL-31, lesions at the buttock (**Fig 2a**) and neck (**Fig 2b**) disappeared (**Fig 2c**, buttock and **Fig 2d**, neck), indicating less pruritus-mediated self-inflicted trauma. Of note, the horse was followed-up for in total 4 months and stayed symptom free until then.

Case study 2, included in April 2018
Shetland pony mare (* 2003) with chronic, year-round pruritus since September 2014. Extensive examination by several veterinarians followed. Pituary pars intermedia dysfunction (PPID) was diagnosed in October 2014 after onset of pruritus and the horse received 0.25 mg pergolide/day per os (Prascend, Boehringer Ingelheim). In addition, the horse had a reduced appetite, lost weight and had a colic episode. Subsequently, the horse was dewormed (0.2 mg/kg bwt Ivermectin, Eraquell, Virbac) and received omeprazole (2 mg/kg bwt, GastroGard, Biokema) after having found three small gastric ulcers during endoscopic examination. The pruritus persisted, reduced appetite and weight loss resolved. Allergen-specific IgE serology testing and subsequent desensitisation against pollen (IMOVET EAC) was performed for 15 months without any improvement of pruritus and self-induced alopecia. Further search for offending allergens by clinical and dermatological evaluations such as food and bedding exclusion procedures were unrewarding. As such it was not clear whether the origin of the pruritus was allergic in this horse, nevertheless the horse met criteria of CPUO. Lesions prior to vaccination at the neck (**Fig 3a**), cheek (**Fig 3b**) and thigh (**Fig 3c**) healed after two vaccinations against IL-31 and hair grew back at all body parts, that is, neck (**Fig 3d**), cheek (**Fig 3e**), and thigh (**Fig 3f**). Of note, the horse received a follow-up booster vaccination once the owner noticed reappearing pruritic behaviour (approx. 7 months after the last boost) and stayed symptom free until today.

Case study 3, included September 2018
Swiss Warmblood mare (* 2005) with chronic, nonseasonal pruritus, self-induced alopecia and excoriations. Initially in 2012, the horse was a bit itchy during winter months, but no lesions were visible. The horse moved with the same owner at the end of 2013 to a new stable, however, there was no change in feeding, bedding and housing, and the horse always had an open box and accessible paddock outside. In winter 2014/2015 the pruritus suddenly worsened dramatically with severe wounds on the skin and developed pruritic urticaria twice. The horse was treated with corticosteroids for a couple of weeks (unknown exact duration and concentration). Thereafter, exclusion procedures for feeding, bedding and topical products were performed. Blankets of horses were only washed without laundry detergent. Pruritus

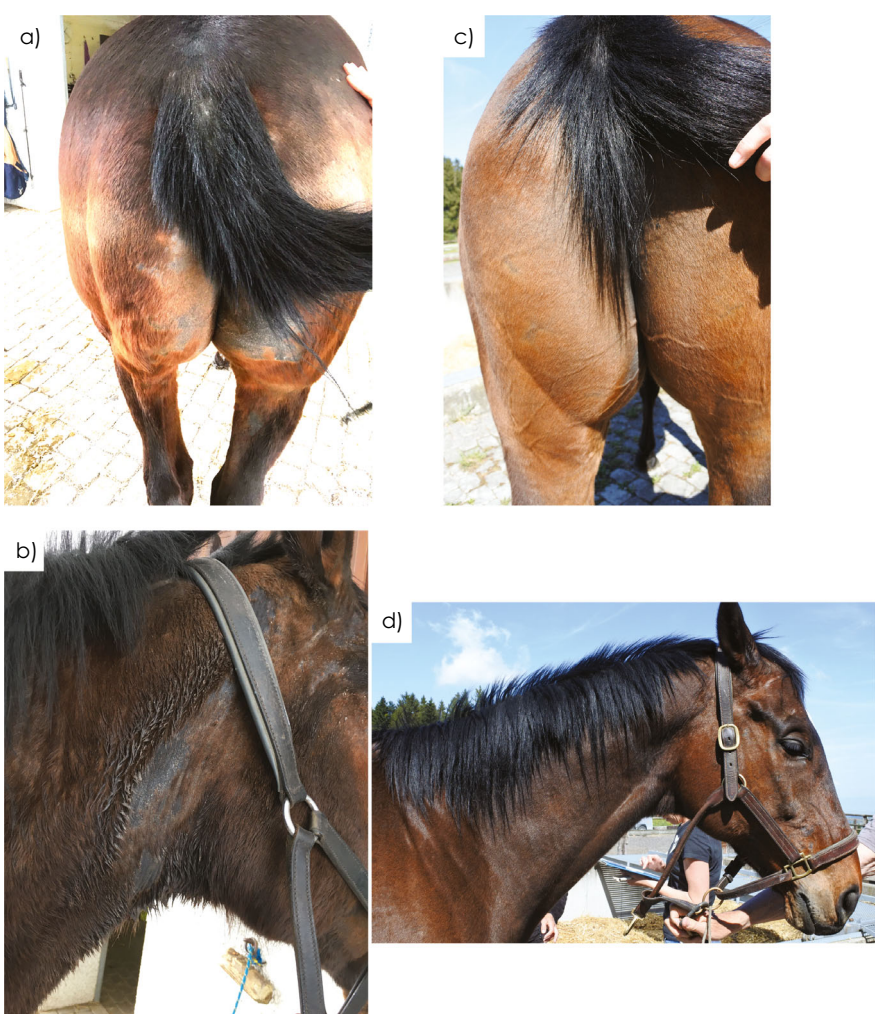

Fig 2: Pruritic skin lesions before and after vaccination, horse 1. a) c) Skin photographs from day of first vaccination. Pruritic skin lesion sites at buttock a) and neck c). b) d) Skin photographs from 5 weeks post second vaccination of the same locations at buttock b) and neck d).

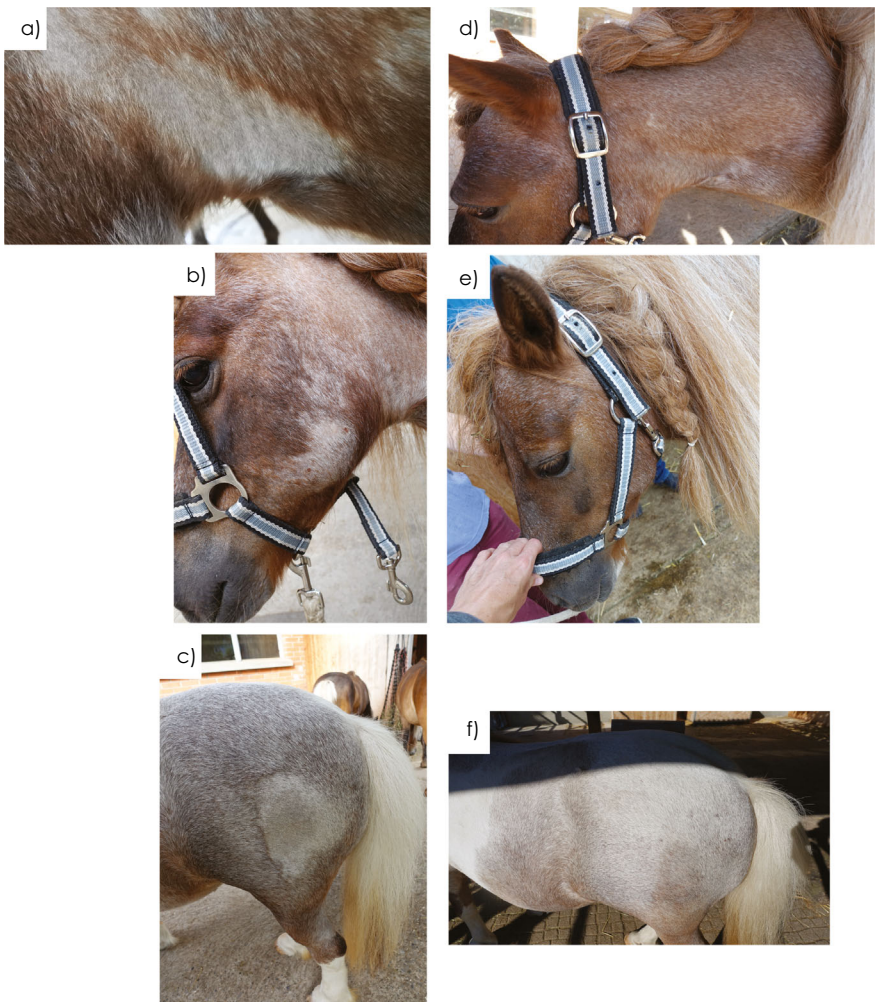

Fig 3: Pruritic skin lesions before and after vaccination, Horse 2. a), b), c) Skin photographs from day of first vaccination. Pruritic sites at neck a), cheek b) and thigh c). d), e), f) Skin photographs from 4 weeks post second vaccination at neck d), cheek e) and thigh f).

did though persist. Interestingly, after the initial pruritus that was limited to winter months only, the horse became all year round pruritic with lesions on the trunk and tail during the winter months and lesions mainly on the head, base of mane and neck during summer months. IgE serology testing and subsequent desensitisation against *D. farinae* and *D. pteronyssinus* (IDEXX Diavet) was performed for 6 months without any improvement of pruritus and skin lesions. Of note, the presence of ectoparasites was tested negative. Although several veterinarians examined this horse, the cause of pruritus remained unidentified and reflects how challenging and frustrating the identification can be. The horse was diagnosed as allergic based on exclusion of other diagnoses. Offending allergens were not identified by exclusion procedures and the pruritus was unresponsive to treatments tried. Two biopsies of lesional and nonlesional skin were taken in December and showed IL-31 expression solely in lesional skin and increased TSLP and MCP-1 expression in lesional skin when compared to nonlesional skin (Horse C, **Fig. 1a**) thus being compatible with allergic pruritus (Olomski *et al*. 2019). This horse was vaccinated three times and photographs of lesions were taken at the time point of first injection, that is, mane (**Fig 4a**), and prior to or at the time point of third booster injection, that is, tail (**Fig 4b**) and back (**Fig 4c**). Photographs post three vaccinations against IL-31 show healing of the lesions, that is, mane at 10 days post third vaccination (**Fig 4d**), tail at 16 weeks (**Fig 4e**) post third injection, and back at 8 weeks post third injection (**Fig 4f**). Of note, the horse receives occasional follow-up booster vaccinations once the owner noticed reappearing pruritic behaviour.

Corticosteroid replaced by active IL-31 vaccination

Case study 4, included June 2018

Icelandic horse gelding (* 2007) born in Iceland and exported in April 2017 showed chronic pruritus and alopecia immediately upon arrival in Switzerland and unchanged during summer and winter time. Subsequently, he was put on prednisolone 750 mg/day per os corresponding to 2 mg/kg bwt (Prednisolon, Vétoquinol). The owner previously tried to reduce the corticosteroid dose several times, however, he had to go back to the old dose immediately due to severe pruritus upon reduction. The horse was dewormed regularly, received common prophylactic vaccinations and also

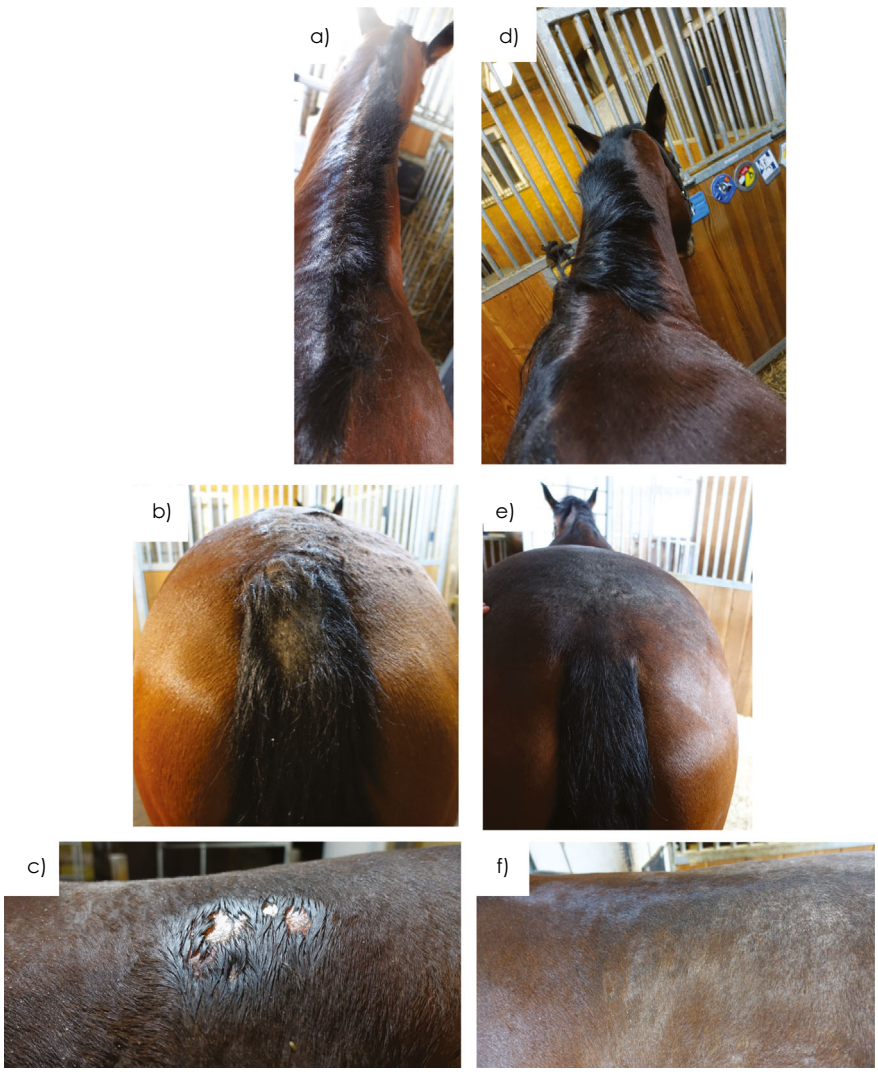

Fig 4: Pruritic skin lesions before and after vaccination, Horse 3. a) Skin photograph from day of first vaccination at mane a). b), c) Skin photograph prior to or at the time point of third booster injection at tail b) and back c). d) Skin photographs 10 days post third vaccination at mane. e) Skin photograph 16 weeks post third injection at tail. f) Skin photographs 8 weeks post third injection at back.

vaccination against dermatophytosis (Insol® Dermatophyton, Boehringer Ingelheim) without influencing the need for corticosteroids. At the time of the first vaccination (Week 0), we slightly reduced the prednisolone dose to 500 mg/day per os, in order to facilitate a proper immune response towards our vaccine, leading to a mild onset of pruritus. Following the second, third and fourth vaccination (Weeks 4, 18, and 35) we continuously decreased the daily amount of prednisolone, i.e. to 375 mg/day (per os) 4 weeks post second vaccination, to 250 mg/day (per os) 3 weeks post third vaccination, to 125 mg/day (per os) 8.5 weeks post third vaccination, to 62.5 mg/day (per os) 18.5 weeks post third vaccination, and discontinued it 7 weeks after the fourth vaccination (**Supplementary item 6**). Of note, the horse received one follow-up booster vaccination approximately 6 months after the last booster and stayed symptom-free for the duration of the study. The horse was excluded from the study after it was moved to a 3-month holiday in the Alps (exclusion criteria of the study).

Discussion

All four horses suffering from persistent chronic allergic pruritus and alopecia showed relief of pruritic symptoms on vaccination against IL-31 using the VLP-based eIL-31-CuMV$_{TT}$ vaccine. Of note, no other horses were included in the study. Due to the reduction of pruritus, the skin lesions healed, the hair coat grew back and one horse dependent on corticosteroids was able to discontinue its medication. Quantification of IL-31, TSLP and MCP-1 levels in skin biopsies of pruritic lesions showed increased expression when compared to autologous healthy skin, in which IL-31 and TSLP were not detectable at all. We previously quantified mRNA expression of IL-31 in the skin lesion of horses with IBH with similar findings (Olomski *et al*. 2019). Despite the herein presented small case numbers in the absence of a placebo group, increased expression of IL-31 including the co-associated molecules TSLP and MCP-1 and the reduction of clinical signs after IL-31-vaccination, may indicate a key role

for IL-31 in the development of chronic pruritus, most likely caused by an allergic trigger. Of note, ectoparasitoses may also lead to IL-31 upregulation as seen in horse A affected with bird mites.

Blocking of IL-31 has been suggested for the treatment of pruritic skin diseases such as atopic dermatitis in humans and dogs. Monoclonal antibodies (mAbs) against the human IL-31 Receptor (Nemolizumab, Chugai Pharma) and against human IL-31 (Bristol-Myers Squibb) were tested in clinical trials with patients since 2012 or 2015, respectively (clinicaltrials.gov). Comparably, a canine anti-IL-31 mAb (Lokivetmab, Cytopoint®, Zoetis) has been registered for the treatment of atopic dermatitis in dogs. The clinical studies in both species have revealed that the antibodies significantly reduced pruritus and to date no adverse reactions have been recorded (Michels *et al*. 2016; Moyaert *et al*. 2017; Ruzicka *et al*. 2017; Tamamoto-Mochizuki *et al*. 2019). Given firstly the weight and size of a horse, and secondly the frequency of injections requiring a veterinarian each time, a mAb therapy would be very costly indeed. In contrast, a vaccine can be applied at low dose and is injected three times in the first year with a yearly follow-up booster (Fettelschoss-Gabriel *et al*. 2019). Comparable to the mAbs in humans, dogs and mice, the VLP-based vaccines show a good safety profile and no side effects were observed throughout our studies (Fettelschoss-Gabriel *et al*. 2018, 2019; Olomski *et al*. 2019; Jonsdottir *et al*. 2020). Also, VLP-based vaccines are already licensed for human papilloma virus (Gardasil®, MSD; Cervarix®, GlaxoSmithKline), hepatitis B virus (Recombivax HB®, Merck; Engerix-B®, GlaxoSmithKline; Elovac B®, Human Biologicals Institute; Genevac B®, Serum Institute; Shanvac B®, Shanta Biotechnics; and others), and hepatitis C virus (in China only) (Chroboczek *et al*. 2014).

In order to develop antibodies against a self-protein, it is required to overcome B cell unresponsiveness towards self (Bachmann and Dyer 2004; Bachmann and Jennings 2010). This can be achieved by so-called hapten-carrier complexes. One requirement is a very immunogenic carrier such as a virus-like particle (VLP) with a particulate structure and small size to freely drain to the lymph nodes, a repetitive surface for B cell crosslinking and complement fixation, and pathogen associated molecular patterns (PAMPs) for innate immune activation. The second requirement is the native structure of the hapten molecule in order to bind to naïve B cell receptors. The third and last requirement is the linkage of the carrier and hapten to ensure an uptake by the same antigen-presenting cell (APC). This will enable a fulminant T cell response against the VLP carrier and a B cell response against the VLP carrier and the self-protein as hapten through carrier specific bystander T help (Landsteiner 1924; Avery and Goebel 1929; Goebel and Avery 1931; Jennings and Bachmann 2009; Jonsdottir *et al*. 2020). We have previously shown that a $CuMV_{TT}$ VLP-based vaccine can induce a B cell response against the self-protein eIL-5, hence fulfilling requirement one (Fettelschoss-Gabriel *et al*. 2018, 2019). For the second requirement, the native structure of the target protein, we show comparable secondary structure patterns of the *E. coli*-produced and refolded eIL-31 and the mammalian HEK cell-produced eIL-31, thus indicating a correct native structure of the eIL-31, which was used in the vaccine. Finally, we show coupling of the VLP $CuMV_{TT}$ and the refolded eIL-31 protein. Indeed, all horses developed anti-IL-31 antibodies on the second and third vaccination using eIL-31-$CuMV_{TT}$. This is comparable to our eIL-5-$CuMV_{TT}$ vaccine (Fettelschoss-Gabriel *et al*. 2018, 2019). Nevertheless, a single horse (Horse 4) receiving high dose of corticosteroids produced lower levels of antibody titres against both the IL-31 self-molecule and the foreign VLP molecule. The low anti-VLP antibody response suggests the general suppression of immunity by the corticosteroids. All recruited horses had a long history of chronic allergic pruritus. All horse owners noticed a substantial reduction in the pruritic behaviour of their horse after the third vaccination, which was also visible on the photographs comparing lesions before and after vaccination. According to the analysis of the owners' questionnaire horses improved on average by 76.4% in pruritic behaviour with initial pruritus score before vaccination ranging between 8 and 9 and post vaccine scores of mild pruritus ranging between 1 and 3 in absence of any other concurrent medication. Of note, Horse 4, that was weaned off corticosteroids, reached a post vaccine pruritus score of 1. Furthermore, the high dose of corticosteroids could gradually be reduced until complete cessation following vaccination. In line with these findings, we recently showed that the same vaccine was able to reduce clinical signs in horses with insect bite hypersensitivity (IBH), suggesting a general role during allergic pruritus in horses (Olomski *et al*. 2019).

In general, vaccination against self-targets has to fulfil certain safety aspects, (i) vaccine-induced anti-self antibody responses must be reversible, (ii) anti-self antibodies are not induced by endogenous self-protein, (iii) vaccine does not induce self-reactive T cells. We recently published a long-term safety study in horses using our anti-IL-5 antibody inducing eIL-5-$CuMV_{TT}$ vaccine and found that antibody titres were indeed reversible when tested over four consecutive years. In addition endogenous IL-5 protein was not able to boost anti-IL-5 antibody titres, and no peripheral autoreactive T cells were found upon *in vitro* restimulations in horses receiving vaccine for up to five consecutive years (Jonsdottir *et al*. 2020).

Currently, treating a chronic pruritic horse or CPUO is a challenge. Antihistamines solely treat symptoms of the histaminergic itch via histamine-sensitive C nerve fibres. The majority of pruritic nerves are histamine-independent cowhage fibres and therefore do not benefit from antihistamines. The choice here is a nonspecific corticosteroid treatment, which dampens the amount of effector molecules that activate the nerves. However, corticosteroids can be associated with significant adverse effects; for example, they might cause laminitis in horses (Johnson *et al*. 2002; Ryu *et al*. 2004). For a minority of cases where causative allergen could have been identified, ASIT can be applied, however it requires 1–2 years of treatment until obtaining efficacy (Stepnik *et al*. 2012; Loeffler *et al*. 2018; Radwanski *et al*. 2019). Allergy management so far does not only lack straight forward curative treatment options but also lacks reliable and sensitive diagnostic tools. Serological testing for the presence of allergen-specific IgE antibodies generates variable results with unsatisfactory sensitivity and specificity (Hellberg *et al*. 2006; Peeters *et al*. 2013; Fettelschoss-Gabriel *et al*. 2018; Torsteinsdottir *et al*. 2018). Initial diagnostic improvements have been made with the functional *in vitro* histamine-release test (HRT) and the cellular antigen stimulation test (CAST) (Baselgia *et al*. 2006). However, despite their cellular basis, these tests only take the contribution of IgE into

account and do not affect IgG-mediated type II and III hypersensitivities or type I late phase- and type IV DTH-mediated effects that also can contribute to the clinical signs and pathology of an allergic disease. Also, the more functional intracutaneous 'prick test' in humans or the intradermal test in animals involving an *in vivo* reaction is limited by varying sensitivity and specificity and also needs training of the conducting veterinarian (Quinn *et al*. 1983; Halldorsdottir *et al*. 1989; Kolm-Stark and Wagner 2002; Lebis *et al*. 2002). In addition, all tests strongly depend on the quality of allergens used, meaning when using allergen extract the chances of cross-reactivities are increasing and well-known quality differences from batch to batch lead to variable and less comparable results (Nelson *et al*. 1996; Esch 1997; Brunetto *et al*. 2010; Focke *et al*. 2010; Blank *et al*. 2011; Curin *et al*. 2011; Sturm *et al*. 2011; van Damme *et al*. 2020). In addition, recombinant allergens need a correctly folded three-dimensional structure in order to detect structural binding antibodies. Due to the very active research in the field of IBH most of the data on diagnostics is best characterised for insect allergens. However, it is likely that many of those facts also apply to environmental allergens such as pollen, mould or mites. In summary, the differentiation of healthy sensitised individuals and allergic individuals can be challenging. Given the challenges of identification of the allergic trigger by the limitations of diagnostics, an allergen-independent treatment option is greatly desirable for horses. Even if this is only a small number of cases without a placebo group, we can show promising results with the eIL-31-CuMV$_{TT}$ vaccine for the treatment of CPUO, to date without notified side effects. An anti-IL-31 therapy would be independent of the allergen, enabling a broad use in allergic diseases with only limited diagnostics required.

Authors' declaration of interests

V. Fettelschoss, K. Birkmann, T. Kündig and A. Fettelschoss-Gabriel are involved in the development of active immunotherapies. The authors F. Olomski and K. Bergvall have no conflicts of interest to disclose.

Ethical animal research

The studies involving animals were performed according to ethical standards in Switzerland and all animal studies have been approved by the cantonal animal authorities of Switzerland. All horse owners signed informed consent.

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Authorship

V. Fettelschoss and F. Olomski planned, performed and analysed experiments. K. Birkmann and T. Kündig contributed to discussions. K. Bergvall contributed to discussions and supported writing the manuscript. A. Fettelschoss-Gabriel planned and analysed experiments, performed the clinical studies, interpreted the data and wrote the manuscript.

Manufacturers' addresses

[1]Thermo Fisher, Waltham, Massachusetts, USA.
[2]Promega, Madison, Wisconsin USA.
[3]Roche, Basel, Switzerland.
[4]IDT, Coralville, Iowa, USA.
[5]NEB, Ipswich, Massachusetts, USA.
[6]PEI Max 40K, Polysciences, Warrington, USA.
[7]IBA Lifesciences, Göttingen, Germany.
[8]Jasco, Tokyo, Japan.
[9]Lonza, Basel, Switzerland.
[10]Thermo Scientific, Waltham, Massachusetts, USA.
[11]Jackson ImmunoResearch, West Grove, Pennsylvania, USA.
[12]Tecan, Männedorf, Switzerland.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary item 1. SDS-PAGE analysis of eIL-31-C-His.

Supplementary item 2. Comparison of E.coli- and HEK-derived eIL-31.

Supplementary item 3. Vaccine production. SDS-PAGE of eIL-31-CuMV$_{TT}$.

Supplementary item 4. Vaccine production. WB of eIL-31-CuMV$_{TT}$.

Supplementary item 5. Coupling efficiency eIL-31-CuMV$_{TT}$.

Supplementary item 6. Corticosteroid reduction upon eIL-31-CuMV$_{TT}$ vaccination.

Supplementary item 7. qPCR primer.