



Safety Evaluation of PQ Birch Allergy Immunotherapy to Support Product Development

Paul Baldrick^a, James W. Hutchings^{b,*}, Matthew D. Heath^b, Murray A. Skinner^b

^a Nonclinical Strategic Product Development, Covance Laboratories Ltd, Otley Road, Harrogate, North Yorkshire, HG3 1PY, England, UK

^b Allergy Therapeutics (UK) Ltd, Dominion Way, Worthing, West Sussex, BN14 8SA, England, UK

ARTICLE INFO

Keywords:

Birch pollen
Allergy vaccine
PQ birch
Toxicology study
Genotoxicity

ABSTRACT

PQ Birch represents an allergen-specific immunotherapy for the treatment of birch pollinosis. It consists of native birch pollen extract chemically modified with glutaraldehyde adsorbed to L-tyrosine in its microcrystalline form with addition of the adjuvant Monophosphoryl Lipid A (MPL®). A nonclinical safety testing strategy was designed based upon interpretation of current legislation and regulatory intelligence and comprised genotoxicity studies (bacterial reverse mutation and Chinese hamster ovary micronucleus assays), a rat repeat dose toxicology study and a rabbit local tolerance study. No safety findings of concern were found. Thus, no evidence of genotoxicity was found. Relatively minor, immunostimulatory effects were seen following repeated subcutaneous dosing (once every 2 weeks for 13 weeks) as reversible increased white cell count (notably neutrophils), increased globulin level (resulting in decreased albumin/globulin [A/G] ratio) and increased fibrinogen, as well as minor dose site reaction in the form of inflammatory cell infiltrate. These findings are likely due to the immunostimulatory nature of MPL® and/or the presence of L-tyrosine within the adjuvanted vaccine. Similar dose site inflammatory changes to the injected formulation were also noted in the rabbit local tolerance study.

1. Introduction

Birch tree pollinosis is a form of seasonal allergic rhinitis caused by allergy to birch tree pollen. Allergic rhinitis is reported as affecting 10–20% of the population (Verlato et al., 2003; Small et al., 2018) and symptoms are characterised by nasal itching, sneezing, watery rhinorrhoea and nasal obstruction (Bousquet et al., 2008; Van Cauwenberge et al., 2000). Additional symptoms such as headache, impaired smell, and conjunctival symptoms can also occur. While the symptoms of allergic rhinitis are often mild, many subjects suffer from moderate and severe symptoms and they have an increased chance of progressing over time and developing to more serious conditions such as allergic asthma. The allergic response arises from pollen (allergen) binding to immunoglobulin (Ig)E antibodies and further interaction with mast cells and basophils via a high-affinity IgE receptor (Akdis and Akdis, 2015; Akkoc et al., 2011; Niederberger, 2009). Subsequent degranulation of the cells elicits allergic symptoms of type I hypersensitivity reactions. After successful immunotherapy, allergen-specific T regulatory (Treg) cells are generated and they suppress proliferative and cytokine responses against allergens. In addition, antibody class-switching occurs in B cells that secrete allergen-specific IgG4 instead of IgE which has blocking capacity, inhibiting binding between allergen and IgE on

mast cells and basophils. Allergen Immunotherapy (AIT) has been practised for many years in patients suffering from pollen allergy and involves administration of increasing quantities of specific allergen extracts to induce a state of allergic non-responsiveness upon subsequent exposure (Akkoc et al., 2011; Bousquet et al., 1998; Ebner, 1999; Larche, 2006).

PQ Birch represents an AIT for the treatment of rhinitis and/or rhinoconjunctivitis with or without bronchial asthma in adults, adolescents and children over the age of 5, caused by an IgE mediated allergy against birch pollen. It consists of birch (*Betula pendula*) pollen extract chemically modified with glutaraldehyde (forming an allergoid) adsorbed to L-tyrosine, a naturally occurring amino acid in its microcrystalline form, with addition of the adjuvant MPL®, a detoxified derivative of lipid A of *Salmonella minnesota* R595. Modification of allergen to allergoid reduces IgE reactivity with retention of IgG binding epitopes as shown from immunoreactivity and structural analysis testing with single or multiple mix grass allergoids (Moran et al., 1977; Starchenka et al., 2017). Adjuvant use of L-tyrosine (by forming a depot) allows controlled slow release of allergoid resulting in reduced incidence of local IgE mediated allergic responses, while prolonging the antigenic response of the therapy (Leuthard et al., 2018). Work with L-tyrosine showed that it protects from IgE-mediated reactivity in mouse

* Corresponding author.

E-mail addresses: paul.baldrick@covance.com (P. Baldrick), James.Hutchings@AllergyTherapeutics.com (J.W. Hutchings).

<https://doi.org/10.1016/j.yrtph.2019.104441>

Received 12 April 2019; Received in revised form 9 August 2019; Accepted 12 August 2019

Available online 16 August 2019

0273-2300/ © 2019 Published by Elsevier Inc.

models but acts independently of inflammasome (specific innate) and Toll-Like Receptor (TLR) signalling. Furthermore, testing in mice sensitised by injection of birch pollen extract with the same allergen adsorbed on L-tyrosine with up to 5 weekly doses (giving a total extract protein dose of 264 µg) showed IgG1 and IgG2a antibody responses were induced. The adjuvant activity of MPL® arises through promotion of a T helper type I (Th1) response through stimulation of the innate immune response by binding to the TLR complex (Casella and Mitchell, 2008; Evans et al., 2003). Thus, switching away from an IgE mediated allergic response, as evidenced by an IgG titre, occurs. Studies in mice with allergoid adsorbed to L-tyrosine and with MPL® showed a Th1 skewed effect with induced IgG2a and IgG2b responses but no IgE change (Wheeler et al., 2001). More recently, it was shown that allergoid (glutaraldehyde-modified ragweed pollen)-L-tyrosine with MPL® enhanced allergen specific IgG1 and IgG2a antibody titres in mice, with the combination of L-tyrosine and MPL® generating a high and sustained IgG antibody response (Hutchings et al., 2017). A randomised, double-blind, parallel group, multi-centre clinical study with PQ Birch showed increases in IgG and IgG4 immunoglobulin subtypes but no change in IgE (Worm et al., 2018).

To support clinical development, PQ Birch has undergone non-clinical evaluation comprising genotoxicity, rat repeat dose toxicology and rabbit local tolerance testing. Relevant regulatory guidelines were referenced to support this evaluation (EMA, 1998; ICH M3(R2), 2009; WHO, 2013) and regulatory agency guidance was sought on the actual studies needed. The design of the repeat dose toxicology study was based upon experience available for other allergoids (grass pollen, ragweed and house dust mite)-L-tyrosine MPL® products (Baldrick et al., 2002a, 2004; 2007). The toxicology study design covered the proposed clinical regimen of 6 subcutaneous injections given at intervals of up to 14 days as 900 Standardised Units (SU)/mL (1st injection), 2400 SU/mL (2nd injection) and 6000 SU/mL (3rd to 6th injections) to give a cumulative dose of 27300 SU. It was highlighted that a study of longer duration (eg. 6 months), greater dosing interval (> 2 weeks) or large number of injections (proposed clinical number of doses [n] + 1, giving 7 in total were used) would be unrepresentative and excessive compared to the clinical situation and not in keeping with the WHO guideline. The n + 1 dosing scheme follow current regulatory thinking (Matsumoto et al., 2016; WHO, 2013).

All studies were performed at Covance Laboratories Ltd, Harrogate, UK in 2015–2018 in compliance with Good Laboratory Practice (GLP).

2. Materials and methods

2.1. Test material and formulation

The PQ Birch 6000 SU/mL formulation is birch tree pollen extract modified by glutaraldehyde that is adsorbed to L-tyrosine, by co-precipitation, with addition of MPL®. The composition of drug product is given in Table 1.

The test material used for the genotoxicity, repeat dose toxicology and local tolerance studies was supplied as vials containing 6000 SU/mL of birch pollen allergoid, 50 µg/mL MPL® adjuvant and 20 mg/mL tyrosine. The control material was supplied as vials of “2% Tyrosine suspension” (repeat dose toxicology study) or “2% Tyrosine Blank” (genotoxicity and local tolerance studies) and was identical to the test material except that birch pollen allergoid and MPL® were not present. This control material was used for dilution of the supplied 6000 SU/mL allergoid strength to give concentrations used in genotoxicity testing.

Animal care and use was compliant with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) recommendations and study designs underwent internal ethical review.

Table 1

Composition of drug product.

Ingredient	Function	Reference to Quality Standard	Quantity per mL of drug product proposed for clinical use		
			6 injections; cumulative dose: 27300 SU		
Active Pharmaceutical Ingredient					
Modified Birch Extract ^a	Active	In house	900 SU/mL	2400 SU/mL	6000 SU/mL
Other components ^b					
L-Tyrosine	Adsorbant base	USP/Ph. Eur.	20 mg	20 mg	20 mg
MPL®	Adjuvant	In house	0.05 mg	0.05 mg	0.05 mg

^a SU - Standardised Units/mL.

^b Further components include dipalmitoyl phosphatidylcholine, phenol, sodium chloride, disodium phosphate dodecahydrate, sodium dihydrogen phosphate dehydrate and water for injection.

2.2. Bacterial reverse mutation assay

PQ Birch was evaluated for the induction of reverse mutations and cytotoxicity in bacterial cells using 5 strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) using a standard plate incorporation method. Strains TA98, TA100, TA1535 and TA1537 were originally obtained from the National Collection of Type Cultures (NCTC), United Kingdom. Strain TA102 was derived from cultures originally obtained from Covance Laboratories Inc., USA. Testing was performed in the absence and presence of exogenous metabolic activation (S9) at concentrations of 3, 9.49, 30, 94.9, 300, 949.0 or 3000 SU/plate, with the latter being a maximum practicable concentration based on the supplied vial concentration of 6000 SU/mL of PQ Birch and a maximum volume of 0.5 mL of test article solution to allow the agar to set sufficiently. The S9 was obtained from Molecular Toxicology Incorporated, USA, where it was prepared from male Sprague-Dawley rats induced with Aroclor 1254. Negative controls (untreated or 2% Tyrosine Blank) were tested in quintuplicate, while the different concentrations of PQ Birch and positive control chemicals (obtained from Sigma Aldrich Chemical Co. Poole, United Kingdom) were included as triplicate plates as follows: 5 µg/plate 2-nitrofluorene (2NF for TA98 -S9), 2 µg/plate sodium azide (NaN₃ for TA100 and TA1535 -S9), 50 µg/plate 9-aminoacridine (AAC for TA1537 -S9), 0.2 µg/plate Mytomyacin C (MMC for TA102 -S9), 10 µg/plate benzo[a]pyrene (B[a]P for TA98 + S9) and 2-aminoanthracene (AAN) at 5 µg/plate (for TA100, TA1535 and TA1537 + S9) and 20 µg/plate (for TA102 + S9). Colonies were counted electronically using a Sorcerer Colony Counter (Perceptive Instruments) or manually where confounding factors such as splitting or bubbles in the agar affected the accuracy of the automated counter. The background lawn was inspected for signs of toxicity or evidence of test material precipitation. Individual plate counts were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined. Results were compared to in-study and laboratory historical control ranges. The presence or otherwise of a concentration response was checked by non-statistical analysis. The evaluation criteria for a mutagenic response was if a concentration-related increase in revertant numbers was ≥ 1.5 fold (for TA102), ≥ 2 fold (for TA98 and TA100) or ≥ 3 fold (for TA1535 and TA100).

2.3. Chinese hamster ovary micronucleus assay

The clastogenic and aneugenic potential of PQ Birch was examined by its effects on the frequency of micronucleated cells in cultured Chinese hamster ovary (CHO) cells treated in the absence and presence of rat S9. Cells were exposed to concentrations of 25, 50, 100, 200, 300,

400, 500 or 600 SU/mL for 3 h in the absence and presence of S9 and sampled at 24 h after the beginning of treatment (3 + 21 h treatment). In addition, an extended 24 h in the absence of S9 at concentrations of 5, 10, 20, 40, 60, 80, 100 or 120 SU/mL was included (24 + 0 h treatment). Testing occurred in duplicate and was called Experiment 1. As the data for the 24 + 0 h treatment in the absence of S9 were considered equivocal (see later), a confirmatory experiment was performed using the same testing conditions and was referred to as Experiment 2. Range-finder experiments were used to confirm that PQ Birch at 600 (3 + 21 h treatment) and 120 (24 + 0 h treatment) SU/mL were maximum achievable concentrations based on cytotoxicity and lack of evidence of test material precipitation measurements. The S9 was obtained from Molecular Toxicology Incorporated, USA. Negative controls (untreated or 2% Tyrosine Blank) tested in quintuplicate and positive controls (obtained from Sigma Aldrich Chemical Co. Poole, United Kingdom), tested in duplicate and comprising 0.2 µg/mL mitomycin C (MMC) (for 3 + 21 h treatment -S9), 4 µg/mL cyclophosphamide (CPA) (for 3 + 21 h treatment + S9) and 0.005 µg/mL vinblastine (VIN) (24 + 0 h treatment) were included. Following treatment, cells were processed to slides and one thousand binucleate cells from each culture (2000 per concentration) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei per cell on each slide was noted with measurement of micronucleated binucleate (MNBN) cells. Evaluation for nucleoplasmic bridges (NPBs) between nuclei in binucleate cells was also recorded during micronucleus analysis for the 24 + 0 h treatments in the absence of S-9 in Experiments 1 and 2. The proportions of MNBN cells for each treatment condition were compared with the proportion in negative controls by using Fisher's exact test. A Cochran-Armitage trend test was applied to each treatment condition to determine any relationship between concentration and frequency of MNBN cells. Probability values of $p \leq 0.05$ were accepted as significant.

2.4. Repeat dose toxicology study

The study took the form of a 13-week intermittent dose subcutaneous toxicity study in the rat followed by a 4-week treatment-free period. Han Wistar CrI:WI(Han) rats were obtained from Charles River Laboratories, Margate, United Kingdom and were subjected to a 3 week acclimation period. A group of 15 male and 15 female rats were then given a single subcutaneous dose of PQ Birch at 1 mL/rat once every 2 weeks for 13 weeks (a total of 7 doses). Each dose contained 6000 SU/mL of birch pollen allergoid, giving a cumulative quantity of 42,000 SU, which was well in excess of the proposed clinical cumulative dose of 27300 SU. A further group of 15 males and 15 females received control material at 1 mL/rat. Four injection sites were used in rotation (first dose into left shoulder, second dose into right shoulder, third dose into left hip, fourth dose into right hip and so on). Rats were approximately 6–7 weeks old and had bodyweights of 165–213 g (males) and 132–175 g (females) on study initiation. Animals were maintained in an air-conditioned room with 15–20 air changes/hour. The temperature and relative humidity ranges were maintained in the specified ranges of 20–24 °C and 45–65%, respectively and a 12 h light/dark cycle was used. Standard animal diet and water were available *ad libitum* throughout the study. Rats were housed 2 or 3 to a cage.

Following the last dose, 10 male and 10 female animals from the control and test groups were sacrificed after 13 weeks; remaining rats were retained for a 4-week recovery period. The following study parameters were measured: mortality and clinical observations, bodyweights, food consumption, ophthalmoscopy, clinical pathology, organ weights plus macroscopic and microscopic examination. Cage-side observations were made daily, bodyweights were recorded weekly and food consumption was measured twice weekly. An ophthalmoscopic examination was performed predose, at Week 12 and at the end of the recovery period; examination was carried out using an ophthalmoscope after induction of mydriasis. During Week 13 and at the end of the

recovery period, blood samples for haematology, coagulation and clinical chemistry assessment were collected from all animals after fasting overnight. Samples were collected in EDTA, trisodium citrate and lithium heparin tubes, respectively. Haematology parameters measured comprised: haemoglobin, red blood cell count, packed cell volume, mean cell volume mean cell haemoglobin, mean cell haemoglobin concentration, reticulocyte count, red cell distribution width, total and differential white cell count and platelet count. Coagulation parameters measured comprised: activated prothrombin time, partial thromboplastin time and fibrinogen. Clinical chemistry parameters measured comprised: aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase, alkaline phosphatase, total cholesterol, triglycerides, total bilirubin, total protein, albumin, globulin, albumin:globulin (A/G) ratio, sodium, potassium, chloride, calcium, inorganic phosphate, creatinine, urea, glucose and creatine kinase. The following urine parameters were measured: volume, colour, turbidity, specific gravity, pH, protein, glucose, ketones, urobilinogen, bilirubin, blood and microscopy of sediment.

Rats were sacrificed after 13 weeks of treatment (Day 86) or, on Day 29 of the recovery period, by isoflurane anaesthesia prior to exsanguination and then examined grossly for pathological changes. Selected organs were weighed and comprised: adrenal, brain, epididymis, heart, kidney, liver, ovary, pituitary, prostate, spleen, testis, thymus, thyroid with parathyroid, uterus with cervix. Following processing to 5 µm sections and staining with haematoxylin and eosin for light microscopy, the following organs/tissues were examined histopathologically for all animals of the terminal control and test groups: adrenal, aorta, brain, caecum, colon, duodenum, epididymis, oesophagus, eyes, femur with bone marrow and femorotibial joint, gut-associated lymphoid tissue/Peyer's patch, gross lesions, heart, ileum, injection sites, jejunum, kidney, liver, lungs with mainstem bronchi and bronchioles, lymph node (axillary, mandibular and mesenteric), mammary gland, muscle (biceps femoris), nerve (optic and sciatic), ovary, pancreas, pituitary, prostate, rectum, salivary gland (mandibular and sublingual), seminal vesicle, skin and subcutis, spinal cord (at 3 levels), spleen, sternum with bone marrow, stomach, testis, thymus, thyroids with parathyroids, tongue, trachea, urinary bladder, uterus with cervix and vagina. Injection sites and any gross lesions were examined for all recovery group animals.

Statistical analysis of bodyweight, food consumption, clinical pathology parameters and organ weights occurred with comparison of the control group with those receiving the PQ Birch. Data for each sex were analysed separately and Levene's test was performed to test for equality of variances between groups. When Levene's test was significant ($P \leq 0.05$), a rank transformation (to stabilize the variances) was applied to the data prior to analysis. Data were analysed using a two-sample *t*-test. Probability values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically significant.

2.5. Local tolerance study

In this study, PQ Birch was injected subcutaneously once, at a dose level of 1 mL/animal, into shaven left and right anterior skin sites of each of 8 New Zealand White rabbits (4 males and 4 females) on Day 1. The control formulation was similarly injected into posterior skin sites on the left and right flanks of each animal. Rabbits were obtained from Harlan UK Ltd, Bicester, United Kingdom and subjected to a 5 day acclimation period. At dosing, rabbits were weighed 2.5–3.0 kg. Animal husbandry was similar to that described for the repeat dose toxicology study, although rabbits were housed singly. Groups of 2 males and 2 females were sacrificed on Day 5 by intravenous injection of sodium pentobarbitone and the remaining animals were terminated on Day 10. Assessment of clinical signs, bodyweights (Day -1, 1 and prior to necropsy) and local dose site reaction was made throughout the study; dose sites were evaluated macroscopically and histologically following the Day 5 and 10 sacrifice. Injections sites, the surrounding tissue and

an area of untreated skin from all animals were taken, processed to 5 µm sections and stained with haematoxylin and eosin. Specific observations for erythema and oedema of all animals for up to 96 h after being dosed comprised:

- Erythema & eschar (no erythema - grade 0, very slight erythema (barely perceptible) - grade 1, well-defined erythema - grade 2, moderate to severe erythema - grade 3 and severe erythema (beef redness) to eschar formation preventing grading of erythema - grade 4).
- Oedema (no oedema - grade 0, very slight oedema (barely perceptible) - grade 1, slight oedema (edges of area well-defined by definite raising) - grade 2, moderate oedema (raised approximately 1 mm) - grade 3 and severe oedema (raised more than 1 mm and extending beyond the test site) - grade 4).

3. Results

3.1. Bacterial reverse mutation assay

No evidence of toxicity was observed, as would normally be manifest as a thinning of the background bacterial lawn or a marked reduction in revertant bacterial colonies. No precipitation was observed on the test plates following incubation. Revertant numbers are shown in Table 2. PQ Birch did not induce mutation in any of the 5 *Salmonella typhimurium* strains in both the absence and presence of S9, when tested using a range of concentrations up to a maximum practicable level of 3000 SU/plate in comparison with the vehicle control. The mean number of revertants per plate was comparable between the negative controls (untreated and vehicle control) treatments. For each strain, the negative controls showed the expected background levels of mutant colonies and positive control chemicals induced significant increases in mutant colonies, both in the absence and presence of S9, demonstrating the validity of the assay.

3.2. Chinese hamster ovary micronucleus assay

Key findings are shown in Table 3. Treatment of cells with PQ Birch for 3 + 21 h treatment in the absence and presence of S9 in Experiment 1 resulted in mean frequencies of MNBN cells that were significantly higher ($p \leq 0.05$) than the concurrent vehicle controls at the lowest concentration analysed (400 SU/mL) for both treatment conditions. However, the MNBN cell frequencies fell within the historical control ranges at all concentrations analysed and the Cochran-Armitage linear trend test was not significant under both treatment conditions. The statistically significant increases were therefore considered not biologically relevant under both treatment conditions. Treatment of cells for 24 + 0 h in the absence of S9 in Experiment 1 resulted in mean frequencies of MNBN cells that were significantly higher ($p \leq 0.05$) than the concurrent vehicle controls at the lowest concentration analysed (80 SU/mL). The MNBN cell frequencies exceeded the normal range in single cultures at the 3 highest concentrations analysed (80, 100 and 120 µg/mL) and the mean MNBN cell frequencies at 80 and 120 SU/mL (2% and 1.7%, respectively) marginally exceeded the normal range of 0.3–1.58%, but the Cochran-Armitage linear trend test was not significant. These findings were considered equivocal and followed up in another assay. Treatment of cells for 24 + 0 h in the absence of S9 in Experiment 2 resulted in mean frequencies of MNBN cells that were similar to and not significantly higher than the concurrent vehicle controls at all concentrations analysed. The MNBN cell frequencies fell within the normal range in all treated cultures and the Cochran-Armitage linear trend test was not significant. Thus, the equivocal micronucleus data for the 24 + 0 h treatment in the absence of S9 in Experiment 1 were followed up with a clear negative result for micronucleus induction under the same treatment condition in Experiment 2. Recommendations for assessing weight of evidence and

Table 2
Bacterial reverse mutation test – mean revertant numbers per plate.

Concentration (SU/plate)	TA98		TA100		TA1535		TA1537		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
VHC ^a	17.2 ± 5.2	36.2 ± 6.9	138.6 ± 7.0	117.4 ± 11.5	10.2 ± 5.3	16.8 ± 5.2	8.4 ± 3.2	22.4 ± 5.7	290.0 ± 14.1	210.6 ± 33.4
UTC	19.0 ± 6.4	ND	137.2 ± 4.0	127.6 ± 16.0	8.8 ± 3.9	15.2 ± 1.9	15.2 ± 1.3	20.4 ± 4.2	288.8 ± 6.3	225.6 ± 11.5
3	20.0 ± 4.0	36.3 ± 2.9	133.7 ± 12.4	128.7 ± 20.7	12.7 ± 2.5	12.3 ± 6.0	7.0 ± 2.6	19.7 ± 7.0	274.7 ± 20.0	188.7 ± 12.5
9.49	18.7 ± 0.6	34.3 ± 9.1	138.7 ± 15.1	112.3 ± 1.2	12.7 ± 4.6	14.3 ± 1.2	4.3 ± 2.9	17.0 ± 7.5	261.3 ± 9.0	195.3 ± 27.0
30	21.7 ± 3.2	41.0 ± 10.4	146.3 ± 8.5	117.0 ± 13.2	13.3 ± 5.8	16.3 ± 2.5	9.3 ± 4.5	25.0 ± 1.0	258.3 ± 49.4	200.7 ± 10.7
94.9	17.7 ± 6.5	30.0 ± 4.0	153.3 ± 9.1	109.7 ± 7.8	12.3 ± 4.0	22.0 ± 7.9	8.7 ± 1.2	21.7 ± 3.2	280.0 ± 18.4	182.7 ± 11.0
300	20.0 ± 1.0	34.0 ± 3.5	131.3 ± 5.7	121.3 ± 13.5	12.0 ± 1.7	16.7 ± 3.2	12.3 ± 7.1	27.3 ± 3.8	289.0 ± 13.7	172.0 ± 9.6
947	22.7 ± 3.2	34.7 ± 0.6	146.3 ± 20.3	124.3 ± 14.2	14.3 ± 5.1	20.0 ± 3.5	7.3 ± 1.2	18.0 ± 3.6	306.7 ± 14.2	178.0 ± 6.6
3000	17.7 ± 4.2	35.7 ± 7.6	150.3 ± 8.7	119.7 ± 18.0	14.0 ± 6.1	16.7 ± 2.1	8.7 ± 0.6	14.0 ± 6.1	296.0 ± 32.2	165.7 ± 39.3
Positive control (µg/plate)	2NF (5)	BlaIP (10)	Na ₂ S (2)	AAN (5)	Na ₂ S (2)	AAN (5)	AAC (50)	AAN (5)	MMC (0.2)	AAN (20)
	786.0 ± 155.5	403.0 ± 48.5	946.0 ± 55.7	1683.3 ± 211.1	696.7 ± 6.8	245.3 ± 10.7	85.7 ± 14.3	111.3 ± 9.5	689.3 ± 65.7	732.0 ± 62.5

UTC Untreated control.

ND No data obtained.

^a Vehicle control (2% Tyrosine Blank).

Table 3MNBN cell frequencies from *in vitro* micronucleus testing using CHO cells.

Treatment	Concentration (SU/mL)	Cytotoxicity (%) ^d	Mean MNBN Cell Frequency (%)	Historical Control Range (%) ^c	Statistical Significance
3 + 21 h -S-9 Experiment 1	Vehicle ^a	–	0.50	0.21–1.39	–
	400.0	0	0.95		p ≤ 0.05
	500.0	0	0.45		NS
	600.0	0	0.70		NS
	^b MMC, 0.20	19	8.50		p ≤ 0.001
3 + 21 h + S-9 Experiment 1	Vehicle ^a	–	0.70	0.11–1.79	–
	400.0	0	1.25		p ≤ 0.05
	500.0	0	1.00		NS
	600.0	0	0.45		NS
	^b CPA, 4.00	15	7.65		p ≤ 0.001
24 + 0 h -S-9 Experiment 1	Vehicle ^a	–	1.20	0.30–1.58	–
	UTC	–	0.55		NS
	80.00	2	2.00		p ≤ 0.05
	100.0	0	1.45		NS
	120.0	5	1.70		NS
	^b VIN, 0.005	0	4.35		p ≤ 0.001
24 + 0 h -S-9 Experiment 2	Vehicle ^a	–	0.35	0.30–1.58	–
	UTC	–	0.60		NS
	80.00	0	0.20		NS
	100.0	0	0.70		NS
	120.0	0	0.40		NS
	^b VIN, 0.005	0	1.90		p ≤ 0.001

NS Not significant.

UTC Untreated control.

^a Vehicle control (2% Tyrosine Blank) added at 10% v/v for the 3 + 17 h - and +S-9 treatments and at 2% v/v for the 24 + 0 h -S-9 treatment.^b Positive control, µg/mL.^c 95th percentile of the observed range.^d Based on replication index (the relative number of nuclei compared to vehicle controls).

follow-up testing for positive or equivocal genotoxicity results, proposed by the International Workshop for Genotoxicity Testing (IWGT) (Thybaud et al., 2007) suggest that as the equivocal *in vitro* result for micronucleus induction in Experiment 1 was not reproduced in Experiment 2, the weight of evidence indicates a lack of genotoxic potential. Finally, all positive control compounds induced statistically significant increases in the proportion of cells with micronuclei, demonstrating the validity of the assay.

As can be seen from Table 4, increases in the numbers of binucleate cells with NPBs (indicating misrepair of double-strand DNA breaks) were seen in all treated cultures and the vehicle controls for the 24 + 0 h treatment in the absence of S-9, compared to the untreated (culture medium) controls, in both Experiment 1 and 2 and was particularly evident in Experiment 1.

3.3. Repeat dose toxicity studies

Single subcutaneous doses of 1 mL PQ Birch at 6000 SU/mL once every 2 weeks for 13 weeks were well tolerated with no obvious signs of systemic toxicity and study findings were limited to a few clinical pathology parameters and dose site histopathological change. Slightly higher bodyweight gain (2%) at termination for females (resulting in a 9% increase in bodyweight) compared to the control group was not seen after the recovery period and was considered co-incidental. Haematology and coagulation parameter changes at termination are given in Table 5. Thus, there were minor increases in reticulocyte count (mainly in females), red cell distribution width (mainly in males) and activated partial thromboplastin time (increased in males and decreased in females), which may well have been co-incidental along with increased white blood cell count (both sexes), notably for neutrophils and large unstained cells; lymphocytes were decreased (both sexes) and fibrinogen level was slightly increased (both sexes). Table 6 shows that clinical chemistry changes were associated with increased globulin level (both sexes) resulting in decreased A/G ratio and minor alterations in albumin (decreased in both sexes) and total protein (increased in both sexes). None of these clinical pathology changes were seen after

Table 4Binucleate cells with NPBs from *in vitro* micronucleus testing using CHO cells.

Treatment (SU/mL)	Replicate	Experiment 1 NPBs	Experiment 2 NPBs
Vehicle ^a	A	133	27
	B	129	33
	Total	262	60
UTC	A	44	25
	B	37	16
	Total	81	41
80.00	A	125	34
	B	147	34
	Total	272	68
100.0	A	176	31
	B	189	33
	Total	365	64
120.0	A	146	29
	B	149	32
	Total	295	61
^b VIN, 0.005	A	57	15
	B	32	15
	Total	89	30

UTC Untreated control.

NPB Nucleoplasmic bridge.

^a Vehicle control was 2% Tyrosine Blank.^b Positive control, µg/mL.

the recovery period. Macroscopic examination showed occasional red areas/reddening or thickening/raised areas among animals of control and test groups at the left hip dose site, while gelatinous appearance

Table 5

Noteworthy haematology and coagulation findings after 13 weeks of treatment with PQ Birch compared to control.

Parameter (Units)	Control males	Test males	Control females	Test females
Reticulocyte (10E9/L)	184.7 ± 21.81	191.0 ± 22.33 (+3%)	167.5 ± 31.56	204.3 ± 33.35 (+22%)*
Reticulocyte (%)	2.0 ± 0.24	2.1 ± 0.23 (+5%)	2.0 ± 0.37	2.5 ± 0.43 (+25%)*
Red cell distribution width (%)	12.2 ± 0.46	13.3 ± 0.56 (+9%)*	10.9 ± 0.43	11.2 ± 0.33 (+3%)
White blood cell (10E9/L)	6.3 ± 0.47	9.1 ± 2.37 (+44%)*	4.2 ± 0.55	5.2 ± 1.55 (+24%)
Neutrophil (10E9/L)	1.56 ± 0.368	4.80 ± 2.050 (+208%)*	1.12 ± 0.286	2.62 ± 0.995 (+134%)*
Large unstained cell (10E9/L)	0.06 ± 0.017	0.13 ± 0.024 (+117%)*	0.04 ± 0.021	0.06 ± 0.017 (+50%)
Lymphocyte (%)	71 ± 4.8	45 ± 8.9 (-37%)*	68 ± 6.3	46 ± 8.0 (-32%)*
APTS (sec)	18.9 ± 0.72	19.9 ± 1.40 (+5%)	21.7 ± 8.28	19.4 ± 1.03 (-11%)
Fibrinogen (g/L)	1.68 ± 0.117	2.47 ± 0.109 (+47%)*	1.32 ± 0.156	2.09 ± 0.162 (+58%)*

APTS = Activated partial thromboplastin time.

*p < 0.05; ***p < 0.001.

Note: Values in parenthesis are percent change from control (2% Tyrosine suspension).

was noted for a small number of animals administered PQ Birch. Histopathology findings are shown in Table 7. At the left hip dose site, inflammation was observed in both control and test groups and was characterised by a zone of inflammatory cells (predominantly neutrophils) surrounding a central amorphous area of injected material. In general, the severity of this finding was marginally higher for animals administered PQ Birch compared with the control group. At the other dose sites (right shoulder, left shoulder and right hip), focal areas of fibrosis were noted for a small number of animals in both control and test groups. After the recovery period, focal areas of fibrosis were observed at dose sites in single animals administered PQ Birch.

3.4. Local tolerance study

Single subcutaneous injections of 1 mL PQ Birch at 6000 SU/ml to the rabbit resulted in no clinical signs, minor skin reactions and no macroscopic or vaccine-related histopathological changes. Skin reactions on Day 5 were limited to very slight to slight oedema and/or slight to well defined erythema at the site of injection but only in 4 out of 8 animals and in 3 animals, the signs were restricted to only one of the 2 dose sites (data not shown). No dermal observations were recorded at any of the dose sites where the control formulation was administered. Histopathology findings at Day 5 were comparable between dose sites administered PQ Birch and the control formulation and included subacute focal/localised inflammatory changes such as mixed inflammatory cell accumulation and fibroblast/blood vessel proliferation often with central necrosis and foreign (test or control) material (data not shown). At Day 10, findings were again similar for the 2 groups, frequently less pronounced than at Day 5 and included chronic focal/localised inflammatory changes with variable, sometimes minor accumulation of mononuclear cells, particularly epithelioid and foreign body giant cells with accompanying granulation/fibrous tissue, sometimes with central pseudocyst containing foreign material. There were also occasions of central necrosis usually with accompanying active inflammation.

Table 6

Noteworthy clinical chemistry findings after 13 weeks of treatment with PQ Birch compared to control.

Parameter (Units)	Control males	Test males	Control females	Test females
Total Protein (g/L)	68 ± 2.7	73 ± 2.4 (+7%)*	73 ± 3.3	76 ± 3.6 (+4%)
Albumin (g/L)	42 ± 1.2	39 ± 1.6 (-7%)*	49 ± 2.5	46 ± 1.1 (-6%)*
Globulin (g/L)	27 ± 2.1	34 ± 2.7 (+26%)*	24 ± 1.8	30 ± 3.4 (+25%)*
A:G ratio	1.6 ± 0.12	1.2 ± 0.11 (-25%)*	2.1 ± 0.17	1.6 ± 0.19 (-24%)*

A:G = Albumin:globulin.

p < 0.01; *p < 0.001.

Note: Values in parenthesis are percent change from control (2% Tyrosine suspension).

4. Discussion

PQ Birch is an allergoid adsorbed to L-tyrosine with MPL® AIT for the treatment of birch pollinosis. An array of nonclinical data are available supporting the safety of allergoid L-tyrosine with MPL® products and their components (Baldrick et al., 2002a, 2002b; 2002c, 2004; 2007, 2011; Reeve et al., 2012). The present investigation was to further characterise the safety of PQ Birch to support clinical development as discussed and agreed following regulatory agency interaction. Not unexpectedly given its nature as a pollen extract, PQ Birch was not genotoxic. Testing in a bacterial reverse mutation assay showed no mutation at concentrations up to 3000 SU/plate, in the absence and presence of S9. It was shown that PQ Birch did not induce micronuclei in Chinese hamster ovary (CHO) cells when tested up to the maximum practicable concentrations of 600 SU/mL for 3 + 21 h in the absence and presence of S9 and 120 SU/mL for 24 + 0 h in the absence of S9. Increases in the frequency of NPBs were observed in both 2% Tyrosine Blank and PQ Birch treated cultures following incubation for 24 + 0 h in the absence of S-9 in comparison with untreated control cultures. NPBs between nuclei in binucleate cells recorded during micronucleus analysis can provide an indication of chromosome rearrangement and originate from dicentric chromosomes, possibly by misrepair of double-strand DNA breaks or telomere end fusions (Thomas et al., 2003). The significance of this finding is unclear, especially as it was only seen in one of the treatment regimens but may suggest that some component part/s of the vehicle used may not be suitable for use in the test system for an extended treatment period. Confirming the results from studies with PQ Birch, previous genotoxicity testing with allergoid L-tyrosine with MPL® products (including *in vivo* micronucleus and Comet assay studies in rat with the related PQ Grass) and their components have also given negative results (Baldrick et al., 2002a, 2002b; 2007; Reeve et al., 2012).

Subcutaneous doses of PQ Birch every second week on 7 occasions in the rat over 13 weeks showed no obvious signs of systemic toxicity, with minor study findings which reflected use of an adjuvanted vaccine. Thus, increased white cell count (notably neutrophils), increased globulin level (resulting in decreased A/G ratio) and increased fibrinogen were seen with PQ Birch as well as minor dose site reaction in the form

Table 7

Incidence of injection site findings after 13 weeks of treatment and 4 weeks of recovery with PQ Birch compared to control.

		Control male	Test male	Control female	Test female
Tissue and finding	Level (SU/mL)	0	6000	0	6000
Terminal sacrifice					
Subcutaneous, Left Shoulder	No. examined:	10	10	10	10
Fibrosis	Grade - 1	10 0	10 0	9 1	10 0
Subcutaneous, Right Shoulder	No. examined:	10	10	10	10
Fibrosis	Grade - 1	10 0	10 0	10 0	8 2
Subcutaneous, Left Hip	No. examined:	10	10	10	10
Inflammation	Grade - 1	3 3	2 2	2 6	3 2
	2	4	0	2	3
	3	0	6	0	2
Subcutaneous, Right Hip	No. examined:	10	10	10	10
Fibrosis	Grade - 1	9 1	8 1	10 0	9 1
	2	0	1	0	0
Recovery sacrifice					
Subcutaneous, Right Shoulder	No. examined:	5	5	5	5
Fibrosis	Grade - 1	5 0	4 1	5 0	5 0
Subcutaneous, Left Hip	No. examined:	5	5	5	5
Fibrosis	Grade - 1	5 0	5 0	5 0	4 1
Subcutaneous, Right Hip	No. examined:	5	5	5	5
Fibrosis	Grade - 1	5 0	4 1	5 0	4 1

- = Finding not present; 1 = Minimal; 2 = Slight; 3 = Moderate; F = Female; M = Male.

Control = 2% Tyrosine suspension.

of inflammatory cell infiltrate surrounding a central amorphous area of injected material at a marginally higher severity in animals administered PQ Birch and/or a few cases of focal areas of fibrosis which was also seen in controls (same vaccine formulation except birch pollen allergoid and MPL® were not present). Recovery after the 4 week non-dose period was generally seen for study findings. This lack of toxicity was also supported in other work (not published) with a related allergy vaccine ("Tree MATA MPL"). As for PQ Birch, this vaccine was composed of a pollen extract (in this case, a combination of pollen from birch, hazel and alder) which was chemically modified with

glutaraldehyde to form an allergoid, then adsorbed to L-tyrosine with further addition of MPL®. Twice weekly subcutaneous doses to groups of 10 male and 10 female rats using 2000 SU/mL at 0.1 or 1 mL occurred with a 2 week recovery period (using 5 males and 5 females). The test material contained 20 mg/mL L-tyrosine and 0.05 mg/mL MPL®, while a control group received the same formulation but without allergoid or MPL®. Notable clinical pathology findings, compared to control, were only seen at the 1 mL dose and comprised increased neutrophils and/or white blood cell count and lymphocytes along with increased plasma total protein and globulin and/or reduced albumin; all changes showed recovery. Dose site histopathological findings of haemorrhage, cellulitis/fibrosis and granulomas and/or abscesses were seen across all groups (including controls) but were more prominent in the 1 mL vaccine-treated group. Evidence of recovery was seen. Finally, previous toxicology work with other allergoid L-tyrosine with MPL® products (with household mite and ragweed pollen extracts) and their components have further shown consistent findings associated with immunostimulation (including increased white blood cell values and altered globulin levels) in the rat (Baldrick et al., 2002a, 2002b; 2002c, 2004; 2007, 2011). Thus, both systemic and dose site effects are likely due to the immunostimulatory nature of MPL® and/or the presence of L-tyrosine as a response from use of an adjuvanted vaccine. More recently, a review of toxicology studies with 30 different adjuvanted vaccines showed common study findings of altered clinical pathology parameters (commonly raised blood neutrophil count and increased globulin levels, but also raised fibrinogen) and evidence of inflammation at the dose site (Baldrick, 2016). It was concluded that clinical pathological and injection site changes can be interpreted as related to an expected reaction after vaccine dosing, with generation of an immune response largely as a result of the presence of adjuvant, although direct vaccine antigen involvement was also occasionally demonstrated by the presence of a slightly increased inflammatory response seen over adjuvant treatment only.

Local tolerance testing confirmed the dose site effects with PQ Birch. Thus, specific examination following a single subcutaneous injection of the human dose (1 mL PQ Birch) in the rabbit gave minor skin reactions of very slight to slight oedema and/or slight to well defined erythema in 50% of the animals, with signs generally restricted to one of the 2 PQ Birch-treated sites. Histopathology evaluation showed findings at Day 5 that were comparable between dose sites administered PQ Birch and the control (lacking birch pollen allergoid and MPL®) formulation and included subacute focal/localised inflammatory changes which were considered consistent with a non-specific inflammatory response to the injected formulations. Findings at Day 10 were frequently less pronounced than at Day 5 and included chronic focal/localised inflammatory changes and were considered consistent with the reparative phase of an inflammatory response to foreign material. Further local tolerance testing (not published) of the related allergy vaccine ("Tree MATA MPL") mentioned earlier gave similar results. Treatment comprised a single subcutaneous dose to a group of 8 rabbits at 1 mL with vaccine at 2000 SU/mL, vaccine without allergoid or physiological saline. Dose site reactions comprised microscopic findings of cellulitis/fibrosis and granulomas as examined on Day 5 for both vaccine and vaccine without allergoid groups. Evidence of partial recovery (decreased inflammation and greater fibrotic content at dose sites) was seen on Day 10.

Overall, repeated subcutaneous doses of PQ Birch in the rat revealed no safety concerns at a cumulative quantity of 42,000 SU birch pollen extract, which was well in excess of the proposed clinical cumulative dose of 27300 SU. This lack of toxicity has been supported by a large clinical database involving use of PQ Birch or related birch allergoid vaccine (an earlier form without the presence of MPL®) which have been shown to be well tolerated with the majority of adverse drug reactions being resolvable local reactions [in-house data; 18].

In conclusion, a specific nonclinical safety testing strategy developed to support clinical use of PQ Birch and comprising 2 *in vitro*

genotoxicity studies (bacterial reverse mutation and Chinese hamster ovary micronucleus assays), a repeat dose toxicology study in rat and a local tolerance study in rabbit has shown that there were no safety findings of concern. Thus, no evidence of genotoxicity was found. Relatively minor, immunostimulatory effects were seen following repeated dosing (once every 2 weeks for 13 weeks) in the form of reversible increased white cell count (notably neutrophils), increased globulin level (resulting in decreased A/G ratio) and increased fibrinogen were seen as well as minor dose site reaction in the form of inflammatory cell infiltrate. These findings are likely due to the immunostimulatory nature of MPL® and/or the presence of L-tyrosine as a response from use of an adjuvanted vaccine, or a combination of all of these. Similar dose site inflammatory changes to the injected formulation material were also noted in the rabbit local tolerance study.

Funding body information

Safety Evaluation of PQ Birch Allergy Immunotherapy to Support Product Development by Paul Baldrick, James W Hutchings, Matthew D Heath and Murray A Skinner.

No funding body was involved in supporting this work.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflicts of interest

Paul Baldrick assisted in preparation of the manuscript under contract with Allergy Therapeutics Ltd.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yrtph.2019.104441>.

Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.yrtph.2019.104441>.

References

- Akdis, C.A., Akdis, M., 2015. Mechanisms of allergen-specific immunotherapy and immune tolerance to allergens. *World Allergy Organ. J.* 8, 1–12.
- Akkoc, T., Akdis, M., Akdis, C.A., 2011. Update in the mechanisms of allergen-specific immunotherapy. *Allergy Asthma Immunol. Res.* 3 (1), 11–20.
- Baldrick, P., Richardson, D., Wheeler, A.W., 2002a. Safety evaluation of a glutaraldehyde modified tyrosine adsorbed household mite extract containing monophosphoryl lipid A (MPL®) adjuvant: a new allergy vaccine for dust mite allergy. *Vaccine* 20, 737–743.
- Baldrick, P., Richardson, D., Wheeler, A.W., 2002b. Review of L-tyrosine confirming its safe human use as an adjuvant. *J. Appl. Toxicol.* 22, 333–344.
- Baldrick, P., Richardson, D., Elliott, G., Wheeler, A.W., 2002c. Safety evaluation of monophosphoryl lipid A (MPL®): an immunostimulatory adjuvant. *Regul. Toxicol. Pharmacol.* 35, 398–413.
- Baldrick, P., Richardson, D., Wheeler, A.W., Woroniecki, S.R., 2004. Safety evaluation of a new allergy vaccine containing the adjuvant Monophosphoryl Lipid A (MPL®) for the treatment of grass pollen allergy. *J. Appl. Toxicol.* 24, 261–268.
- Baldrick, P., Richardson, D., Woroniecki, S.R., Lees, B., 2007. Pollinex® Quattro Ragweed: safety evaluation of a new allergy vaccine adjuvanted with Monophosphoryl Lipid A (MPL®) for the treatment of ragweed pollen allergy. *J. Appl. Toxicol.* 27, 399–409.
- Baldrick, P., Hewings, S., Skinner, M., 2011. Reproduction and juvenile animal toxicology studies in the rat with a new allergy vaccine adjuvanted with monophosphoryl lipid A (MPL®) for the treatment of grass pollen allergy. *Reprod. Toxicol.* 32 (3), 322–328.
- Baldrick, P., 2016. Dose site reactions and related findings after vaccine administration in safety studies. *J. Appl. Toxicol.* 36, 980–990.
- Bousquet, J., Lockey, R., Malling, H.J., 1998. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J. Allergy Clin. Immunol.* 102 (4 Pt 1), 558–562.
- Bousquet, J., Khaltaev, N., Cruz, A.A., Denburg, J., Fokkens, W.J., et al., 2008. Allergic rhinitis and its impact on asthma (ARIA) 2008 update (in collaboration with the world health organization, GA(2)LEN and AllerGen). *Allergy* 63 (Suppl. 86), 8–160.
- Casella, C.R., Mitchell, T.C., 2008. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell. Mol. Life Sci.* 65 (20), 3231–3240.
- Ebner, C., 1999. Immunological mechanisms operative in allergen-specific immunotherapy. *Int. Arch. Allergy Immunol.* 119, 1–5.
- EMA, 1998. European agency for the evaluation of medicinal products. In: Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines (CPMP/SWP/465/95). http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500004004.pdf, Accessed date: April 2019.
- Evans, J.T., Cluff, C.W., Johnson, D.A., Lacy, M.J., Persing, D.H., Baldridge, J.R., 2003. Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribis. *Expert Rev. Vaccines* 2 (2), 219–229.
- Hutchings, J.W., Heath, M.D., Skinner, M.A., 2017. Immunogenicity of a modified microcrystalline tyrosine (MCT®)-adsorbed Ragweed immunotherapeutic product with Monophosphoryl Lipid A in a murine model. *ARC J. Immun. Vacc.* 2 (1), 22–27.
- ICH.M3(R2), 2009. In: International Conference on Harmonization: ICH M3(R2) Guideline on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorisation for Pharmaceuticals. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/M3_R2_Guideline.pdf Accessed April 2019.
- Larche, M., 2006. Immunoregulation by targeting T cells in the treatment of allergy and asthma. *Curr. Opin. Immunol.* 18, 745–750.
- Leuthard, D.S., Duda, A., Freiburger, S.N., Weiss, S., Dommann, I., Fenini, G., Contassot, E., Kramer, M.F., Skinner, M.A., Kundig, T.M., Heath, M.D., Johansen, P., 2018. Microcrystalline tyrosine and aluminum as adjuvants in allergen-specific immunotherapy protect from IgE-mediated reactivity in mouse models and act independently of inflammasome and TLR signalling. *J. Immunol.* 200, 3151–3159.
- Matsumoto, M., Komatsu, S., Matsui, H., Shimomura, K., Watanabe, K., Amakasu, K., Fueki, O., Sawada, J., Maki, K., Shinoda, K., Onodera, H., 2016. Note on regulatory toxicology requirements for adjuvants and vaccines; in view of the newly established WHO guidelines. *J. Med. Tox. Clin. Forensic Med.* 1 (2), 1–5.
- Moran, D.M., Wheeler, A.W., Overell, B.G., Woroniecki, S.R., 1977. Chemical modification of crude timothy grass pollen extract. III. The effect of glutaraldehyde-induced aggregation on antigenic and immunogenic properties. *Int. Arch. Allergy Appl. Immunol.* 54 (4), 315–321.
- Niederberger, V., 2009. Allergen-specific immunotherapy. *Immunol. Lett.* 122 (2), 131–133.
- Reeve, L., Hewings, S., Skinner, M., 2012. A battery of genotoxicity studies with an allergy vaccine adjuvanted with monophosphoryl lipid A (MPL®) for the treatment of grass pollen allergy. *J. Appl. Toxicol.* 32, 608–616.
- Small, P., Keith, P.K., Kim, H., 2018. Allergic rhinitis. *Allergy asthma. Clin. Immunol.* 14 (Suppl. 2), 31–41.
- Starchenka, S., Bell, A.J., Mwange, J., Skinner, M.A., Heath, M.D., 2017. Molecular fingerprinting of complex grass allergoids: size assessments reveal new insights in epitope repertoires and functional capacities. *World Allergy Organ. J.* 10, 1–13.
- Thomas, P., Umegaki, K., Fenech, M., 2003. Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay. *Mutagenesis* 18, 187–194.
- Thybaud, V., Aardema, M., Clements, J., Dearfield, K., Galloway, S., Hayashi, M., Jacobson-Kram, D., Kirkland, D., MacGregor, J.T., Marzin, D., Ohshima, W., Schuler, M., Suzuki, H., Zeiger, E., 2007. Strategy for genotoxicity testing: hazard identification and risk assessment in relation to *in vitro* testing. *Mutat. Res.* 627, 41–58.
- Van Cauwenberge, P., Bachert, C., Passalacqua, G., Bousquet, J., Canonica, G.W., Durham, S.R., Fokkens, W.J., Howarth, P.H., Lund, V., Malling, H.J., Mygind, N., Passali, D., Scadding, G.K., Wang, D.Y., 2000. Consensus statement on the treatment of allergic rhinitis. *European academy of allergology and clinical immunology. Allergy* 55 (2), 116–134.
- Verlato, G., Corsico, A., Villani, S., Cerveri, I., Migliore, E., Accordini, S., Carolei, A., Piccioni, P., Bugiani, M., Cascio, V.L., Marinoni, A., Poli, A., De Marco, R., 2003. Is the prevalence of adult asthma and allergic rhinitis still increasing? Results of an Italian study. *J. Allergy Clin. Immunol.* 111, 1232–1238.
- Wheeler, A.W., Marshall, J., Ulrich, J.T., 2001. A Th1-inducing adjuvant, MPL®, enhances antibody profiles in experimental animals suggesting it has the potential to improve the efficacy of allergy vaccines. *Int. Arch. Allergy Immunol.* 126, 135–139.
- WHO, 2013. In: World Health Organization. Guidelines on the Nonclinical Evaluation of Vaccine Adjuvants and Adjuvanted Vaccines. http://www.who.int/biologicals/areas/vaccines/ADJUVANTS_Post_ECBS_edited_clean_Guidelines_NCE_Adjuvant_Final_17122013.WEB.pdf Accessed April 2019.
- Worm, M., Higenbottam, T., Pfaar, O., Mosges, R., Aberer, W., Gunawardena, K., Wessiepe, D., Lee, D., Kramer, M.F., Skinner, M., Lees, B., Zielen, S., 2018. Randomized controlled trials define shape of dose response for Pollinex Quattro Birch allergoid immunotherapy. *Allergy* 73 (9), 1812–1822.