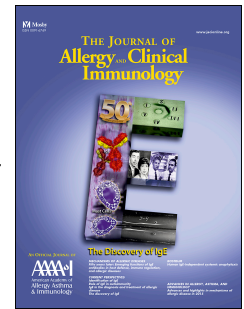


Accepted Manuscript

Lolium perenne peptides for treatment of grass pollen allergy: A randomized, double-blind, placebo-controlled clinical trial

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Title: *Lolium perenne* Peptides for the Treatment of Grass Pollen Allergy: A

Randomized Double Blind Placebo-Controlled Clinical Trial

Capsule summary

- Peptide hydrolysate from *Lolium Perenne* (LPP) has reduced allergenicity and maintained immunogenicity
- Subcutaneous administration of peptide hydrolysate from *Lolium Perenne* (LPP) is safe and tolerated.
- Peptide hydrolysate from *Lolium Perenne* (LPP) induces allergen-specific IgG4 and other blocking antibodies.

Keywords

Seasonal Allergic Rhinitis, allergen immunotherapy, grass pollen peptide immunotherapy, DnaK, HSP70, safety, IgG4, blocking antibodies.

Abbreviations

AIT, allergen immunotherapy; ELISA, enzyme-linked immunosorbent assay; LP, *Lolium perenne*; LPP, *Lolium perenne* peptides formulated for subcutaneous injection, Ig, immunoglobulin; NS, not statistically significant; PBMC, peripheral blood mononuclear cells; TEAE, treatment-emergent adverse events; SAR, seasonal allergic rhinitis; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; IL, interleukin; CD, clusters of differentiation; IC₅₀, concentration at which the inhibitor response is reduced by half; ACS, ; RTSS, rhinoconjunctivitis total symptom scores; RMS, rescue medication scores; TSS, total symptom scores; FAB, facilitated allergen binding.

26 **To the Editor:**

27 Allergen immunotherapy (AIT), first used more than one century ago,¹ is an effective
28 treatment in patients with seasonal allergic rhinitis (SAR). Allergen extract can be
29 administered by subcutaneous (SCIT) or sublingual (SLIT) route. It is important to note that
30 allergen extract preparations used for AIT vary between manufacturers' batches and may
31 contain cross-reacting allergens, which can increase the risk of systemic reactions.² As the
32 antigen allergenicity is associated with resistance to proteolysis,³ several innovative strategies
33 are currently being instigated, such as replacing whole allergen with allergen-derived
34 peptides. The advantages over the whole-protein allergens, peptides do not bind to IgE and
35 cross-link FcεRI on the surface of mast cells and basophils, therefore they do not release
36 mediators such as tryptase and histamine. T-cell epitope peptides AIT is being developed for
37 the treatment of several allergies, including cat,⁴ peanut,⁵ and birch allergy.⁶ This method
38 uses a mixture of a limited number of synthetic peptides known to contain the
39 immunodominant T-cell epitope of major allergen component. Another approach involves the
40 use of proteolytic hydrolysates of allergens which has the advantage over synthetic peptides
41 by providing more epitopes and therefore can be used in a wider population. We have
42 developed a formulation of purified peptidic fragments of rye grass (*Lolium perenne*
43 peptides) (LPP) suitable for subcutaneous AIT administration. Due to the extensive cross-
44 reactivity of allergenic components of grass pollen from different species, the *L. perenne*
45 allergen can be used to treat allergic rhinitis induced by other grasses.⁷

46

47 The purpose of this study was to assess the safety and the immunologic effect of LPP in
48 adults with grass pollen-induced SAR. In addition, this study examined the adjuvant effect of
49 a bacterial homolog of the heat shock protein 70 family (HSP70, DnaK).⁸ DnaK have been
50 shown to suppress inflammation in animal models of arthritis by induction of IL-10

51 producing cells and has been tested in clinical trials.⁸ LPP (ranging between 1 and 10 kDa)
52 was generated by enzymatic hydrolysis of purified proteins from *Lolium perenne* (see the
53 Methods section in this article's Online Repository at www.jacionline.org for further details).
54 Recombinant DnaK was produced in *Escherichia coli* and purified as an aqueous solution.⁹
55 LPP and DnaK were reproducibly manufactured in gram quantities according to Good
56 Manufacturing Practice guidelines. The study was approved by the local ethics committees
57 (Leuven University Hospital) and all subjects gave written informed consent.

58

59 The binding capacity of LPP to IgE was assessed by immunoblot analysis using IgE-rich
60 serum (*Lolium Perenne* specific IgE >100kU/L) obtained from grass pollen allergic patients
61 (see the Methods section in this article's Online Repository at www.jacionline.org for further
62 details). Purified pollen proteins contained major (Lol p 1 and p 5) and minor allergens (Lol p
63 11, p 2, and p 3) from *L. perenne* (Figure 1A, lane 2). These proteins were not detectable in
64 the corresponding peptide preparations and no IgE binding was detectable (Figure 1A, lane
65 1). Purified *L. perenne* proteins inhibited serum IgE binding to proteins with an IC₅₀ of 0.1
66 µg/mL (Figure 1B). The IC₅₀ for the corresponding peptides, in contrast, was 100-fold higher
67 (10 µg/mL). Reproducibility of LP peptides batch to batch variability was also assessed by
68 ELISA (Figure 1C). Allergenicity of LPP was investigated using blood samples from 12
69 allergic and 12 non-allergic subjects (Table E1). A dose-dependent expression of CD63, a
70 marker of basophil activation and degranulation, was observed in allergic patients (Figure
71 1D) but not in non-allergic subjects (Figure 1E, Table E2A-B) when basophils were
72 stimulated with LP proteins. This stimulation was significantly higher than the one induced
73 by the same concentration of LPP. A 100-fold higher concentration of LP peptides was
74 required to elicit basophil degranulation. DnaK alone had no effect on basophil activation and
75 degranulation. Adding DnaK to the peptides (1:1 w/w) reduced their ability to induce

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degranulation ($P < .01$ at 1000 ng/mL, compared to LPP) (Figure 1D). Moreover, the immunogenicity of LP peptides was demonstrated when PBMCs obtained from grass pollen-allergic and non-allergic subjects were stimulated with LP proteins, LP peptides, DnaK and LP peptides/DnaK. LP peptides induced dose-dependent proliferation which is smaller than the one of whole proteins and is restricted to allergic subjects (Figure S1A, Table E3A-B). LP proteins and LP peptides both stimulated, to a similar extent, the production of IL-5 in PBMCs from allergic subjects (Figure S1B-C, Table E3A-B).

We tested the safety of LPP and LPP/DnaK in a randomized, double-blind, placebo-controlled, parallel group trial of AIT (www.clinicaltrials.gov, NCT01111279). Study participants ($n=27$) were sequentially randomized 1:1:1 to receive 5 subcutaneous injections (weekly) of placebo, increasing doses of LPP, or LPP/DnaK (Figure S2-3, Table E4). The primary endpoint was to assess the safety of LPP and LPP/DnaK. In the placebo group, all local reactions were mild (≤ 5 cm). No clinically relevant differences were observed in the local reactions between the LPP and LPP/DnaK groups, and reaction size did not increase with successive increase of LPP or LPP/DnaK dose (Figure S4, Table E5). During treatment two unsolicited AEs were reported in the placebo group as related to treatment, 16 AEs were reported in the LPP group, and 12 AEs in the LPP/DnaK group. The frequency of AE of special interest (especially headache) was higher in the LPP/DnaK group. All AEs were mild or moderate and those of special interest were mild and resolved without corrective therapy. No differences in the hematological and biochemistry parameters were observed between groups (Table E6). The first patient who received LPP developed anaphylaxis at the first injection (50 μ g). Following this event, the treatment schedule was reviewed and the initiation dose was reduced to 5 μ g. The new revised treatment schedule was tolerated by all

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the patients (see the Methods section in this article's Online Repository at www.jacionline.org for further details).

This study was not powered to demonstrate clinical efficacy of LPP and/or LPP/DnaK. No difference was observed between treated-groups and placebo when considering the full analysis set. However, when considering the patients having received at least a cumulative dose of 105 µg of LPP peptides (78% of the cumulative dose per protocol), a reduction of 35% was observed for ACS, 30% for RTSS, 43% for RMS and 32% for TSS and mean number of well days was higher in patients treated with LPP compared to placebo over the grass pollen season (Table E7). In contrast, ACS, RTSS, RMS and TSS were higher and mean number of well days was lower in the LPP/DnaK group than in the placebo group (Table E7). The higher frequency of AEs in LPP/DnaK and the apparent lack of efficacy suggested that the proportion of LPP and DnaK may be not optimal. A post-hoc analysis of the relation between cumulative dose of LPP peptides and clinical scores showed that the dose of LPP peptides was positively correlated with the number of well days (Figure 1I-J) and negatively correlated with the average combined score (data not shown). An inverse relationship was observed in the LPP/DnaK group. These results suggested that DnaK might have an adjuvant effect at very low cumulative doses (45 µg). Further investigations are required to confirm this hypothesis. The secondary endpoint was to assess the impact of LPP and LPP/DnaK on IgE, IgG4 and blocking antibody responses. Grass pollen-specific IgE levels were increased in LPP and LPP/DnaK treated groups at week 5, 14 and 25 compared to baseline (Figure 1F). A natural course of sIgE induction was observed in the placebo-treated group at week 14 (pollen season) and persisted until week 25. Grass pollen-specific IgG4 levels were increased in LPP and LPP/DnaK groups after treatment and remained elevated until week 25 (all, $P < .01$). No significant change in the placebo groups was observed (Figure

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1G). IgG-associated blocking antibodies as measured by the IgE-FAB assay (see the Methods section in this article's Online Repository at www.jacionline.org for further details) were induced in both treated groups. Allergen-IgE binding to B cells was reduced following treatment with LPP and LPP/DnaK and reached a -30.1% decrease compared to baseline ($P<.01$) in LPP group and -21.3% (NS) in LPP/DnaK group at the end of the study (Figure 1I).

In summary, we report for the first time the characterization of LPP tested as candidates for AIT. The administration of only 5 subcutaneous injections of increasing doses of LPP was safe and well-tolerated. The treatment was associated with the induction of immune reactive and IgG-associated blocking antibodies that persisted for at least 24 weeks. Consequently, LPP alone may be a potential candidate for AIT and is being further validated in AIT trials (NCT01111279).

Figure 1. A) IgE binding to *L. perenne* proteins and processed peptides by western blot (2.3 µg of proteins and peptides have been loaded on SDS gel) . B) Capacity of the LP proteins and LPP to inhibit the binding of sIgE to pollen. C) Batch-to-batch reproducibility of LLP peptides. D-E) *Ex-vivo* basophil responsiveness to LP proteins, LPP, DnaK, LPP/DnaK in grass pollen allergics (n=12) and non-allergics (n=12). Wilcoxon rank test compared to corresponding concentration of LPP. F-H) sIgE, sIgG4 and blocking antibody responses following of subcutaneous LLP, LLP peptides/DnaK or placebo. I-J) Number of well days and cumulative dose of LPP and LPP /DnaK.

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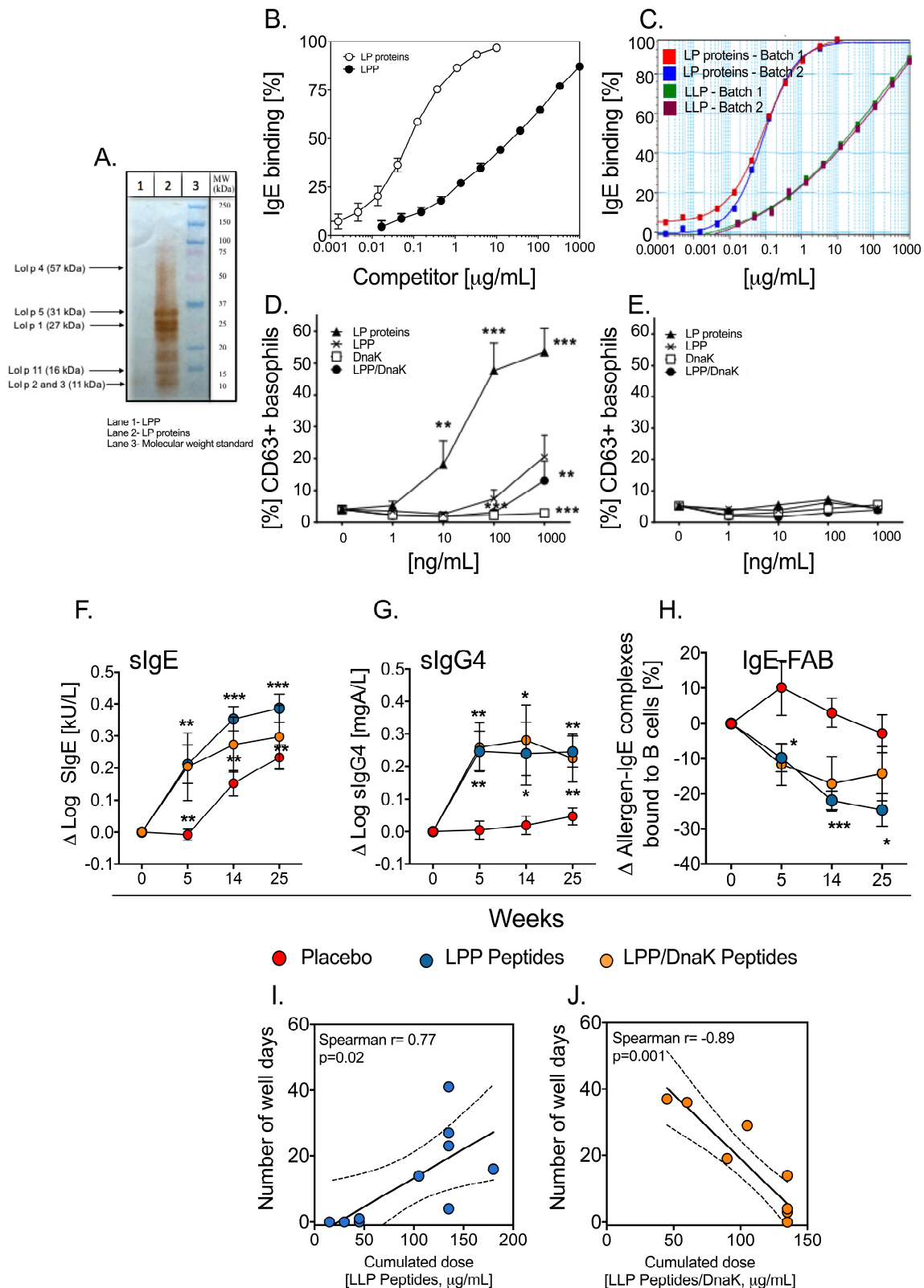


Figure 1. A) IgE binding to L perenne proteins and processed peptides by western blot (2.3 μg of proteins and peptides have been loaded on SDS gel). B) Capacity of the LP proteins and LPP to inhibit the binding of sIgE to pollen. C) Batch-to-batch reproducibility of LLP peptides. D-E) Ex-vivo basophil responsiveness to LP proteins, LPP, DnaK, LPP/DnaK in grass pollen allergics (n=12) and non-allergics (n=12). Wilcoxon rank test compared to corresponding concentration of LPP. (F-H) sIgE, sIgG4 and blocking antibody responses following of subcutaneous LLP, LLP peptides/DnaK or placebo. * $<.05$, ** $.01$ and *** $<.001$. I-J) Number of well days and cumulative dose of LPP and LPP /DnaK.

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METHODS AND MATERIALS

***Lolium perenne* Peptides for the Treatment of Grass Pollen Allergy: A Randomized Double Blind Placebo-Controlled Clinical Trial**

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31

32

33 METHODS & MATERIALS**34 Ethics**

35 Both studies were approved by the local ethics committees and were conducted in
36 compliance with the Declaration of Helsinki (as amended in Tokyo, Venice, Hong Kong,
37 South Africa, Edinburgh, Washington and Tokyo), International Conference for
38 Harmonization Guideline for Good Clinical Practice (January 1997), and all international and
39 national laws and regulations. All subjects gave written informed consent before being
40 included in the studies.

41

42 Investigational product

43 The mixture of grass pollen peptides in LPP was generated by enzymatic hydrolysis of
44 purified proteins from *Lolium perenne*. The production process for the pollen peptides
45 involved the following steps: 1) Extraction of a natural source of allergens of *Lolium perenne*
46 to obtain a crude allergen extract. 2) Purifying the crude extract to remove non-protein
47 components to obtain a purified extract. 3) Denaturing the proteins present in this purified
48 extract. 4) Enzymatically hydrolyzing the denatured proteins to generate allergen fragments.
49 5) Performance of a size selection to obtain allergen fragments with a MW comprised
50 between 10kDa and 1kDa. The amount of enzyme used and condition of hydrolysis were
51 defined to guarantee hydrolysis of all allergens as well as batch to batch consistency (figure
52 1C below). The enzyme was then removed to ensure the ratio enzyme/peptide was less than
53 0.01%. Both proteins and peptides quantities were assessed by a validated BCA protein assay
54 (bicinchoninic acid). Recombinant DnaK was produced in *Escherichia coli* and purified as
55 an aqueous solution.¹ Both LPP and DnaK were produced according to Good Manufacturing
56 Practice guidelines. The determination of purity rate was made by SDS-PAGE and
57 Coomassie Blue coloration. This purity rate concerns only the degradation products or the
58 truncated forms of DnaK. The purity of GMP DnaK bulk was estimated by visual comparison
59 of the impurity bands contained in the 10 µg load lane and the main band of GMP DnaK bulk
60 in several dilutions. The electrophoretic purity of the GMP DnaK is > 98.25%.

61

62 Study design

63 This was a randomized, double-blind, placebo-controlled, parallel group trial of AIT (Eudract
64 no. 2010-018648-15; NCT01111279) performed at the Department of Allergy and Clinical

Immunology, UZ, Leuven, Belgium. The study included 27 patients of both genders from 18 to 50 years of age with moderate to severe allergic rhinitis to grass pollen during at least the 2 previous years (according to ARIA classification),² a positive skin prick test (wheal diameter ≥ 3 mm) to standardized grass pollen mixture extract (ALK-Abelló, Hørsholm, Denmark), and a grass pollen-specific immunoglobulin IgE level >0.7 kU/L. Patients were sequentially randomized 1:1:1 to receive a total of 5 subcutaneous injections of placebo, increasing doses of LPP, or increasing doses of LPP/DnaK. The randomization list was prepared in blocks of three treatments with a 1:1:1 blocking scheme. Patients were injected every 7 days (weeks 0-4) approximately 3 months prior the grass pollen season (Figure S1). Treatment schedule was initially designed to start with an injection of 50 μ g of LPP. The first patient receiving this dosage developed an immediate local reaction of more than 5 cm within 5 min of injection, which was followed by urticaria on the face, irritation of the eye and nose, and coughing. The patient's blood pressure remained normal, and no pulmonary wheezing occurred. The patient received desloratadine, and the event resolved within 2 h of treatment. This event was described as systemic allergic reaction grade 2 according WAO classification and reported as SAE by the Investigator. The study protocol was amended to reduce the doses of LPP and LPP/DnaK administered to all patients as follows: 5 μ g was administered at week 0, 10 μ g at week 1, 20 μ g at week 2, and 50 μ g at weeks 3 and 4, resulting in a cumulative dose of 135 μ g of each product. All patients completed the study, although 3 in the LPP group and 3 in the LPP/DnaK group were excluded from the per-protocol analysis set because they received less than 78% (105 μ g) of the planned treatment dose. Treatments were provided in identical numbered glass vials so that both the patient and investigator were blinded to the treatment administered. Patients were observed closely for 2 h after each injection for immediate allergic or hypersensitivity reactions. Patients were followed up through the grass pollen season.

Endpoints

The primary endpoint was the assessment of the safety and clinical tolerability of LPP when administered by subcutaneous injection alone or with DnaK at 1:1 weight ratio.

The secondary endpoints were the reduction of symptom scores and rescue medication intake during the grass pollen season; and improvement of quality of life as well as the induction of grass pollen-specific IgE, IgG (sIgG), IgG4 (sIgG4), and IgA (sIgA) levels and IgG4-associated blocking antibody levels.

99 Clinical tolerability and safety assessments

100 Solicited local adverse events AEs (redness, swelling, induration, and pain at the injection
101 site) following each injection were recorded on diary cards by all participants during 7 days.
102 Patients recorded the diameter of redness, swelling, and induration as well as whether they
103 experienced pain. Severity of allergic reactions was classified according to the World Allergy
104 Organization recommendations.³ AEs of special interest were defined as headache, feeling of
105 fever, muscle pain, fatigue, digestive discomfort such as nausea/vomiting, diarrhea, anorexia,
106 difficulty of breathing, urticaria, and swelling of the face. The causal relationship of the
107 unsolicited AE to the investigational product was evaluated by the investigator and the
108 sponsor according to ICH Good Clinical Practice (GCP) guidelines. Clinical chemistry
109 parameters (blood and urine) were analyzed at screening, after treatment, and at the end of
110 the study.

112 Clinical effect assessments

113 Patients recorded nose symptoms (runny nose, blockage, sneezing, and itching), eye
114 symptoms (itching and watering), and lung symptoms (cough, wheeze, chest tightness, and
115 exercise-induced dyspnea) on diary cards throughout the grass pollen season, using a 4-point
116 scale as described previously.⁴ The average rhinoconjunctivitis total symptom score (RTSS)
117 was calculated over the grass pollen season as the average of the daily sum of the 6 nose and
118 eye symptom scores totals.⁴ The total symptom score (TSS) was calculated as the average of
119 the daily sum of the RTSS and 4 lung symptoms. The daily rescue medication score (RMS)
120 was the highest score for medications taken during the day to relieve rhinoconjunctivitis
121 symptoms, wherein 3 was assigned for oral corticosteroids, 2 for nasal corticosteroids and no
122 oral corticosteroids, 1 for antihistamines or cromoglycate and no oral nor nasal
123 corticosteroids, and 0 for no medications taken.⁴ Average Combined scores (ACS) was
124 calculated as according Clarck and Shall using the formula $(RTSS/6 + RMS)/2$.⁴ A well-day
125 was defined as a day with $TSS < 2$ without intake of rescue medication. The pollen season
126 was defined as the period between the first of 3 consecutive days during the follow-up period
127 with a pollen count ≥ 10 grains/m³ air and the last day with a pollen count ≥ 10 grains/m³
128 preceding 3 consecutive days with a pollen count < 10 grains/m³.

130 Allergenicity and Immunogenicity assessments**131 Western blotting**

132 Pollen proteins (2.3 µg) and pollen peptides (2.3 µg) were analyzed by SDS-PAGE and transferred

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on a nitrocellulose membrane. The nitrocellulose membrane pre-coated with casein hydrolysate was incubated for 18 hrs at 4 °C with pool of sera containing IgE from allergic patients (PlasmaLab International, Everett, USA). The nitrocellulose membrane was washed and incubated with the secondary antibody anti-human IgE coupled to HRP (1:10.000; Merck, BE) for 1 h at Room temperature, followed by the staining in presence of 3,3'-diaminobenzidine (HRP substrate).

Competitive ELISA

The presence and activity of allergens has been tested by a competitive ELISA developed and optimized using a pool of commercialized IgE rich sera from grass pollen allergic patients (PlasmaLab International, Everett, USA). Briefly, pooled of sera of grass pollen allergic patients was preincubated with pollen purified proteins (0-10 µg/ml) and the pollen peptides (0-1000 µg/ml) at different concentrations. The mixture containing free antibodies and antigen-antibody complexes was transferred to a pollen protein-coated ELISA plate (0.7 µg/ml). The preformed antibody-antigen complexes were washed off before adding anti IgE- HRP antibody (1:200; Merck, BE). Only free pollen-specific IgE would bind to coated grass pollen protein and were detected by the ELISA. Signal amplitude was inversely proportional to the protein or peptide quantity present in the pre-incubation step.

Basophil activation assays

The Basotest assay (ORPEGEN Pharma, Becton Dickinson, Erembodegem, Belgium) was performed according to the manufacture' instructions . Briefly, heparinized whole blood was incubated for 10 minutes at 37°C with stimulation buffer and then in the presence of various concentrations of test products or test controls for 20 minutes at 37°C. The test was performed in triplicates with increasing doses of pollen proteins, pollen peptides, DnaK and Peptides/DnaK (1, 10, 100, 1000 ng/ml). fMPL was used as positive control. The degranulation process was stopped by incubating the blood samples for 5 minutes on ice. The cells were labelled with antihuman IgE-Phycoerythrin. Activated basophils were detected using antihuman CD63-FITC (Fluorescein). After staining of the basophils, the erythrocytes were lysed and washed by centrifugation. The percentage of activated basophils were determined by flow cytometry.

PBMC proliferation assays and cytokine measurements

The PBMCs were seeded in 96-well plates (500.000 cells/well) and incubated at 37°C (5% CO₂), in the presence of increasing doses (0, 3, 10, 30 µg/ml) of pollen proteins, pollen peptides, DnaK or peptides/DnaK. Phytohemagglutinin (4µg/ml) was used as positive control. After 5 days of culture, 100 µl of cell supernatants were harvested for cytokine measurement. After the removal of the supernatant, 1 µCi [3H]-thymidine-containing medium (100 µl) was added to the cell cultures for 16 -

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18 hours. On day 6, incorporation of [3H]-thymidine incorporation was measured with a beta-counter using the principle of liquid scintillation and proliferation rates were expressed as ratios between stimulated and unstimulated cultures, denominated as the stimulation index. IFN- γ (TH1) and IL-5 (TH2) cytokines in cell supernatants were analyzed by ELISA (BD OptEIA Human IFN- γ ELISA Kit II (Becton Dickinson, Erembodegem, Belgium and Biosource EASIA IL-5 kit (Thermo Fisher, Merelbeke, Belgium) according to the instructions of the manufacturer).

174 Immunologic assessment of LPP and LPP/DnaK

175 Blood samples were collected at screening (time 0), after treatment (week 5), end of the grass pollen season (week 14) and after grass pollen season (week 25). sIgE, sIgG, IgG4, and sIgA levels were measured by ImmunoCAP[®] (Pharmacia, Phadia AB, Uppsala, Sweden). IgG4-associated blocking antibody levels were measured by IgE allergen-facilitated binding assay.⁵⁻⁷

180 IgE allergen-facilitated binding assays

181 As described above, the Facilitated allergen binding assay which measures allergen-IgE binding to B cells has been previously described.⁵⁻⁷ Briefly, Stock indicator serum (20 μ l) containing high concentration of grass pollen- (*P. pratense*) specific IgE (>100 IU/ml), was pre-incubated with 0.3 μ g/ml allergen (5 μ l) at 37 °C for 1 h to form allergen-IgE complexes. To test for inhibition of facilitated allergen binding we mixed an equal volume of indicator serum (20 μ l) with test (post-immunotherapy) serum (20 μ l) or an equal volume of RPMI as a control. EBV-transformed B cells (5 μ l) were then added to the allergen-IgE mixture and incubated for 1 h at 4 °C. Cells were washed and bound complexes were detected using a polyclonal human anti-IgE PE-labelled antibody (using 20 μ l of a 1/30 dilution for 30 min) by flow cytometry (FACS Canti II; BD Biosciences). B cells were gated using forward scatter/side scatter parameters and a positive marker was set using cells incubated with indicator serum only. Five thousand cells were analyzed and all samples were measured in triplicate. When analyzing the results for longitudinal studies of grass pollen immunotherapy it is possible to account for IgE present in the immunotherapy 'test' sample which may increase the binding of allergen-IgE complexes to B cells above that of the indicator serum alone. Therefore, data from the clinical trial was expressed as the percentage relative binding to allergen-IgE complexes where binding with indicator serum alone was normalized to 100%. The following formula was used to calculate the percentage relative B cell binding: % relative allergen - IgE complex binding to B cells - (%IgE - FAB using indicator and immunotherapy serum)/(% IgE- FAB using indicator serum only)* 100.

201 Statistical analysis

The safety set (defined as all patients that received at least one treatment dose) was equivalent to the full analysis set (FAS) and was used to assess safety and tolerability as well as immunogenicity. No formal statistical sample size calculations were made because the primary objective of the study was the analysis of safety and tolerability. Clinical effect was assessed in both full analysis set and per-protocol analysis set, defined as all patients without any protocol deviation interfering with the evaluation of either the immunogenicity and/or the efficacy endpoints. Immunogenicity variables were compared between groups by analysis of variance with treatment as a factor and, if available, baseline assessment as a covariate. For immunogenicity variables, a Bonferroni test for multiple comparisons was performed. For non-normally distributed data, an appropriate transformation was performed (*e.g.* log₁₀). A two-sided p-value below 0.05 was considered to indicate statistical significance. Statistical analysis was performed using SAS version 9.1 (SAS Institute, Cary, NC) for all parameters except for comparison between group of PBMCs proliferation, cytokine responses and IgE-facilitated allergen binding assay analyses (Graph PAD Prism version 7.01).

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ONLINE REPOSITORY:

***Lolium perenne* Peptides for the Treatment of Grass Pollen Allergy: A Randomized
Double Blind Placebo-Controlled Clinical Trial**

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Table E1. Demographic characteristics of blood sample donors for *ex vivo* study

Variable	Non allergic N=12	Allergic N=12
Age (y), mean $\pm$ SD	29.6 $\pm$ 5.8	28.9 $\pm$ 8.2
Gender (Male:Female)	9:3	5:7
Skin Prick Test to grass pollen (mm), median and IQR	0.00 (0.0 - 0.0)	5.50 (4.3 - 6.8)

Abbreviations: IQR, interquartile range.

Table E2A. Allergenicity of LP Proteins, LPP, DnaK and LPP/DnaK in allergic subjects

Concentration (ng/μL)	mean ± SEM			
	LP Proteins	LPP	DnaK	LPP/DnaK
0	5.222 ± 2.257	5.222 ± 2.257	5.222 ± 2.257	5.222 ± 2.257
1	5.023 ± 1.346	3.177 ± 0.7998	2.079 ± 0.4251	1.858 ± 0.4536
10	17.84 ± 7.584	2.358 ± 0.5004	1.683 ± 0.2736	1.588 ± 0.3409
100	47.28 ± 8.597**	7.284 ± 2.753	2.076 ± 0.429	2.733 ± 0.8959
1000	52.75 ± 7.557**	20.3 ± 6.755	2.569 ± 0.7748	12.95 ± 6.122

*signifies comparison with 0; ** $P < 0.01$

Table E2B. Allergenicity of LPP, DnaK and LPP/DnaK compared to LP Proteins in allergic subjects

Concentration (ng/μL)	mean ± SEM			
	LP Proteins	LPP	DnaK	LPP/DnaK
0	5.222 ± 2.257	5.222 ± 2.257	5.222 ± 2.257	5.222 ± 2.257
1	5.023 ± 1.346	3.177 ± 0.7998	2.079 ± 0.4251	1.858 ± 0.4536 ^o
10	17.84 ± 7.584	2.358 ± 0.5004 ^o	1.683 ± 0.2736 ^{oo}	1.588 ± 0.3409 ^{ooo}
100	47.28 ± 8.597	7.284 ± 2.753 ^{ooo}	2.076 ± 0.429 ^{ooo}	2.733 ± 0.8959 ^{oooo}
1000	52.75 ± 7.557	20.3 ± 6.755 ^{ooo}	2.569 ± 0.7748 ^{oooo}	12.95 ± 6.122 ^{oooo}

^osignifies comparison with LP Proteins; ^o $P < 0.05$, ^{oo} $P < 0.01$, ^{ooo} $P < 0.001$

91 **Table E3A. Immunogenicity of LP Proteins, LPP, DnaK and LPP/DnaK in allergics and non-allergics**

Concentration (ng/mL)	mean $\pm$ SEM							
	Allergics				Non-Allergics			
	LP Proteins	LPP	DnaK	LPP/DnaK	LP Proteins	LPP	DnaK	LPP/DnaK
Proliferation (SI)								
0	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
3	5.482 $\pm$ 1.557***	1.509 $\pm$ 0.2855	1.109 $\pm$ 0.09091	2.1 $\pm$ 0.4016**	3.075 $\pm$ 0.6256**	1.15 $\pm$ 0.1525	1.042 $\pm$ 0.1209	1.475 $\pm$ 0.1907*
10	9.242 $\pm$ 1.355***	2.083 $\pm$ 0.5337**	1.208 $\pm$ 0.1111	2.858 $\pm$ 0.5159***	8.4 $\pm$ 1.367***	1.15 $\pm$ 0.1555	1.225 $\pm$ 0.1737	1.525 $\pm$ 0.3451
30	12.25 $\pm$ 1.918***	2.683 $\pm$ 0.7799**	1.6 $\pm$ 0.1927*	4.592 $\pm$ 1.016***	8.75 $\pm$ 1.013***	1.158 $\pm$ 0.1485	1.625 $\pm$ 0.2837	2.858 $\pm$ 0.6103***
IL-5 (pg/mL)								
0	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307
3	40.55 $\pm$ 14.61**	16.91 $\pm$ 3.329**	9.957 $\pm$ 2.114*	15.85 $\pm$ 5.097**	9.548 $\pm$ 1.007*	11.43 $\pm$ 1.765***	15.92 $\pm$ 2.139***	8.765 $\pm$ 1.081*
10	22.64 $\pm$ 6.798*	21.13 $\pm$ 7.331	5.686 $\pm$ 0.9235	30.28 $\pm$ 12.6	9.1 $\pm$ 2.285	6.267 $\pm$ 0.9554	6.074 $\pm$ 0.8506	6.092 $\pm$ 0.7137
30	17.36 $\pm$ 5.903	27.02 $\pm$ 12.32	5.328 $\pm$ 1.079	25.28 $\pm$ 12	7.745 $\pm$ 1.19	4.337 $\pm$ 0.3504	7.029 $\pm$ 0.7648	6.708 $\pm$ 0.7701
IFN-γ (pg/mL)								
0	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368
3	103.9 $\pm$ 32.94***	10.29 $\pm$ 3.011***	10.87 $\pm$ 4.652*	15.4 $\pm$ 5.667**	90.19 $\pm$ 30.55***	5.628 $\pm$ 1.823	10.08 $\pm$ 6.278	12.65 $\pm$ 6.428*
10	238.3 $\pm$ 24.96***	8.135 $\pm$ 3.578	6.385 $\pm$ 1.701*	18.62 $\pm$ 8.648**	244.4 $\pm$ 29.59***	4.648 $\pm$ 1.59	15.83 $\pm$ 12.64	13.27 $\pm$ 5.148
30	293.5 $\pm$ 6.468***	21.77 $\pm$ 12.28***	31.98 $\pm$ 13.04**	41.74 $\pm$ 21.37***	298.4 $\pm$ 1.594***	14.77 $\pm$ 9.746	22.9 $\pm$ 10.53**	36.01 $\pm$ 24.28**

92 *signifies comparison with 0 per group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

93 **Table E3B. Immunogenicity of LP Proteins, LPP, DnaK and LPP/DnaK in non-allergics compared to allergics**

Concentration (ng/mL)	mean $\pm$ SEM							
	Allergics				Non Allergics			
	LP Proteins	LPP	DnaK	LPP/DnaK	LP Proteins	LPP	DnaK	LPP/DnaK
Proliferation (SI)								
0	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00	1.00 $\pm$ 0.00
3	5.482 $\pm$ 1.557	1.509 $\pm$ 0.2855	1.109 $\pm$ 0.09091	2.1 $\pm$ 0.4016	3.075 $\pm$ 0.6256	1.15 $\pm$ 0.1525	1.042 $\pm$ 0.1209	1.475 $\pm$ 0.1907
10	9.242 $\pm$ 1.355	2.083 $\pm$ 0.5337	1.208 $\pm$ 0.1111	2.858 $\pm$ 0.5159	8.4 $\pm$ 1.367	1.15 $\pm$ 0.1555 ^o	1.225 $\pm$ 0.1737	1.525 $\pm$ 0.3451 ^o
30	12.25 $\pm$ 1.918	2.683 $\pm$ 0.7799	1.6 $\pm$ 0.1927	4.592 $\pm$ 1.016	8.75 $\pm$ 1.013	1.158 $\pm$ 0.1485 ^{oo}	1.625 $\pm$ 0.2837	2.858 $\pm$ 0.6103
IL-5 (pg/mL)								
0	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307	4.5 $\pm$ 1.307
3	40.55 $\pm$ 14.61	16.91 $\pm$ 3.329	9.957 $\pm$ 2.114	15.85 $\pm$ 5.097	9.548 $\pm$ 1.007	11.43 $\pm$ 1.765	15.92 $\pm$ 2.139	8.765 $\pm$ 1.081
10	22.64 $\pm$ 6.798	21.13 $\pm$ 7.331	5.686 $\pm$ 0.9235	30.28 $\pm$ 12.6	9.1 $\pm$ 2.285	6.267 $\pm$ 0.9554	6.074 $\pm$ 0.8506	6.092 $\pm$ 0.7137
30	17.36 $\pm$ 5.903	27.02 $\pm$ 12.32	5.328 $\pm$ 1.079	25.28 $\pm$ 12	7.745 $\pm$ 1.19	4.337 $\pm$ 0.3504	7.029 $\pm$ 0.7648 ^{ooo}	6.708 $\pm$ 0.7701
IFN-g (pg/mL)								
0	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368	3.167 $\pm$ 0.6368
3	103.9 $\pm$ 32.94	10.29 $\pm$ 3.011	10.87 $\pm$ 4.652	15.4 $\pm$ 5.667	90.19 $\pm$ 30.55	5.628 $\pm$ 1.823 ^o	10.08 $\pm$ 6.278	12.65 $\pm$ 6.428
10	238.3 $\pm$ 24.96	8.135 $\pm$ 3.578	6.385 $\pm$ 1.701	18.62 $\pm$ 8.648	244.4 $\pm$ 29.59	4.648 $\pm$ 1.59	15.83 $\pm$ 12.64	13.27 $\pm$ 5.148
30	293.5 $\pm$ 6.468	21.77 $\pm$ 12.28	31.98 $\pm$ 13.04	41.74 $\pm$ 21.37	298.4 $\pm$ 1.594	14.77 $\pm$ 9.746	22.9 $\pm$ 10.53	36.01 $\pm$ 24.28

94 ^osignifies comparison with Allergics; ^o $P < 0.05$, ^{oo} $P < 0.01$, ^{ooo} $P < 0.001$

Table E4. Demographic characteristics of Phase I study

Characteristic	Placebo N=9	LPP N=9	LPP/DnaK N=9
Age (y), mean $\pm$ SD	25.6 $\pm$ 6.8	32.2 $\pm$ 11.4	36.1 $\pm$ 7.7*
Gender, n (%)			
Male	3 (33.3)	4 (44.4)	2 (22.2)
Female	6 (66.7)	5 (55.6)	7 (77.8)
Ethnicity, n (%)			
Caucasian/White	9 (100.0)	9 (100.0)	8 (88.9)
Black	0 (0.0)	0 (0.0)	1 (11.1)
Height (cm), mean $\pm$ SD	172.7 $\pm$ 7.3	176.0 $\pm$ 5.9	172.2 $\pm$ 10.1
Weight (kg), mean $\pm$ SD	63.56 $\pm$ 8.05	71.28 $\pm$ 11.51	72.06 $\pm$ 16.10
Grass pollen specific IgE (kU/L), median and IQR)	15.2 (8.4 – 47.3)	16.1 (4.7 – 43.7)	12.0 (3.9 – 22.7)
Skin Prick Test to grass pollen (mm), median and IQR	10.0 (9.5 – 12.5)	10.5 (9.0 – 12.5)	10.0 (8.0 – 12.5)
Other immune system disorders			
Allergic respiratory symptoms	1 (11.1%)	0	0
Allergy to animal	1 (11.1%)	0	3 (33.3%)
Allergy to plants	1 (11.1%)	0	0
Dermatitis allergic	0	1 (11.1%)	0
Dermatitis contact	0	0	1 (11.1%)
Drug hypersensitivity	1 (11.1%)	0	0
Food allergy	1 (11.1%)	0	2 (22.2%)
House dust mite allergy	1 (11.1%)	0	1 (11.1%)
Oral allergy syndrome	1 (11.1%)	0	0
Other disease			
Depression	0	0	2 (22.2%)
Asthma	2 (22.2%)	0	1 (11.1%)
Exercise-induced asthma	1 (11.1%)	0	0
Dyspnoea	1 (11.1%)	0	0
Eczema	2 (22.2%)	1 (11.1%)	3 (33.3%)
Hypertension	0	1 (11.1%)	0

Values are for the full analysis set. Abbreviations: SD, standard deviation; IQR, interquartile range. * $P = 0.005$. Mann Whitney U test.

Table E5. Adverse Events related to Treatment

Event	Placebo N=9	LPP N=9	LPP/DnaK N=9
Nber of unsolicited AEs, n AE	2	16	12
Nber of patient reporting unsolicited AE, n patient (%)	1 (11.1)	7 (77.8)	5 (55.6)
Leukopenia	0 (0.0)	1 (11.1)	0 (0.0)
Palpitations	1 (11.1)	0 (0.0)	0 (0.0)
Injection site pruritus	0 (0.0)	5 (55.6)	3 (33.3)
Allergic cough	0 (0.0)	0 (0.0)	1 (11.1)
Anaphylactic reaction	0 (0.0)	1 (11.1)	0 (0.0)
Seasonal allergy	0 (0.0)	1 (11.1)	0 (0.0)
Neutrophil count decreased	0 (0.0)	1 (11.1)	0 (0.0)
Hypoaesthesia	1 (11.1)	0 (0.0)	1 (11.1)
Cough	0 (0.0)	1 (11.1)	0 (0.0)
Pruritus	0 (0.0)	2 (22.2)	1 (11.1)
AEs of special interest, n patient (%)	3 (33.3)	4 (44.4)	7 (77.8)
Headache	1 (11.1)	1 (11.1)	4 (44.4)
Muscle pain	0 (0.0)	0 (0.0)	2 (22.2)
Fatigue	0 (0.0)	3 (33.3)	2 (22.2)
Diarrhea	1 (11.1)	0 (0.0)	0 (0.0)
Difficulty breathing	1 (11.1)	0 (0.0)	0 (0.0)
Urticaria	0 (0.0)	1 (11.1)	1 (11.1)
AEs leading to study discontinuation, n patient (%)	0 (0.0)	0 (0.0)	0 (0.0)
Deaths, n (%)	0 (0.0)	0 (0.0)	0 (0.0)

Values are for the full analysis set (identical to Safety Set)

120 Table E6. Safety parameters

	Placebo (mean $\pm$ SD) N=9		LPP (mean $\pm$ SD) N=9		LPP / DnaK (mean $\pm$ SD) N=9	
	Visit 1	Visit 6	Visit 1	Visit 6	Visit 1	Visit 6
Display of Haematology data						
Haematology - Values over Time - Safety Population						
Hemoglobin (g/dL)	137.7 $\pm$ 15.3	135.8 $\pm$ 15.7	145.8 $\pm$ 13.9	143.2 $\pm$ 15.1	138.2 $\pm$ 14.1	137.8 $\pm$ 12.5
Hematocrit (Fraction of 1)	0.4086 $\pm$ 0.0408	0.4002 $\pm$ 0.0413	0.4316 $\pm$ 0.0307	0.4266 $\pm$ 0.0347	0.4094 $\pm$ 0.0369	0.4053 $\pm$ 0.0331
Erythrocytes (10**12/L)	4.591 $\pm$ 0.478	4.513 $\pm$ 0.380	4.980 $\pm$ 0.455	4.889 $\pm$ 0.435	4.731 $\pm$ 0.459	4.673 $\pm$ 0.375
Leukocytes (10**9/L)	5.72 $\pm$ 0.90	6.11 $\pm$ 1.14	5.86 $\pm$ 2.04	5.87 $\pm$ 1.89	6.13 $\pm$ 1.41	6.24 $\pm$ 1.23
Basophils (%)	0.444 $\pm$ 0.194	0.500 $\pm$ 0.308	0.656 $\pm$ 0.354	0.700 $\pm$ 0.604	0.733 $\pm$ 0.527	0.589 $\pm$ 0.232
Eosinophils (%)	3.111 $\pm$ 2.103	4.800 $\pm$ 3.744*	2.267 $\pm$ 1.531	3.922 $\pm$ 3.124	3.878 $\pm$ 3.068	3.956 $\pm$ 2.470
Monocytes (%)	7.256 $\pm$ 1.700	7.600 $\pm$ 1.904	7.589 $\pm$ 2.322	6.378 $\pm$ 1.690	6.111 $\pm$ 1.043	6.678 $\pm$ 1.516
Lymphocytes (%)	32.133 $\pm$ 7.498	28.911 $\pm$ 5.216	27.967 $\pm$ 5.554	29.556 $\pm$ 7.560	32.033 $\pm$ 9.244	32.978 $\pm$ 8.266
Neutrophils (%)	57.056 $\pm$ 8.883	58.189 $\pm$ 7.241	61.522 $\pm$ 5.746	59.444 $\pm$ 10.488	57.356 $\pm$ 10.565	55.800 $\pm$ 7.770
Platelets (10**9/L)	253.1 $\pm$ 66.2	258.1 $\pm$ 54.2	236.0 $\pm$ 74.9	248.8 $\pm$ 74.2	271.1 $\pm$ 37.3	281.6 $\pm$ 59.4
Display of Biochemistry data						
Biochemistry - Values over Time - Safety Population						
Glucose (mmol/L)	5.186 $\pm$ 0.892	4.724 $\pm$ 0.570	5.081 $\pm$ 0.583	5.100 $\pm$ 0.559	5.828 $\pm$ 1.344	5.063 $\pm$ 0.710
Creatinine (umol/L)	72.783 $\pm$ 10.772	72.193 $\pm$ 11.424	74.747 $\pm$ 15.061	74.551 $\pm$ 13.738	71.899 $\pm$ 11.138	68.854 $\pm$ 8.227
Urea (mmol/L)	11.11 $\pm$ 3.23	9.68 $\pm$ 2.80	10.43 $\pm$ 1.96	10.79 $\pm$ 2.99	10.75 $\pm$ 2.44	10.12 $\pm$ 1.10
Cholesterol (mmol/L)	4.849 $\pm$ 0.710	4.616 $\pm$ 0.772	4.607 $\pm$ 0.659	4.504 $\pm$ 0.556	4.748 $\pm$ 0.654	4.812 $\pm$ 0.820
Triglycerides (mmol/L)	1.141 $\pm$ 0.372	1.115 $\pm$ 0.346	1.225 $\pm$ 0.929	1.084 $\pm$ 0.425	1.224 $\pm$ 0.518	1.130 $\pm$ 0.723
Sodium (mmol/L)	141.90 $\pm$ 1.29	140.24 $\pm$ 1.11	142.52 $\pm$ 1.94	141.61 $\pm$ 1.78	142.14 $\pm$ 1.78	140.32 $\pm$ 1.75
Potassium (mmol/L)	4.050 $\pm$ 0.340	3.970 $\pm$ 0.351	3.890 $\pm$ 0.245	4.177 $\pm$ 0.256	3.907 $\pm$ 0.156	4.223 $\pm$ 0.268
Chloride (mmol/L)	105.41 $\pm$ 2.09	104.61 $\pm$ 2.04	105.47 $\pm$ 1.22	105.27 $\pm$ 1.38	105.50 $\pm$ 1.50	105.29 $\pm$ 2.53
Calcium (mmol/L)	2.320 $\pm$ 0.061	2.280 $\pm$ 0.072	2.349 $\pm$ 0.057	2.309 $\pm$ 0.079	2.324 $\pm$ 0.035	2.290 $\pm$ 0.054
Total protein (g/L)	74.9 $\pm$ 3.1	71.8 $\pm$ 3.6	75.7 $\pm$ 2.0	73.1 $\pm$ 3.1	75.0 $\pm$ 3.4	73.9 $\pm$ 2.8
Albumin (g/L)	49.46 $\pm$ 3.14	47.27 $\pm$ 3.40	48.30 $\pm$ 2.44	48.22 $\pm$ 3.99	47.89 $\pm$ 2.48	47.72 $\pm$ 2.31
Aspartate Aminotransferase (IU/L)	21.6 $\pm$ 5.3	21.0 $\pm$ 6.1	20.2 $\pm$ 4.8	19.9 $\pm$ 4.1	17.9 $\pm$ 2.4	19.9 $\pm$ 2.8
Alanine Aminotransferase (IU/L)	19.1 $\pm$ 7.9	17.4 $\pm$ 7.8	18.6 $\pm$ 9.8	16.2 $\pm$ 7.3	15.9 $\pm$ 3.8	17.0 $\pm$ 6.2
Gamma Glutamyl Transferase (IU/L)	17.0 $\pm$ 6.2	14.9 $\pm$ 6.9	17.3 $\pm$ 4.6	16.0 $\pm$ 4.5	15.3 $\pm$ 4.0	15.7 $\pm$ 4.9

Total bilirubin (μmol/L)	7.429 ± 4.205	6.726 ± 3.440	12.027 ± 8.920	11.742 ± 10.715	10.469 ± 4.979	12.065 ± 8.548
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Display of Immunological parameter data

Immunological parameters - Total IgE and Total IgG - Values over Time - Safety Population

Total IgE (kU/L)	159.6 ± 128.9	162.0 ± 140.1	182.6 ± 160.2	192.4 ± 166.8	197.8 ± 349.3	170.4 ± 242.1
Total IgG (g/L)	11.888 ± 2.821	11.083 ± 2.747	11.411 ± 1.885	11.076 ± 2.428	12.153 ± 2.711	11.494 ± 2.239

Display of Inflammatory parameter data

Inflammatory parameters - Values over Time - Safety Population

Erythrocyte sedimentation rate (mm/hour)	5.1 ± 4.1	5.2 ± 3.4	10.6 ± 10.3	7.7 ± 6.6	7.7 ± 4.1	8.6 ± 4.9
C-reactive protein (mg/L)	2.42 ± 3.21	1.93 ± 1.88	2.88 ± 3.24	2.11 ± 3.05	1.90 ± 2.20	2.04 ± 1.58

*P < 0.05 compared to Visit 1 (paired T test)

Table E7. Clinical assessments over the grass pollen season

Measure	Placebo N=9	LPP N=6	LPP/DnaK N=6
Average combined score (ACS)			
Mean $\pm$ SD	0.479 $\pm$ 0.298	0.312 $\pm$ 0.284	0.747 $\pm$ 0.511
Relative change vs. placebo		-34.8%	55.8%
P-value		0.316	0.376
Rhinoconjunctivitis score (RTSS)			
Mean $\pm$ SD	3.49 $\pm$ 1.95	2.46 $\pm$ 2.19	5.45 $\pm$ 4.13
Relative change vs. placebo		-29.7%	56.1%
P-value		0.331	0.458
Rescue medication score (RMS)			
Mean $\pm$ SD	0.377 $\pm$ 0.330	0.215 $\pm$ 0.213	0.585 $\pm$ 0.414
Relative change vs. placebo		-42.8%	55.4%
P-value		0.464	0.365
Total symptom score (TSS)			
Mean $\pm$ SD	3.89 $\pm$ 2.22	2.63 $\pm$ 2.22	6.45 $\pm$ 5.98
Relative change vs. placebo		-32.4%	65.6%
P-value		0.234	0.659
Well days			
Mean $\pm$ SD	14.67 $\pm$ 9.79	20.83 $\pm$ 12.67	8.33 $\pm$ 11.36
Relative change vs. placebo		42.1%	-43.2%
P-value		0.414	0.220

Values are for the per-protocol analysis set. Abbreviation: SD, standard deviation.

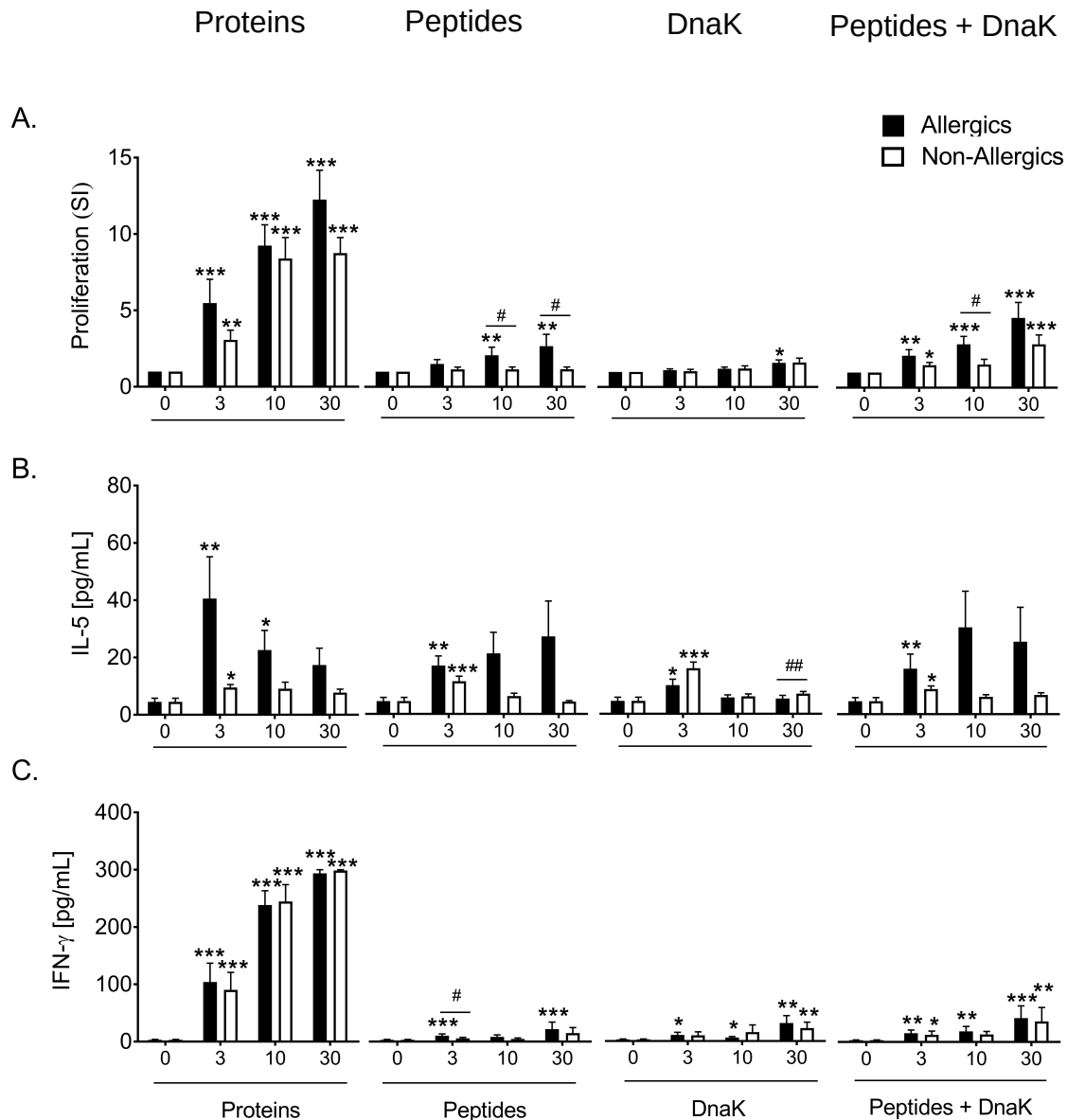


Figure E1. Allergen - driven PBMCs proliferation and cytokine responses

PBMCs from non-allergic (n=12) and allergic subjects (n=12) were stimulated with increasing concentrations (3, 10 and 30 $\mu\text{g/ml}$) of LP proteins, LP peptides, DnaK or the mixture LP peptides + DnaK. **A)** T cell proliferation was quantified by $[^3\text{H}]$ -thymidine incorporation and is presented as Stimulation Index compared to non stimulated culture. **B)** IL-5 and **C)** IFN- γ released in the culture medium were quantified by ELISA. Results are presented as means $\pm$ SEM. Mean basal production of IL-5 (negative control) was 4.51 pg/ml in allergic and 8.52 pg/ml in non-allergic subjects. Mean basal production of IFN- γ was 3.17 pg/ml in allergic and 4.25 pg/ml in non-allergic subjects. * <.05, ** <.01 and *** <.001 as compared to unstimulated cells. # <.05 and # <.01 as indicated (Allergics vs non allergics).

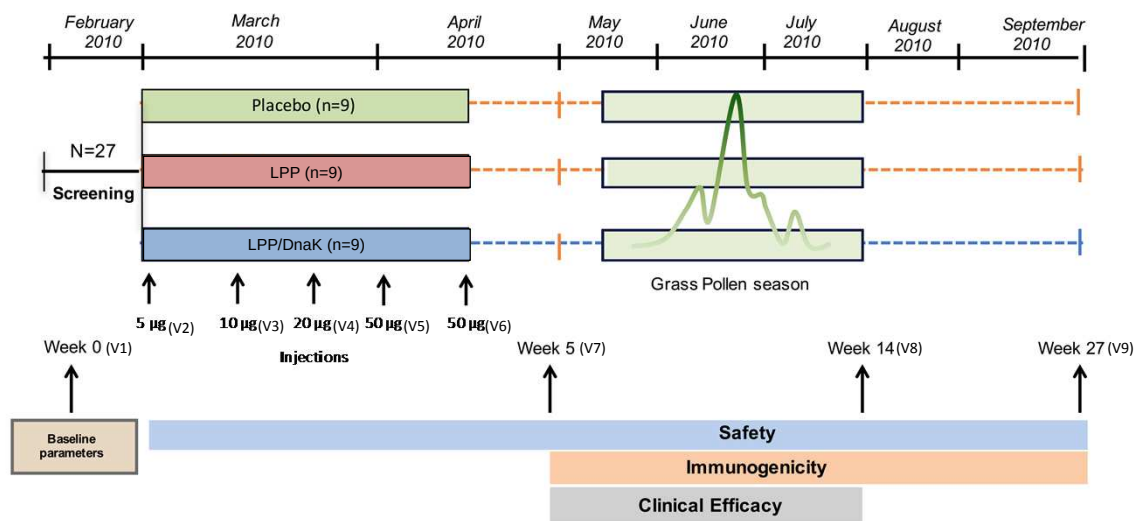


Figure E2. Study design.

Safety was measured through out the study period. Immunogenicity was measured at baseline, week 5, 14 and 27. Clinical effect was assessed during the grass pollen season.

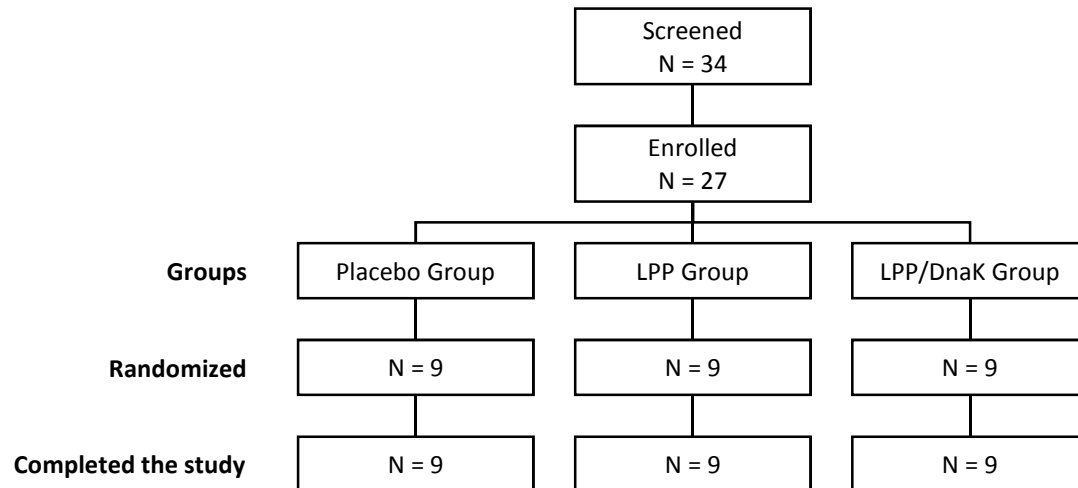


Figure E3. numbers at enrolment, randomization, treatment, and for analysis of the primary endpoint.

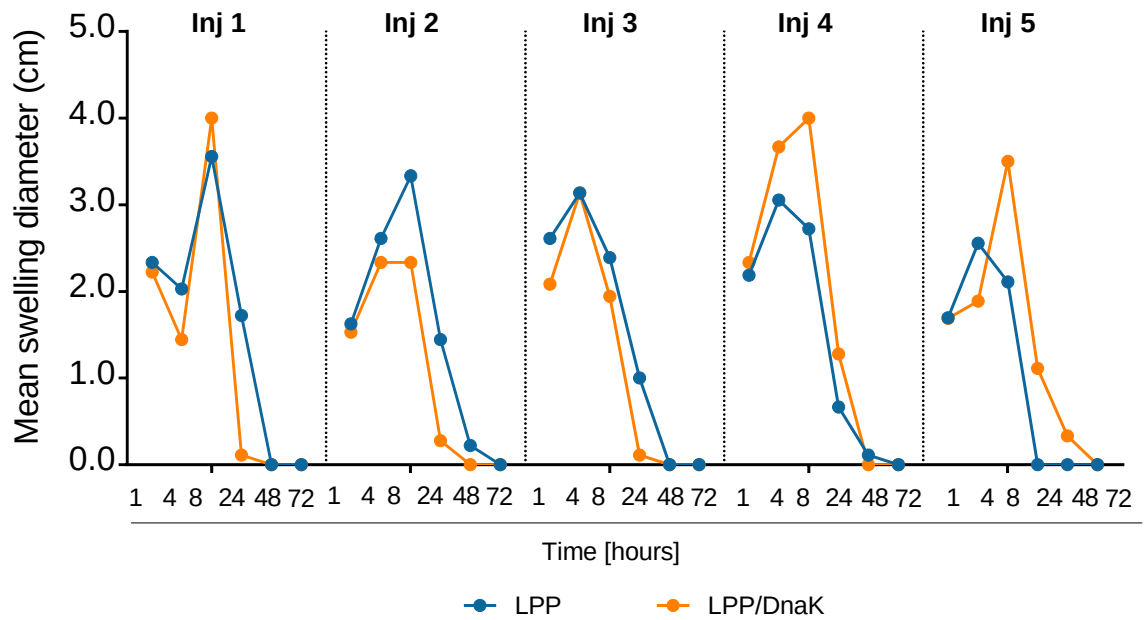


Figure E4: Mean wheal swelling in LPP and LPP/DnaK treated groups over time. Mean swelling diameter was measured at 1hr, 4hrs, 8-12hrs, 24hrs, 48hrs and 72hrs after LPP peptides (n=9), LPP/DnaK (n=9) and placebo (n=9) injections. Data is as mean.