

A randomised placebo-controlled trial investigating efficacy and mechanisms of low-dose intradermal allergen immunotherapy in treatment of seasonal allergic rhinitis

*Anna Slovic, Abdel Douiri, Rachel Muir, Andrea Guerra,
Konstantinos Tsioulos, Evie Haye, Emily PS Lam, Joanna Kelly,
Janet L Peacock, Sun Ying, Mohamed H Shamji, David J Cousins,
Stephen R Durham and Stephen J Till*



**National Institute for
Health Research**

A randomised placebo-controlled trial investigating efficacy and mechanisms of low-dose intradermal allergen immunotherapy in treatment of seasonal allergic rhinitis

Anna Slovic,^{1,2} Abdel Douiri,³ Rachel Muir,⁴
Andrea Guerra,¹ Konstantinos Tsioulos,¹ Evie Haye,¹
Emily PS Lam,¹ Joanna Kelly,⁵ Janet L Peacock,³
Sun Ying,¹ Mohamed H Shamji,⁶ David J Cousins,^{1,2,7}
Stephen R Durham⁶ and Stephen J Till^{1,2*}

¹Division of Asthma, Allergy and Lung Biology, King's College London, School of Medicine, Guy's Hospital, London, UK

²Medical Research Council (MRC)–Asthma UK Centre for Allergic Mechanisms of Asthma, London, UK

³Division of Health and Social Care Research, King's College London, London, UK

⁴Clinical Research Facility, NIHR Biomedical Research Centre, Guy's Hospital, London, UK

⁵King's Clinical Trials Unit, King's College London, Institute of Psychiatry, London, UK

⁶Allergy and Clinical Immunology, National Heart and Lung Institute, Faculty of Medicine, Imperial College, London, UK

⁷Department of Infection, Immunity and Inflammation, National Institute for Health Research (NIHR) Leicester Respiratory Biomedical Research Unit, Leicester Institute for Lung Health, University of Leicester, Leicester, UK

*Corresponding author

Declared competing interests of authors: Stephen J Till reports personal fees and grants from ALK Abelló, and personal fees from Thermofisher Scientific, outside the submitted work. Mohamed H Shamji reports grants from BioTech Tools and Regeneron USA, outside the submitted work. David J Cousins reports grants from GlaxoSmithKline, Asthma UK, and the Medical Research Council, outside the submitted work. Stephen R Durham reports grants from ALK Abelló, grants and personal fees from Merck, grants from Regeneron USA, personal fees from Biomay Austria and personal fees from Circassia UK, outside the submitted work; in addition, Stephen R Durham has a patent pending. Emily Lam is a Health Technology Assessment Primary Care, Community and Preventive Interventions panel member.

Published December 2016

DOI: 10.3310/eme03100

This report should be referenced as follows:

Slovick A, Douiri A, Muir R, Guerra A, Tsioulos K, Haye E, *et al.* A randomised placebo-controlled trial investigating efficacy and mechanisms of low-dose intradermal allergen immunotherapy in treatment of seasonal allergic rhinitis. *Efficacy Mech Eval* 2016;**3**(10).

Efficacy and Mechanism Evaluation

ISSN 2050-4365 (Print)

ISSN 2050-4373 (Online)

This journal is a member of and subscribes to the principles of the Committee on Publication Ethics (COPE) (www.publicationethics.org/).

Editorial contact: nihredit@southampton.ac.uk

The full EME archive is freely available to view online at www.journalslibrary.nihr.ac.uk/eme. Print-on-demand copies can be purchased from the report pages of the NIHR Journals Library website: www.journalslibrary.nihr.ac.uk

Criteria for inclusion in the *Efficacy and Mechanism Evaluation* journal

Reports are published in *Efficacy and Mechanism Evaluation* (EME) if (1) they have resulted from work for the EME programme, and (2) they are of a sufficiently high scientific quality as assessed by the reviewers and editors.

EME programme

The Efficacy and Mechanism Evaluation (EME) programme was set up in 2008 as part of the National Institute for Health Research (NIHR) and the Medical Research Council (MRC) coordinated strategy for clinical trials. The EME programme is broadly aimed at supporting 'science driven' studies with an expectation of substantial health gain and aims to support excellent clinical science with an ultimate view to improving health or patient care.

Its remit includes evaluations of new treatments, including therapeutics (small molecule and biologic), psychological interventions, public health, diagnostics and medical devices. Treatments or interventions intended to prevent disease are also included.

The EME programme supports laboratory based or similar studies that are embedded within the main study if relevant to the remit of the EME programme. Studies that use validated surrogate markers as indicators of health outcome are also considered.

For more information about the EME programme please visit the website: <http://www.nets.nihr.ac.uk/programmes/eme>

This report

The research reported in this issue of the journal was funded by the EME programme as project number 11/20/05. The contractual start date was in September 2012. The final report began editorial review in September 2015 and was accepted for publication in March 2016. The authors have been wholly responsible for all data collection, analysis and interpretation, and for writing up their work. The EME editors and production house have tried to ensure the accuracy of the authors' report and would like to thank the reviewers for their constructive comments on the final report document. However, they do not accept liability for damages or losses arising from material published in this report.

This report presents independent research. The views and opinions expressed by authors in this publication are those of the authors and do not necessarily reflect those of the NHS, the NIHR, the MRC, NETSCC, the EME programme or the Department of Health. If there are verbatim quotations included in this publication the views and opinions expressed by the interviewees are those of the interviewees and do not necessarily reflect those of the authors, those of the NHS, the NIHR, NETSCC, the EME programme or the Department of Health.

© Queen's Printer and Controller of HMSO 2016. This work was produced by Slovic *et al.* under the terms of a commissioning contract issued by the Secretary of State for Health. This issue may be freely reproduced for the purposes of private research and study and extracts (or indeed, the full report) may be included in professional journals provided that suitable acknowledgement is made and the reproduction is not associated with any form of advertising. Applications for commercial reproduction should be addressed to: NIHR Journals Library, National Institute for Health Research, Evaluation, Trials and Studies Coordinating Centre, Alpha House, University of Southampton Science Park, Southampton SO16 7NS, UK.

Published by the NIHR Journals Library (www.journalslibrary.nihr.ac.uk), produced by Prepress Projects Ltd, Perth, Scotland (www.prepress-projects.co.uk).

Efficacy and Mechanism Evaluation Editor-in-Chief

Professor David Crossman Bute Professor of Medicine and Dean and Head of Faculty of Medicine, University of St Andrews, and Honorary Consultant Cardiologist, NHS Fife Health Board, UK

NIHR Journals Library Editor-in-Chief

Professor Tom Walley Director, NIHR Evaluation, Trials and Studies and Director of the EME Programme, UK

NIHR Journals Library Editors

Professor Ken Stein Chair of HTA Editorial Board and Professor of Public Health, University of Exeter Medical School, UK

Professor Andree Le May Chair of NIHR Journals Library Editorial Group (EME, HS&DR, PGfAR, PHR journals)

Dr Martin Ashton-Key Consultant in Public Health Medicine/Consultant Advisor, NETSCC, UK

Professor Matthias Beck Chair in Public Sector Management and Subject Leader (Management Group), Queen's University Management School, Queen's University Belfast, UK

Professor Aileen Clarke Professor of Public Health and Health Services Research, Warwick Medical School, University of Warwick, UK

Dr Tessa Crilly Director, Crystal Blue Consulting Ltd, UK

Dr Eugenia Cronin Senior Scientific Advisor, Wessex Institute, UK

Ms Tara Lamont Scientific Advisor, NETSCC, UK

Professor William McGuire Professor of Child Health, Hull York Medical School, University of York, UK

Professor Geoffrey Meads Professor of Health Sciences Research, Health and Wellbeing Research Group, University of Winchester, UK

Professor John Norrie Chair in Medical Statistics, University of Edinburgh, UK

Professor John Powell Consultant Clinical Adviser, National Institute for Health and Care Excellence (NICE), UK

Professor James Raftery Professor of Health Technology Assessment, Wessex Institute, Faculty of Medicine, University of Southampton, UK

Dr Rob Riemsma Reviews Manager, Kleijnen Systematic Reviews Ltd, UK

Professor Helen Roberts Professor of Child Health Research, UCL Institute of Child Health, UK

Professor Jonathan Ross Professor of Sexual Health and HIV, University Hospital Birmingham, UK

Professor Helen Snooks Professor of Health Services Research, Institute of Life Science, College of Medicine, Swansea University, UK

Professor Jim Thornton Professor of Obstetrics and Gynaecology, Faculty of Medicine and Health Sciences, University of Nottingham, UK

Professor Martin Underwood Director, Warwick Clinical Trials Unit, Warwick Medical School, University of Warwick, UK

Please visit the website for a list of members of the NIHR Journals Library Board:
www.journalslibrary.nihr.ac.uk/about/editors

Editorial contact: nihredit@southampton.ac.uk

Abstract

A randomised placebo-controlled trial investigating efficacy and mechanisms of low-dose intradermal allergen immunotherapy in treatment of seasonal allergic rhinitis

Anna Slovick,^{1,2} Abdel Douiri,³ Rachel Muir,⁴ Andrea Guerra,¹ Konstantinos Tsioulos,¹ Evie Haye,¹ Emily PS Lam,¹ Joanna Kelly,⁵ Janet L Peacock,³ Sun Ying,¹ Mohamed H Shamji,⁶ David J Cousins,^{1,2,7} Stephen R Durham⁶ and Stephen J Till^{1,2*}

¹Division of Asthma, Allergy and Lung Biology, King's College London, School of Medicine, Guy's Hospital, London, UK

²Medical Research Council (MRC)–Asthma UK Centre for Allergic Mechanisms of Asthma, London, UK

³Division of Health and Social Care Research, King's College London, London, UK

⁴Clinical Research Facility, NIHR Biomedical Research Centre, Guy's Hospital, London, UK

⁵King's Clinical Trials Unit, King's College London, Institute of Psychiatry, London, UK

⁶Allergy and Clinical Immunology, National Heart and Lung Institute, Faculty of Medicine, Imperial College, London, UK

⁷Department of Infection, Immunity and Inflammation, National Institute for Health Research (NIHR) Leicester Respiratory Biomedical Research Unit, Leicester Institute for Lung Health, University of Leicester, Leicester, UK

*Corresponding author stephen.till@kcl.ac.uk

Background: We previously reported that repeated low-dose grass pollen intradermal allergen injection suppresses allergen-induced cutaneous late-phase responses, comparable with conventional high-dose subcutaneous and sublingual immunotherapy.

Objective: To evaluate the efficacy and mechanism of grass pollen intradermal immunotherapy for treatment of allergic rhinitis.

Design: A Phase II, double-blind, randomised controlled parallel-group trial.

Setting: Single-centre UK study.

Participants: Adults aged 18–65 years, with grass pollen-induced allergic rhinoconjunctivitis.

Interventions: Seven 2-weekly intradermal injections were given into the forearm, containing either *Phleum pratense* soluble grass pollen extract (7 ng of the major allergen Phl p 5) or histamine control.

Main outcome measures: The primary outcome was a combined symptom and medication score (CSMS) during the 2013 grass pollen season. Secondary clinical outcomes were overall symptom scores; individual symptoms scores for nose, mouth, eyes and lungs; overall medication scores; CSMSs during the peak season; visual analogue scale (VAS) scores for nose and eye symptoms; Mini Rhinitis Quality of Life Questionnaire scores; health-related quality-of-life scores (European Quality of Life-5 Dimensions, 5-levels); a global evaluation of symptoms, number of symptom-free and medication-free days; number of days when prednisolone was used; and adverse events. Mechanistic studies included measurement of late-phase skin response sizes, allergen-specific antibody titres, analysis of skin biopsies and basophil activation tests.

Results: There was no significant difference in CSMSs between treatment arms [difference in median area under curve (AUC) 14, 95% confidence interval (CI) –172.5 to 215.1; $p = 0.80$]. Paradoxically, among the secondary outcomes, nasal symptoms measured with daily scores were higher in the active arm (difference in median AUC 35, 95% CI 4.0 to 67.5; $p = 0.03$), with a trend for higher nasal symptoms measured by VASs (difference in median AUC 53, 95% CI –11.6 to 125.2; $p = 0.05$). No differences were seen in other clinical outcomes in the main intention-to-treat analysis. In mechanistic studies, active treatment increased *P. pratense*-, Phl p 1- and Phl p 5-specific immunoglobulin E (all $p = 0.001$) compared with the control. T cells cultured from skin biopsies of active intradermal immunotherapy subjects showed higher T helper type 2 cell (Th2) marker CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) expression ($p < 0.05$) and lower T helper type 1 cell marker CXCR3 [chemokine (C-X-C Motif) receptor 3] expression ($p < 0.05$), respectively. Interleukin 5 messenger ribonucleic acid, measured by microarray, was more highly expressed by cultured skin T cells in the active arm ($p < 0.05$). Late-phase skin responses to grass pollen were still inhibited up to 7 months after intradermal immunotherapy ($p = 0.03$), but not at 10–13 months' time points.

Limitations: Grass pollen doses were not increased during the course, as our proof-of-concept trial showed that repeating the same doses was sufficient to achieve almost complete late-response suppression. Injections were not continued throughout the season, as previous subcutaneous grass pollen immunotherapy trials have demonstrated preseasonal regimen efficacy.

Conclusions: Intradermal immunotherapy suppressed late-phase skin responses to allergen, but was not clinically effective. The intervention appeared to have an immunological priming effect and exacerbated certain seasonal symptoms, notably in the nose.

Future work: Further studies on low-dose intradermal grass pollen immunotherapy are not recommended because of our demonstrated worsening of allergic rhinitis symptoms and immunological priming. The findings are of great significance for other novel immunotherapies targeting the skin, such as epicutaneous techniques.

Trial registration: Current Controlled Trials ISRCTN78413121.

Funding: This project was funded by the Efficacy and Mechanism Evaluation programme, a Medical Research Council and National Institute for Health Research partnership.

Contents

List of tables	xi
List of figures	xiii
List of abbreviations	xv
Plain English summary	xvii
Scientific summary	xix
Chapter 1 Introduction	1
Chapter 2 Methods	3
Setting	3
Patient and public involvement	3
Primary objective	3
Secondary objectives	3
Participants	4
<i>Inclusion criteria</i>	4
<i>Exclusion criteria</i>	4
Randomisation	5
Trial medication	5
Intervention	6
Assessment of efficacy	6
Data management	8
Safety	8
Withdrawal criteria and stopping rules	9
Concomitant medications	9
Measurement of skin early- and late-phase responses	9
Skin biopsy	10
Analysis of T cells cultured from skin biopsies	10
Immunohistochemistry	11
Serum antibody measurements	11
Basophil activation tests	11
Statistical analysis	11
Chapter 3 Results	13
Study population	13
Clinical outcomes	13
Safety	19
Immunological end points	22
Intradermal skin challenge responses	23
Chapter 4 Discussion and conclusions	29
Conclusions	31

Acknowledgements	33
References	35
Appendix 1 PollenLITE trial website with prescreening questions	39
Appendix 2 PollenLITE recruitment advertisement panel used on public transport	41
Appendix 3 Example of daily symptom and medication-use diary card	43
Appendix 4 Visual analogue scale	45
Appendix 5 Global Evaluation scores (completed September 2013)	47
Appendix 6 Statistical analysis plan	49
Appendix 7 Effect of intradermal immunotherapy on primary and secondary outcomes (intention to treat): missing data imputed	73
Appendix 8 Effect of intradermal immunotherapy on primary and secondary outcomes (per-protocol analysis)	75
Appendix 9 Effect of intradermal immunotherapy on daily organ symptom scores (intention to treat)	77
Appendix 10 Effect of intradermal immunotherapy on individual visual analogue scale scores (intention to treat)	79

List of tables

TABLE 1 Baseline characteristics of study participants	15
TABLE 2 Verification of participant blinding	16
TABLE 3 Effect of intradermal immunotherapy on primary and secondary outcomes (ITT)	19
TABLE 4 Frequency of AEs reported from first intradermal allergen immunotherapy or control injection until end of pollen season	21
TABLE 5 Microarray gene expression profiles of activated CD4 ⁺ T cells derived from skin biopsy explants	25

List of figures

FIGURE 1 Study design	7
FIGURE 2 Consolidated Standards of Reporting Trials diagram	14
FIGURE 3 Primary outcome and daily symptom and daily medication scores in the primary intention-to-treat analysis	17
FIGURE 4 Nasal symptoms	20
FIGURE 5 Immunological outcomes	22
FIGURE 6 Immunological outcomes	23
FIGURE 7 Expression of (a) CRTH2 (Th2 marker); (b) CXCR3 (Th1 marker); and (c) ratio of CRTH2 to CXCR3 expression on CD4 ⁺ cells expanded from skin biopsies (24 hours post-skin challenge)	24
FIGURE 8 Immunohistochemistry analysis of skin biopsies	26
FIGURE 9 Basophil activation tests	27
FIGURE 10 Late-phase skin responses	28

List of abbreviations

AE	adverse event	IgE	immunoglobulin E
ANCOVA	analysis of covariance	IgG	immunoglobulin G
ANOVA	analysis of variance	IL-2	interleukin 2
AP	alkaline phosphatase	IL-5	interleukin 5
APAAP	alkaline phosphatase anti-alkaline phosphatase	IQR	interquartile range
AUC	area under curve	ITT	intention to treat
CCR6	chemokine (C-C motif) receptor 6	KCL	King's College London
CD3	cluster of differentiation 3	KCTU	King's Clinical Trials Unit
CD3+	cluster of differentiation 3-positive	mAb	monoclonal antibody
CD4	cluster of differentiation 4	Mini-RQLQ	Mini Rhinitis Quality of Life Questionnaire
CD4+	cluster of differentiation 4-positive	MRC	Medical Research Council
cDNA	complementary deoxyribonucleic acid	mRNA	messenger ribonucleic acid
CI	confidence interval	NIHR	National Institute for Health Research
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2 cells	PBS	phosphate-buffered saline
CSMS	combined symptom and medication score	PEF	peak expiratory flow
CXCR3	chemokine (C-X-C Motif) receptor 3	PollenLITE	Pollen Low dose Intradermal Therapy Evaluation
DC	dendritic cell	RCT	randomised controlled trial
eCRF	electronic case report form	RNA	ribonucleic acid
EQ-5D-5L	European Quality of Life-5 Dimensions, 5-levels	SD	standard deviation
FEV ₁	forced expiratory volume in 1 second	SPT	skin prick test
GCP	Good Clinical Practice	Th1	T helper type 1 cell
GMP	Good Medical Practice	Th2	T helper type 2 cell
GP	general practitioner	Treg	regulatory T cell
		VAS	visual analogue scale
		WAO	World Allergy Organization

Plain English summary

Several million people in the UK have hay fever, which significantly affects their quality of life. In such people, an allergy vaccine (called 'immunotherapy') may reduce the allergic response to grass pollen. Although current vaccines are effective, they are expensive and involve frequent visits to specialist clinics for injections or daily self-dosing with tablets or drops for several years.

Based on encouraging results from a pilot study, we undertook a clinical trial of a potentially new and very different form of grass pollen immunotherapy. The new approach involved giving very small grass pollen doses (thousands of times less than existing methods) by injections directly into the topmost skin layer (called the dermis). We recruited 93 participants, who were randomly selected to receive seven such injections every 2 weeks before the 2013 summer grass pollen season, or seven dummy injections. The severity of hay fever symptoms and usage of allergy medications was then recorded. We also performed experiments to see the effect of the new vaccine on the immune system.

The results of the study conclusively showed that the new approach had no benefit in reducing hay fever symptoms or need for medications. Unexpectedly, symptoms in the nose were actually modestly worse in those who had the grass pollen injections. Our experiments also indicated a small stimulation effect on the immune system.

These results have implications for other future research in this area, and also make an important scientific contribution to our understanding of the mechanisms that can drive allergies.

Scientific summary

Background

In the UK an estimated 5 million people suffer moderate/severe persistent symptoms of allergic rhinitis that have an impact on quality of life, including disturbed sleep, disruption of leisure activities and impairment of performance at work/school. There is a substantial unmet need for both therapy and prophylaxis of seasonal allergic rhinitis.

In the UK, immunotherapy is indicated in patients with moderate or severe symptoms who fail to respond to conventional medications. Immunotherapy with grass pollen for treatment of season allergic rhinitis was first described in 1911, and the conventional approach involves the regular subcutaneous administration of allergen extracts at high doses (typically microgram quantities of group 5 grass pollen allergens). A significant body of evidence, including a Cochrane meta-analysis (Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. *Cochrane Database Syst Rev* 2007;**1**:CD001936), exists to support the clinical efficacy of high-dose subcutaneous immunotherapy. Grass pollen allergen may also be administered at high dose as sublingual tablets or drops, an approach that is further supported by a Cochrane meta-analysis (Radulovic S, Calderon MA, Wilson D, Durham S. Sublingual immunotherapy for allergic rhinitis. *Cochrane Database Syst Rev* 2010;**12**:CD002893). Both subcutaneous and sublingual high-dose immunotherapy have significant limitations: the vaccine products are expensive and the need for repeated administration in a specialist clinic (subcutaneous immunotherapy) or daily at home (sublingual immunotherapy) is associated with additional expense and/or inconvenience. Therefore, there is a continuing need to develop new and improved immunomodulatory therapies for allergic rhinitis.

We established 'proof of concept' for a novel low-dose intradermal immunotherapy regimen in subjects with grass pollen-induced allergic rhinitis. A feature of an intradermal allergen injection is the development of local swelling within 6 hours that persists for 24–36 hours. This 'late-phase response' is characterised by infiltration of inflammatory cells, notably activated T cells, eosinophils and basophils. We previously showed that six 2-weekly intradermal injections of grass pollen (containing only 7 ng of major allergen Phl p 5; 10 BU) resulted in a 93% suppression (mean of $n = 10$ subjects) in the cutaneous late-phase response, measured after 24 hours in response to these injections. This effect was systemic and antigen specific, and the magnitude of late-phase response suppression was comparable to that seen following treatment with a conventional high-dose subcutaneous grass pollen vaccine, and greater than that seen following sublingual immunotherapy. The concept of administering low-dose allergen immunotherapy by the intradermal route has been described in the medical literature dating back to 1926, and our own findings suggested the plausibility of this approach. A potential advantage of the novel intradermal regimen was that the effect on skin responses was seen with a low dose of allergen, which was not changed between visits. As adverse reactions to immunotherapy usually occur when doses are increased, this would offer significant clinical advantages over existing vaccines. Based on this, we initiated a randomised controlled trial (RCT) of low-dose intradermal allergen immunotherapy as a treatment for seasonal allergic rhinitis.

Objective

The objective of this study was to investigate the efficacy and mechanism of low-dose intradermal grass pollen immunotherapy in adults with seasonal allergic rhinitis ('hay fever').

Methods

We conducted a Phase II RCT comparing intradermal injection immunotherapy with grass pollen allergen extract or a histamine control.

Eligible participants were aged 18–65 years, with grass pollen-induced allergic rhinitis of at least 2 years' duration, with moderate or severe symptoms despite treatment with antihistamine drugs and/or nasal corticosteroid drugs. Participants were required to have a positive skin prick test response (> 3 mm to *Phleum pratense*, ALK Abelló, Reading, UK), a positive specific immunoglobulin E (IgE; $>$ class 2) against *P. pratense*, and a pre-bronchodilator forced expiratory volume in 1 second (FEV₁) of $> 70\%$ of predicted value. Exclusion criteria included seasonal grass pollen-induced asthma requiring regular treatment and symptomatic seasonal allergic rhinitis and/or asthma caused by tree pollen, weed pollen or a perennial allergen to which the participant was regularly exposed, except for mild intermittent symptoms. Potential participants were also excluded if they had received treatment with grass pollen immunotherapy within the previous 5 years.

The intervention was a series of seven grass pollen or control injections, administered intradermally every 2 weeks into the forearm, before the 2013 grass pollen season. Each active intradermal allergen injection contained 10 BU [33.3 SQ-U (standard quality units)] of *P. pratense* soluble grass pollen extract (Aquagen SQ™ Timothy, ALK Abelló). The control drug was histamine only, administered at concentrations of 100 µg/ml (injections 1 and 2), 30 µg/ml (injections 3 and 4) and 10 µg/ml (injections 5–7). A reducing dose of histamine was used to help preserve blinding. Active and control study medications appeared identical.

The primary end point was a combined symptom and medication score (CSMS) during the grass pollen season period spanning 13 May to 31 August 2013. Daily symptoms (nose, eyes, mouth and lungs) and medication use (antihistamines, nasal steroid drugs, antihistamine eye drops and oral prednisolone) were recorded on diary cards. Symptom scores and medication scores for each participant were calculated as area under curve (AUC).

Secondary clinical end points were:

- overall symptoms during entire pollen season (AUC)
- overall medication scores over entire pollen season (AUC)
- Mini-Rhinitis Quality of Life Questionnaire (Mini-RQLQ) scores (measured three times during, and once after, the pollen season)
- health-related quality-of-life scores, evaluated using the European Quality of Life-5 Dimensions, 5-levels (EQ-5D-5L) questionnaire (measured three times during, and once after, the pollen season)
- visual analogue scale (VAS) scores for nasal and eye symptoms, recorded 2-weekly during the entire pollen season (AUC)
- global evaluation of symptoms, recorded once after the pollen season
- number of general practitioner visits for hay fever during summer 2013
- CSMSs during the peak of the 2013 grass pollen season (peak pollen season days defined in accordance with prespecified criteria)
- number of medication-free days during the grass pollen season
- number of symptom-free days during the grass pollen season
- individual symptoms scores (AUC) for each organ: nose, mouth, eyes and lungs
- total number of days during which prednisolone was taken during the grass pollen season
- frequency of adverse events (AEs).

Mechanistic studies

Sera were collected before and after intradermal grass pollen or control immunotherapy for measurement of grass pollen-specific immunoglobulins. Basophil activation tests were also performed following administration of the final intradermal allergen immunotherapy or control injection (May 2013). All

participants underwent intradermal skin challenge testing 4 months after the final intradermal allergen immunotherapy or control injection (September 2013). Participants were then randomised to undergo repeat follow-up testing 7, 10 or 13 months later to assess persistence of late-response suppression. The procedure for the intradermal skin challenge testing and the dose of allergen used were identical to that for an active intradermal allergen immunotherapy injection. Early- and late-phase responses were measured 15 minutes and 24 hours after challenge, respectively.

Forty participants (20 in each trial arm) were selected at random to undergo 3-mm skin punch biopsies immediately after measurement of late-phase responses (i.e. 24 hours after challenge) at the 4-month time point in September 2013. Biopsies were analysed for inflammatory cell infiltration by immunohistochemistry, and a proportion was also cultured as explants for analysis of cutaneous T cells by flow cytometry and microarray transcriptional profiling.

Statistical analysis

On the basis of data from a previous RCT of subcutaneous grass pollen immunotherapy, we estimated that with 35 participants in each group the study would have a power of 90% ($\alpha = 0.05$) to detect a between-group difference in the primary outcome during the grass pollen season. For the purposes of sample size estimation, the treatment effect was conservatively estimated at only 80% of that observed with subcutaneous immunotherapy. To make allowance for the unknown distribution of the primary outcome, and based on the lower bound for the asymptotic relative efficiency of the Mann–Whitney *U*-test, the sample size was increased by a further 15% to 40 participants in each arm. To account for a post-randomisation dropout rate of up to 10%, a total sample size of 90 (45 each arm) was estimated as required.

Statistical analyses were performed on an intention-to-treat (ITT) basis, with data from all of the participants who could be assessed for the primary outcome. Summary measures for the baseline characteristics of each group were calculated as mean and standard deviation for continuous (approximate) normally distributed variables, medians and interquartile ranges (IQRs) for non-normally distributed variables, and frequencies and percentages for categorical variables. The AUC of the CSMSs was plotted against time as a summary measure of the primary outcome. The primary efficacy analysis, that is, the difference between the two arms in AUC of the CSMSs, was analysed on randomised patients using a stratified Mann–Whitney *U*-test (van Elteren test), adjusted for the baseline stratification factors of size of the skin test to grass pollen and presence or absence of rhinitis symptoms outside the grass pollen season. Median differences between the groups were calculated using the stratified Hodges–Lehmann method. Similar analyses were conducted for symptom scores, medication scores, symptoms in different organs and VASs. Linear mixed models were used to evaluate Mini-RQLQ and EQ-5D-5L scores in order to isolate the effect of the intervention on each arm after adjusting for stratification factors. Differences between the groups were reported with their 95% confidence intervals (CIs). All mechanistic between-group comparisons were performed by Mann–Whitney *U*-test, with the exception of serology and immunohistochemistry comparisons, which were analysed by analysis of covariance. Comparisons of serology between pre and post treatment, and skin biopsy immunohistochemistry between diluent control and allergen challenge were made by Wilcoxon signed-rank test.

Results

Ninety-three participants were enrolled in the study and underwent randomisation. Study arms were well balanced for baseline characteristics. All of the 46 participants who were assigned to intradermal allergen immunotherapy completed the treatment course. Of the 47 participants who were assigned to control injections, one did not complete the treatment course for work-related reasons. Missing diary data for the primary end point were few, with 94% of participants supplying > 90% of daily diary card data. Five participants, all in the control arm, significantly deviated from the protocol in use of rescue medications. There was no evidence that participants were able to identify if they had received the active or control intervention.

Primary outcome

All 93 randomised participants were evaluated for the primary outcome and were included in the ITT analysis. There was no significant difference between the intradermal immunotherapy group (active intervention) and the control group for the primary end point, that is, the CSMS over the whole grass pollen season (difference in median AUC = 14; 95% CI -172.5 to 215.1; $p = 0.80$).

Secondary outcomes

There were no differences between the trial arms in the secondary end points of overall symptom scores (AUC; $p = 0.24$) or rescue medication use (AUC; $p = 0.44$) during the whole season, or the CSMSs during peak season (12 June to 26 July 2013) (AUC; $p = 0.99$). Among other secondary end points, allergic rhinitis symptoms measured by daily nasal symptom scores were 44% higher in the intradermal allergen immunotherapy group than in the control group, with a median difference in AUC values of 35 (95% CI 4.0 to 67.5; $p = 0.03$). There was also a trend for higher nasal symptoms measured by VAS in the intradermal allergen immunotherapy group, with a 28% median difference in AUC values (difference 53; 95% CI -11.6 to 125.2; $p = 0.05$). No significant differences were seen between groups in daily eye or lung symptoms, although there was a trend for mouth symptoms to be higher in the intradermal allergen group (difference in median AUC 10, 95% CI -3.8 to 24; $p = 0.05$). No significant group differences were observed in eye symptoms measured by VAS, or Mini-RQLQ scores, EQ-5D-5L scores, global evaluation of symptoms scores, numbers of symptom-free or medication-free days or number of days during which prednisolone was used as a rescue medication. There were few treatment-related AEs, with no difference between trial arms.

Outcomes of mechanistic studies

A seasonal fall in *P. pratense*-specific IgE occurred in the group that received control histamine injections (median change -5.4 kU/l, IQR -13.6 to -1.3; $p < 0.001$), but IgE levels were maintained in the active intradermal immunotherapy group [median change -1.0 kU/l IQR -7.3 to 2.4; $p = 0.23$ ($p = 0.001$ for between-group comparison)]. The same pattern was observed in levels of IgE that were specific for major allergens Phl p 1 and Phl p 5. A similar treatment effect was also seen on *P. pratense*-specific immunoglobulin G (IgG) titres, which fell in the control group ($p = 0.03$) but not the intradermal allergen group over the same period ($p = 0.26$ and $p = 0.007$ for between-group comparison), although this pattern was not seen with IgG4 (immunoglobulin G subclass 4) responses. Cluster of differentiation 4-positive (CD4⁺) T cells that were expanded from grass pollen-challenged skin showed higher expression of T helper type 2 cell (Th2) surface marker CCR4 (chemoattractant receptor-homologous molecule expressed on Th2 cells) in the intradermal allergen immunotherapy group [median 13.4% (IQR 6.3–25.4), $n = 10$] than those in the control group [6.3% (IQR 1.9–7.6%), $n = 9$] ($p = 0.04$), whereas expression of the T helper type 1 cell (Th1) marker CXCR3 [chemokine (C-X-C Motif) receptor 3] was lower in the intradermal allergen immunotherapy group (33.5%, IQR 24.7–47.3% vs. 56%, IQR 45.8–63.8%; $p = 0.01$). Microarray transcriptional profiling performed on skin T cells also identified higher expression of messenger ribonucleic acid for Th2 cytokine interleukin 5 in the intradermal immunotherapy group ($p = 0.03$).

Immunohistochemistry of skin biopsies showed grass pollen-induced recruitment of eosinophils, neutrophils, cluster of differentiation 3-positive T cells and CD4⁺ T cells but no significant treatment effect. Furthermore, no significant treatment effect was seen on surface expression of peripheral blood basophil activation markers. Late-phase responses in the skin were still suppressed at 4 and 7 months after completing intradermal allergen treatment ($p = 0.03$ for both time points), but not at 10 or 13 months. In comparison with historical data, however, the degree of suppression at these times was less than that observed immediately after completing six injections, suggesting that the suppressive effect on late-phase responses was wearing off within 4 months.

Conclusions

In this study, we have demonstrated that preseasonal treatment with intradermal grass pollen injections was not clinically effective, as measured by the primary end point of a CSMS during the 2013 summer

grass pollen season. Although this trial was not specifically designed or powered to detect worsening of symptoms, analysis of secondary end points indicated that intradermal allergen immunotherapy was associated with worse allergic rhinitis nasal symptoms. Furthermore, we found evidence for immunological priming of IgE and Th2 cell responses. We conclude that novel immunotherapy strategies that promote dermal allergen exposure have the potential to be deleterious, even if local macroscopic responses appear to be suppressed by this approach.

Trial registration

This trial is registered as ISRCTN78413121.

Funding

This project was funded by the Efficacy and Mechanism Evaluation programme, a Medical Research Council and National Institute for Health Research partnership.

Chapter 1 Introduction

Tables, figures and parts of the text of this report are reproduced or adapted from Slovic *et al.*¹ © 2016 The Authors. Published by Elsevier Inc. on behalf of American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Allergic rhinitis caused by grass pollen affects one-quarter of the UK population.² Of these, around 5 million people suffer moderate or severe persistent symptoms that have an impact on quality of life, including disturbed sleep, disruption of leisure activities and impairment of performance at work or school.³ Therefore, there is a substantial unmet need for both therapy and prophylaxis of seasonal allergic rhinitis. In the UK, subcutaneous and sublingual immunotherapy is indicated in patients with moderate or severe symptoms who fail to respond to conventional medications.⁴ Immunotherapy, that is, prophylactic inoculation with grass pollen for treatment of seasonal allergic rhinitis, was first described in 1911.⁵ The conventional approach involves the regular subcutaneous administration of allergen extracts at high doses (typically microgram quantities of group 5 grass pollen allergens).⁴ The most commonly used form of grass pollen immunotherapy is that given by injections into the tissue beneath the skin (i.e. subcutaneously) over a period of 2–3 years, with increasing amounts of allergen administered weekly for 12–15 weeks followed by monthly maintenance injections.⁴ A body of evidence, including a Cochrane meta-analysis,⁶ exists to support the clinical efficacy of high-dose subcutaneous immunotherapy. Grass pollen allergen may also be administered at a high dose as sublingual tablets or drops, an approach further supported by Cochrane meta-analysis.⁷ Both subcutaneous and sublingual high-dose immunotherapy have limitations: the vaccine products are expensive and the need for repeated administration in a specialist clinic (subcutaneous immunotherapy) or daily at home (sublingual immunotherapy) is associated with additional expense and/or inconvenience.

Injections of relatively small quantities of allergen (nanograms of major allergen proteins) into the dermis leads to the development of local swelling within 6 hours, which persists for 24–36 hours. This 'late-phase response' is characterised by infiltration of inflammatory cells – notably activated T helper type 2 cells (Th2), eosinophils and basophils – and has been extensively used as a model for investigating mechanisms of chronic allergic inflammation.⁸ We previously established that when these injections are repeated at 2-weekly intervals there is a progressive and significant decline in the size of cutaneous late-phase response that is antigen-specific and systemic. Administration of six intradermal injections of grass pollen containing only 7 ng of major allergen Phl p 5 resulted in a > 90% suppression in the cutaneous late-phase response measured after 24 hours in response to these injections.⁹ The magnitude of inhibition was comparable to that seen with a conventional high-dose subcutaneous grass pollen vaccine¹⁰ despite equating to over 1000-fold less allergen over the same time period, and significantly exceeded the inhibition seen with sublingual immunotherapy given daily and containing 20,000-fold more group 5 allergen over a 10-week period.¹¹ This observation provided the rationale for progressing to a clinical trial of low-dose intradermal grass pollen immunotherapy as a treatment for allergic rhinitis. The concept of therapeutic intradermal allergen inoculation is not without precedent. In 1926, Phillips, a physician dworking in Arizona, published a preliminary account of his uncontrolled experiences with intradermal grass pollen immunotherapy in 29 patients,¹² extended to 322 patients by 1933,¹³ > 90% of whom obtained 'satisfactory relief'. However, no randomised controlled trial (RCT) has previously addressed the efficacy of this approach.

High-dose subcutaneous and sublingual immunotherapy is associated with induction of regulatory T cells (Tregs),^{14–16} probably through interaction of cluster of differentiation 4-positive (CD4+) T cells with protolerogenic dendritic cells (DCs). These cells are anti-inflammatory and also induce B-cell production of allergen-specific 'blocking' immunoglobulin G (IgG) antibodies.¹⁷ Low-dose intradermal allergen desensitisation is biologically plausible: for example, intradermal injection of radiotracer in animal models results in 100-fold higher rates of drainage to regional lymph nodes than subcutaneous injection,

potentially leading to more efficient pulsing of lymph node DCs.¹⁵ In addition, the dermis is, itself, an immunologically active environment, rich in DCs and lymphatic vessels.^{18,19} In contrast, conventional subcutaneous immunotherapy injections target a compartment consisting mostly of connective and adipose tissue but few DCs. Therefore, in this study we hypothesised that intradermal grass pollen immunotherapy would be a clinically effective treatment for seasonal allergic rhinitis, and that accompanying desensitisation of the late-phase response would be reflected in local suppression of proallergic Th2 responses. To test this hypothesis, we conducted a Phase II RCT, the Pollen Low dose Intradermal Therapy Evaluation (PollenLITE), with embedded mechanistic studies to evaluate the immunological response to treatment.

Chapter 2 Methods

Setting

This single-centre RCT was conducted in the Clinical Research Facility of the National Institute for Health Research (NIHR) Biomedical Research Centre at Guy's Hospital from September 2012. The final study visit was on 27 August 2014. The study was conducted in accordance with the principles of Good Medical Practice (GMP) for clinical trials, and approved by the National Research Ethics Service Committee (London–Harrow; 12/LO/0941), with oversight by King's Health Partners Clinical Trial Office, together with an independent Trial Steering Committee and Data Monitoring and Ethics Committee. The clinical trial protocol was published²⁰ and the statistical analysis plan finalised prior to randomisation. All participants provided written informed consent prior to participation.

Patient and public involvement

The recruitment campaign for this trial involved development of a dedicated advertising campaign and website (developed by Media with Impact Ltd, London, UK) (see *Appendices 1* and *2*). The website contained a number of online prescreening questions. With the assistance of Asthma UK, patient representatives reviewed the design and helped to ensure appropriate engagement with the target audience. Patient representatives also reviewed all advertisement materials, participant information sheets and consent forms. In response to this feedback, substantial changes were made to the branding of the trial website and advertising materials in particular, to ensure appropriate engagement with the target population. Patient representatives also reviewed materials prior to disseminating the results to study participants.

Primary objective

The primary objective was to determine if preseasonal low-dose intradermal grass pollen allergen immunotherapy [seven 2-weekly injections of 10 BU (33.3 SQ-U)] reduces symptoms and requirements for antiallergic drugs in seasonal allergic rhinitis during the 2013 grass pollen season compared with the control intervention (histamine only).

Secondary objectives

The secondary objectives were to:

1. determine if this intervention is associated with improvement in quality of life compared with the control intervention, as assessed during the 2013 grass pollen season
2. evaluate if this intervention is safe and well tolerated
3. investigate immunological changes in response to repeated intradermal allergen injections by examining humoral and cellular responses, both in peripheral blood and in tissue
4. explore if the intradermal late-phase response desensitisation effect is long-lived, that is, persists following cessation of intradermal injections.

Participants

Participants were identified via a recruitment campaign including advertisements in press, online and on public transport. Potential participants were invited to visit the trial website (www.pollenlite.co.uk) to answer seven prescreening questions before registering. Participants passing the prescreening on the trial website were contacted for further telephone screening, and, if considered potentially eligible, they were invited to attend the Clinical Research Facility at Guy's Hospital for a formal screening visit. Full eligibility criteria were as follows.

Inclusion criteria

1. Adults aged 18–65 years.
2. A clinical history of grass pollen-induced allergic rhinoconjunctivitis for at least 2 years, with peak symptoms in May, June or July.
3. A clinical history of moderate or severe persistent rhinoconjunctivitis symptoms interfering with usual daily activities or with sleep.
4. A clinical history of rhinoconjunctivitis that remains troublesome despite treatment with either antihistamine drugs or nasal corticosteroid drugs during the grass pollen season.
5. Positive skin prick test (SPT) response, defined as wheal diameter ≥ 3 mm, to *Phleum pratense*.
6. Positive specific immunoglobulin E (IgE), defined as \geq IgE class 2, against *P. pratense*.
7. For women of childbearing age, a willingness to use an effective form of contraception for the duration of intradermal injections.
8. The ability to give informed consent and comply with study procedures.

Exclusion criteria

1. Pre-bronchodilator forced expiratory volume in 1 second (FEV₁) of < 70% of predicted value at screening visit.
2. A history of seasonal grass pollen-induced asthma requiring regular treatment with salbutamol or inhaled corticosteroids. Patients with mild seasonal grass pollen-induced asthma were included, provided that symptoms were satisfactorily controlled with occasional salbutamol only.
3. A clinical history of symptomatic seasonal allergic rhinitis and/or asthma due to tree pollen or weed pollen, near or overlapping the grass pollen season, although patients with mild intermittent symptoms requiring only occasional antihistamines were included.
4. A clinical history of symptomatic allergic rhinitis and/or asthma caused by a perennial allergen to which the participant is regularly exposed, although patients with mild intermittent symptoms requiring only occasional antihistamines were included.
5. Emergency department visit or hospital admission for asthma in the previous 12 months.
6. History of chronic obstructive pulmonary disease.
7. History of significant recurrent acute sinusitis, defined as two episodes per year for the last 2 years, all of which required antibiotic treatment.
8. History of chronic sinusitis, defined as a sinus symptoms lasting > 12 weeks outside the grass pollen season, that includes two or more major factors, or one major factor and two minor factors. Major factors are defined as facial pain or pressure, nasal obstruction or blockage, nasal discharge or purulence or discoloured postnasal discharge, purulence in nasal cavity, or impaired/loss of smell. Minor factors are defined as headache, fever, halitosis, fatigue, dental pain, cough, and ear pain, pressure or fullness.
9. At randomisation, current symptoms of, or treatment for, upper respiratory tract infection, acute sinusitis, acute otitis media, or other relevant infectious process; serous otitis media was not an exclusion criterion.
10. Current smokers or a history of ≥ 5 pack-years.
11. Previous treatment by immunotherapy with grass pollen allergen within the previous 5 years.
12. History of life-threatening anaphylaxis or angioedema.

13. Ongoing systemic immunosuppressive treatment.
14. History of intolerance of grass pollen immunotherapy, rescue medications or their excipients.
15. For females of childbearing age, a positive serum or urine pregnancy test with sensitivity of < 50 mIU/ml within 72 hours of first administration of study therapy.
16. Lactating females.
17. The use of any investigational drug within 30 days of the screening visit.
18. Ongoing treatment with leukotriene receptor antagonists, beta-blockers, calcium channel blockers, tricyclic antidepressants, monoamine oxidase inhibitors or anti-IgE monoclonal antibody (mAb).
19. The presence of any medical condition that the investigator deemed incompatible with participation in the trial.
20. Individuals with insufficient understanding of the trial.

Randomisation

Randomisation was performed by King's Clinical Trials Unit (KCTU; UK Clinical Research Collaboration registered) at King's College London (KCL) using a 24-hour, web-based randomisation system. Participants were randomised 1 : 1 to active intradermal immunotherapy or the control arm by the method of block randomisation with randomly varying block sizes, stratified by the size of skin test response to grass pollen at screening visit (the cut-off SPT size being the median value of all subjects to be randomised, ≥ 11 mm) and presence/absence of rhinitis symptoms outside the grass pollen season. Study medication was blinded. To minimise bias through accidental unblinding, as a result of common injection site reactions in the active trial arm, the control intervention consisted of a reducing dose of histamine to produce similar clinical effects as the active medication. All physicians, researchers, research nurses, outcome assessors and patients remained blinded to treatment allocation until the primary analysis was completed. The trial statistician was subgroup unblind only. Only the KCTU randomisation service provider and the manufacturing pharmacy had access to the blinding information for the study.

In August 2013, the KCTU also randomly selected participants to be approached to undergo skin biopsies. The first 40 participants who gave agreement then underwent biopsy after giving additional procedure-specific informed consent. Furthermore, in August 2013, the KCTU randomised all participants for a second time to one of three groups. These three groups then underwent repeat intradermal allergen injections, at 7, 10 or 13 months after the final intradermal immunotherapy or control injection, to assess if low-dose intradermal allergen immunotherapy was associated with prolonged suppression skin responses.

Trial medication

Each active intradermal allergen injection contained 10 BU (33.3 SQ-U) of *P. pratense* soluble grass pollen extract (Aquagen SQ™ Timothy Grass Pollen extract, ALK Abelló, Reading, UK) contained in a 20- μ l volume [i.e. 500 BU/ml (1666.7 SQ-U/ml)]. Individual vials for each participant and each visit were preprepared and prelabelled by Guy's Hospital Pharmacy under GMP conditions. In brief, Aquagen SQ Timothy Grass Pollen extract was reconstituted in manufacturer-supplied diluent to the maximum recommended concentration [30,000 BU/ml (100,000 SQ-U/ml), i.e. 60 times the final working strength; shelf-life 6 months at 2–8 °C after reconstitution] and 0.15 ml was aliquoted into glass study vials. At each visit for intradermal injection the investigator added 8.85 ml of clinical grade 0.9% normal saline at ambient temperature to the vial corresponding with that participant's visit, to achieve a 60-fold dilution. Then 20 μ l was aspirated from this vial and administered directly. The allergen required dilution on the day of administration, as the recommended shelf-life of Aquagen SQ Timothy Grass Pollen extract at 500 BU/ml (1666.7 SQ-U/ml) is 14 days. The control drug was histamine only, administered at a concentration of 100 μ g/ml for the first and second injections. To help preserve blinding, histamine concentrations were reduced to 30 μ g/ml for the third and fourth injections, and 10 μ g/ml for fifth, sixth and seventh injections. To match the grass

pollen extract dilution and preserve blinding, histamine was also aliquoted into study vials at 60 times the final working strength in 0.15-ml volumes, for further dilution with 8.85 ml of clinical grade 0.9% normal saline immediately prior to injection. Active and control study medications appeared to be identical.

Following manufacture, vials were packed into individual dispensing packs and dispensed by Guy's Hospital Pharmacy against a single study prescription for each study participant, covering all visits. At randomisation, an e-mail was sent from the randomisation system to the dispensing pharmacy. The blinded dispensed packs were thereafter stored in the Clinical Research Facility in temperature-monitored fridges, in a secure environment. Study drug accountability was assessed and documented by Guy's Hospital Pharmacy. Study vials that had been reconstituted in saline for injection were stored separately at room temperature after use for return to pharmacy for drug accountability to be assessed.

Intervention

A series of seven intradermal active or control histamine injections was administered 2-weekly into the forearm before the 2013 grass pollen season (*Figure 1*). The first injection for each participant was administered between 18 February and 1 March 2013, with the seventh and final injection given between 13 May and 24 May 2013. The injection site was alternated between left and right arms at each visit. Intradermal injections were administered in a 20- μ l volume using a 29 gauge insulin syringe (BD Micro-Fine™, Becton Dickinson, Oxford, UK). In the event of an injection being administered too deeply (i.e. into subcutaneous tissue) to elicit an immediate injection 'bleb' and subsequent characteristic wheal, the injection was repeated 1 cm from the original site. Most participants were not taking antihistamines at the time of intradermal injections, as these were performed before the grass pollen season. Nevertheless, all of the participants were asked to avoid taking antihistamines for 5 days before receiving an intradermal injection, so that the presence of a wheal could be confirmed. Following an intradermal injection, participants were able to take an antihistamine to reduce the local itching and swelling if they so wished.

Assessment of efficacy

The primary end point was the area under curve (AUC) of the combined symptom and medication score (CSMS) during the grass pollen season period spanning 13 May to 31 August 2013 (111 days), the clinical end point recommended by World Allergy Organization (WAO) guidelines for clinical trials of immunotherapy for allergic rhinitis.²¹ Participants were provided with daily diary cards (see *Appendix 3*) to record symptoms in the nose (sneezing, blockage and running), eyes (itching, redness, tears and swelling), mouth and throat (itching and dryness) and chest (breathlessness, cough, wheezing and tightness), on a scale of 0–3 (with a score of '0' indicating no symptoms and '1', '2' and '3' indicating mild, moderate and severe symptoms, respectively). Daily rescue medication was scored as follows: desloratadine (Merck Sharp and Dohme Ltd., Moddesdon, UK), 5 mg, up to one tablet daily (6 points per day); olopatadine eye drops, 1 mg/ml, up to one drop per eye twice daily (1.5 points per drop, up to 6 points per day); fluticasone propionate nasal spray, 50 μ g per spray, up to two sprays per nostril once daily (2 points per spray, up to 8 points per day); and prednisolone, 5 mg per tablet, up to six tablets per day (2 points per tablet, up to 12 points per day). Symptom and medication scores were expressed as AUC for the entire grass pollen season. As maximum scores for symptoms (39) and medications (32) were different in magnitude, these parameters were normalised as per WAO guidelines.²¹

Secondary clinical end points were:

1. symptom scores (AUC) over entire pollen season
2. medication scores (AUC) over entire pollen season.

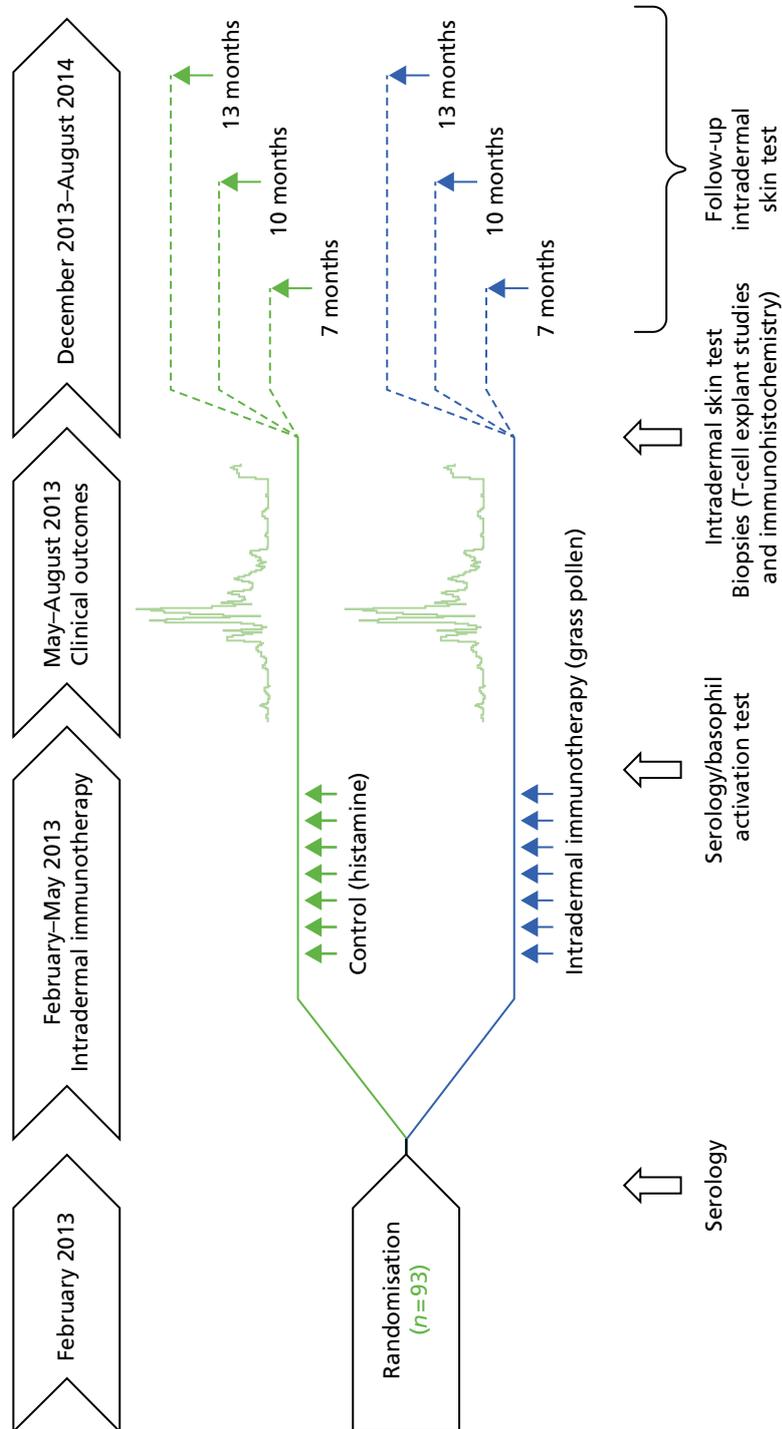


FIGURE 1 Study design.

3. Mini Rhinitis Quality of Life Questionnaire (Mini-RQLQ) scores²² (overall score and domain scores), recorded three times during the pollen season (12 June, 26 June and 10 July) and once after the season in September 2013.
4. Health-related quality of life: evaluated using the European Quality of Life-5 Dimensions, 5-levels (EQ-5D-5L) questionnaire²³ three times during the pollen season (12 June, 26 June and 10 July) and once after the season in September 2013.
5. Visual analogue scales (VASs) for nasal and eye symptoms (see *Appendix 4*). These were recorded every 2 weeks during the pollen season and AUC values calculated.
6. Global evaluation scores (see *Appendix 5*).
7. The number of primary care [i.e. general practitioner (GP)] visits for hay fever during summer 2013.
8. CSMSs during the peak of the 2013 grass pollen season.
9. Number of medication-free days covering the grass pollen season period of 13 May to 31 August 2013.
10. Number of symptom-free days covering the grass pollen season period of 13 May to 31 August 2013.
11. Individual symptoms scores (AUC) for each organ: nose, mouth, eyes and lungs.
12. Total number of days during which prednisolone was used between 13 May and 31 August 2013.
13. Frequency of adverse events (AEs).

The peak of grass pollen season was prospectively defined as starting on the first three consecutive days between 13 May and 31 August 2013, when grass pollen counts in central London were ≥ 30 grains/cm³, using counts supplied by the UK Meteorological Office. The end of the peak season was defined as the first of 3 consecutive days when grass pollen counts were < 30 grains/cm³.

Data management

Data were managed using the regulatory compliant [GCP (Good Clinical Practice), 21CFR11, EC Clinical Trial Directive] InferMed MACRO database system (MACRO 4, Elsevier, Amsterdam, the Netherlands). An electronic case report form (eCRF) was created in collaboration with the trial statisticians and the chief investigator, and maintained by the KCTU. Data were hosted on a dedicated secure server within KCL, and all source data were entered into the eCRF by authorised staff with a full audit trail. Trial data may be obtained from the corresponding author on request.

Safety

Adverse events and side effects were recorded in the eCRF after randomisation and then throughout the study, regardless of their severity or relation to study participation. As a precaution against systemic allergic reactions, all participants were observed after the first intradermal injection for 1 hour and, if there was no systemic reaction, for 30 minutes after subsequent injections. In the event of a participant experiencing a grade 1 reaction, the clinical observation period for that individual was maintained at 1 hour after subsequent injections.

The following AEs were anticipated and not reported:

1. symptoms attributable to aeroallergen exposure: that is, nasal blockage, rhinorrhoea, itching or sneezing; itching, watering, redness or swelling of eyes; itching or dryness of mouth/throat; breathless, cough, wheeze and chest tightness
2. transient discomfort from intradermal injections
3. appearance of an itchy oedematous wheal, with surrounding erythema, after intradermal injection
4. appearance of swelling (oedema) within hours of intradermal injection
5. temporary discomfort, bleeding, bruising, swelling at the needle site following venesection
6. mild localised itching arising from skin prick testing during screening.

Withdrawal criteria and stopping rules

The prespecified criteria for discontinuation of the study therapy (active or control) were as follows.

1. Inability or failure to attend for intervention within 3 weeks of previous administration.
2. Inability or failure to receive seven or eight injections within the dates specified.
3. Two grade 2 systemic reactions, or a single systemic reaction of grade 3 or above after administration of study therapy. Systemic reactions were graded according to the WAO criteria:
 - *Grade 1* Symptoms of 1 organ system (cutaneous, upper respiratory tract, conjunctival, gastrointestinal, other).
 - *Grade 2* Symptoms of more than one organ system present or asthma symptoms/signs [cough, wheezing, shortness of breath but, < 40% drop in peak expiratory flow (PEF) or FEV₁].
 - *Grade 3* Asthma symptoms/signs (with ≥ 40% drop in PEF or FEV₁), upper respiratory tract (laryngeal, uvula, tongue) oedema with or without stridor.
 - *Grade 4* Respiratory failure or hypotension with or without loss of consciousness.
4. An AE that, in the judgement of the principal investigator or the medical monitor, presented an unacceptable consequence or risk to the participant.
5. An illness or infection not associated with the condition under study and which required treatment that was not consistent with protocol requirements or if a participant developed an intercurrent illness that, in the judgement of the principal investigator, in any way justified discontinuation.
6. An inability or unwillingness to comply with the study protocol, with the protocol deviations being sufficient to jeopardise the participant's well-being or the integrity of the study.
7. Pregnancy occurring during study participation.

Predefined study-stopping rules included the occurrence of five grade 3 reactions or a single grade 4 reaction.

Concomitant medications

Rescue medications were provided to participants before and throughout the pollen season. These included: desloratadine (5 mg, up to one tablet daily), olopatadine eye drops (1.0 mg/ml, up to one drop per eye twice daily), fluticasone propionate nasal spray (50 µg per spray, up to two sprays per nostril once daily) and prednisolone (for use at 30 mg per day for up to 5 days). Participants were asked to use only these medications to treat their hay fever symptoms on an 'as required' basis. However, participants who were not experiencing hay fever symptoms were encouraged to try not to use these medications. Participants were asked to use only these medications. A short course of prednisolone was made available for severe symptoms, although participants were instructed to contact a trial doctor prior to starting this treatment. Concurrent treatment with beta-blockers, calcium channel blockers, tricyclic antidepressant drugs, monoamine oxidase inhibitors or anti-IgE monoclonal antibody (mAb) were not permitted.

Measurement of skin early- and late-phase responses

All participants underwent intradermal skin challenge testing 4 months after the final intradermal allergen immunotherapy or control injection (September 2013). Participants were then randomised to undergo a repeat follow-up test at either 7, 10 or 13 months later to assess persistence of late-response suppression by comparing late-phase response sizes in those who had received active intradermal immunotherapy with those who had received the control intervention. The procedure for the intradermal skin challenge testing and the dose of allergen used were identical to that for an active intradermal allergen immunotherapy injection. In brief, grass pollen extract (10 BU, equivalent to 33.3 SQ-U, of *P. pratense* Aquagen ALK Abelló) in a 20-µl volume of allergen diluent was injected intradermally into the extensor aspect of each

forearm. A negative control injection of 20 µl of diluent was injected into the contralateral forearm. Although the trial was not unblinded at this stage, these intradermal injections were performed open label. Participants were asked to refrain from taking antihistamines or oral steroids for a minimum of 5 days and 2 weeks beforehand, respectively. Early phase responses were measured 15 minutes after the intradermal injection. The wheal outline was traced and transferred into the patient record. Late-phase responses were measured after 24 hours by palpating the outline of oedema. The areas of the late response was also traced and transferred to the patient record. A single clinician performed all measurements under double-blind conditions. The early- and late-phase response areas were calculated from scaled scanned images of the tracings with NIS Elements v4.2 software (Nikon Instruments, Surrey, UK). Early- and late-phase response areas were then compared in the intradermal immunotherapy and control arms at each time point.

Skin biopsy

Forty participants (20 in each trial arm) were randomised to undergo 3-mm skin punch biopsies immediately after measurement of late-phase responses (i.e. 24 hours after challenge), 4 months after the final treatment injection, in September 2013. Biopsies were collected from both allergen-challenged and diluent control sites. Local anaesthesia was achieved with 10 mg/ml of lidocaine hydrochloride with 5 µg/ml of adrenaline (1 in 200,000). In the first 20 subjects, biopsies were divided with a scalpel into two pieces and one half piece was fixed in 4% paraformaldehyde (Sigma-Aldrich, Poole, UK) for 2 hours. In the rest of the subjects, entire biopsies were processed for immunohistochemistry by fixation in 4% paraformaldehyde at room temperature for 4 hours. After washing twice in 15% sucrose, biopsies were mounted in Optimal Cutting Temperature compound (OCT) embedding medium (Bayer UK Ltd, Basingstoke, UK) and stored at –80 °C pending analysis. The remaining unfixed half-biopsy pieces were cultured directly for T-cell analysis.

Analysis of T cells cultured from skin biopsies

Skin biopsy tissue was finely dissected and suspended in complete medium [Roswell Park Memorial Institute Medium, Sigma-Aldrich® (RPMI) supplemented with 10% foetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and l-glutamine (2 mM); all Life Technologies, Warrington, UK]. Tissue was then cultured at 37 °C in a humidified atmosphere containing 5% carbon dioxide in the presence of interleukin 2 (IL-2; 50 U/ml). After 3–4 days, cells were passed through a 0.2-µm cell strainer to obtain single cell suspensions, and restimulated with immobilised anti-cluster of differentiation 3 (CD3)/cluster of differentiation 28 antibodies for a further 3 days, followed by expansion for 4 days in the presence of IL-2. Expanded T cells were stained with the viability dye eFluor®780 (eBioscience, Vienna, Austria) prior to surface staining with anti-cluster of differentiation 4 (CD4) PerCP-Cy5.5 (BioLegend, London, UK), anti-cluster of differentiation 8 BV510 (BD Biosciences, Oxford, UK), anti-CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) PE (BioLegend), anti-CXCR3 [chemokine (C-X-C Motif) receptor 3] BV421 (BioLegend), anti-chemokine receptor 6 (CCR6) PE-Cy7 (BD Biosciences) and anti-interleukin 25 receptor AF647 (kind gift of Dr Andrew McKenzie). Samples were resuspended for flow cytometric analysis (FACSCalibur™, BD Biosciences). Data were analysed using FlowJo™ v7.6 software (Tree Star Inc., Ashland, OR, USA). For microarray studies, cells were activated for 4 hours with ionomycin (0.5 µg/ml) and phorbol 12-myristate 13-acetate (5 ng/ml) (both Sigma-Aldrich). Ribonucleic acid (RNA) was isolated from cell pellets using the miRNeasy Mini Kit and RNeasy MinElute Cleanup Kit (both Qiagen, Manchester, UK) in accordance with the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) synthesis and amplification were performed with the Ovation PicoSL WTA Systems V2 kit (NuGEN, Leek, the Netherlands) as per the manufacturer's instructions. Purity and yield was then analysed using the Bioanalyzer Platform (Agilent, Stockport, UK) and NanoDrop 2000 spectrophotometer (Thermo Scientific, Loughborough, UK), respectively, before amplified cDNA was biotin-labelled with the NuGEN Encore BiotinIL Module according to the manufacturer's instructions. Biotin-labelled cDNA was hybridised to an Illumina HumanHT-12 v4 Expression BeadChip (Illumina, Saffron Walden, UK) before scanning with the

iScan System (Illumina) utilising GenomeStudio software. Data analysis was performed with the Partek Genomics Suite™ software (Partek Inc., Chesterfield, MO, USA).

Immunohistochemistry

Immunohistochemical staining of skin biopsies was performed using the modified alkaline phosphatase anti-alkaline phosphatase (APAAP) method to stain for eosinophils, neutrophils, CD4⁺ T cells and CD3-positive (CD3⁺) T cells.^{24,25} In brief, 8- to 10- μ m thickness tissue sections were air dried overnight on poly-L-lysine-coated slides. For immunostaining, slides were incubated at room temperature in a humidified chamber with the primary mouse mAb [neutrophil elastase (Dako, Ely, UK); eosinophil major basic protein (Abcam, Cambridge, UK); CD3 and CD4, both Dako] suspended in 5% human serum/phosphate-buffered saline (PBS) for predetermined optimised incubation times. Sections were then washed in PBS and incubated with rabbit anti-mouse immunoglobulin (Dako) for 30 minutes then washed again. Slides were then incubated with a third layer of soluble complexes of alkaline phosphatase (AP) and mouse anti-APAAP (Serotec, Kidlington, UK) for 30 minutes, washed and developed with Fast Red (Sigma-Aldrich) for a further 20 minutes. Sections were washed extensively in PBS before counterstaining with Harris haematoxylin (BDH, Poole, UK) and mounting in glycerol gel. For negative controls, each primary antibody was substituted with the appropriate isotype-matched irrelevant mAb. Slides were counted blind in random order by two observers. Allergen and diluent biopsy sections were evaluated from each subject. The total number of positive cells was expressed as the number of cells per square millimetre of biopsy. Interobserver variability was 7%, assessed on repeat counts of 19 slides. The difference between the two counts was plotted against the mean of the two counts; all but one of the differences fell within two standard deviations (SDs) of the mean difference, indicating satisfactory agreement between observers.

Serum antibody measurements

Sera were analysed for concentrations of pre- and post-treatment *P. pratense*-specific IgG, IgG4 and IgE, and IgE specific to the major allergens Phl p 5 and Phl p 1 using a commercial assay system (ImmunoCAP™, ThermoFisher Scientific, Horsham, UK) in accordance with the manufacturer's instructions.

Basophil activation tests

Basophil activation tests were performed in 92 participants following administration of the final intradermal allergen immunotherapy or control injection (May 2013). Whole blood was collected and tested within 2 hours of sampling under blinded conditions by a single investigator. Heparinised whole blood was immunostained with anti-human CD3 PE-Cy7 (BD Biosciences), CD294 PE (Miltenyi Biotec, Woking, UK), CD203c PerCP-Cy5.5 (BioLegend), CD303 APC (Miltenyi Biotec), CD107a Brilliant Violet 421 (BioLegend), CD63 FITC (BioLegend) and isotype controls. Basophils were then stimulated with anti-human IgE (1000 ng/ml, positive control; Abcam) or *P. Pratense* extract (ALK Abelló) at 10 ng/ml and 100 ng/ml for 15 minutes at 37 °C. Samples were then lysed (BD FACS Lysing Solution, BD Biosciences), washed and resuspended (CellFIX™, BD Biosciences) for flow cytometric analysis (FACSCalibur™, BD Biosciences). Data were analysed using FlowJo™ v7.6 software (Tree Star), gating on CD3-CD303-CD294⁺ basophils. Basophil activation was expressed as the percentage of CD63⁺, CD203c⁺ or CD107a⁺ basophils of the entire basophil population, and compared between the two groups.

Statistical analysis

Sample size calculations for the primary outcome (CSMS) were performed, based on raw data from a previous clinical trial of subcutaneous grass pollen immunotherapy.²⁶ The power calculation was

conservatively based on the detection of a clinical effect size of 80% of that reported in that trial. Using this method and a two-sided non-parametric test based on a Monte Carlo approach, group sample sizes of 35 and 35 achieved 90% power to detect such a difference in AUC of the CSMSs at a significance level of 0.05. To make allowance for the unknown distribution of the primary outcome, and based on the lower bound for the asymptotic relative efficiency of the Mann–Whitney *U*-test, the sample size was increased by a further 15% to 40 in each arm. Further accounting for a post-randomisation dropout rate of up to 10%, consistent with previous trials of grass pollen immunotherapy, a total sample size of 90 (45 each arm) was estimated as required.

The statistical analysis plan was finalised and agreed before any analysis was undertaken (see *Appendix 6*). Statistical analyses were performed on an intention-to-treat (ITT) basis, with data from all participants who could be assessed for the primary outcome. Summary measures for the baseline characteristics of each group were calculated as mean and SD for continuous (approximate) normally distributed variables, medians and interquartile ranges (IQRs) for non-normally distributed variables, and frequencies and percentages for categorical variables. The AUC of the CSMSs was plotted against time as a summary measure of the primary outcome. The primary efficacy analysis, that is, the difference between the two arms in AUC of the CSMSs, was analysed on randomised patients using a stratified Mann–Whitney *U*-test (van Elteren test), adjusted for the baseline stratification factors of size of the skin test to grass pollen, and presence or absence of rhinitis symptoms outside the grass pollen season. Median differences between the groups were calculated using the stratified Hodges–Lehmann method. Similar analyses were conducted for symptom scores, medication scores, symptoms in different organs and VAS scores. Linear mixed models were used to evaluate Mini-RQLQ and EQ-5D-5L scores in order to isolate the effect of the intervention on each arm after adjusting for stratification factors. Differences between the groups were reported with their 95% confidence intervals (CIs). All mechanistic between-group comparisons were performed by Mann–Whitney *U*-test, with the exception of serology and immunohistochemistry comparisons, which were analysed by analysis of covariance (ANCOVA). Comparisons of serology between pre and post treatment, and skin biopsy immunohistochemistry between diluent control and allergen challenge were made by Wilcoxon signed-rank test.

The primary outcome and secondary outcomes are reported in the ITT population without imputation of missing data. However, a sensitivity analysis was also performed, with missing data imputed for the primary outcome and secondary outcomes in the ITT population. A multiple imputation technique was applied, whereby missing data on a particular date were substituted with the mean CSMS on that date in the corresponding trial arm. Further sensitivity analyses were undertaken for the primary outcome and secondary outcomes in the predefined per-protocol population. Participants who were on holiday outside continental Europe during the daily collection period were considered as ‘missing data’ for the days concerned, in accordance with the Trial Steering Committee and statistical analysis plan. When > 50% of the data were missing, participants were excluded from the per-protocol analysis.

The principal software package was SAS/STAT® version 9.2 (SAS Institute Inc., Cary, NC, USA), with verification of results from syntax for selected analyses analysed in Stata® version 12.1 (StataCorp LP, College Station, TX, USA).

Chapter 3 Results

Study population

From 1660 people who completed initial online prescreening, 150 potential participants attended the Clinical Research Facility for full screening. Of these, 93 were enrolled and randomised to receive intradermal allergen or histamine control injections between 18 February and 1 March 2013 (*Figure 2*). Study arms were well balanced for baseline characteristics (*Table 1*). All 46 participants assigned to intradermal allergen immunotherapy completed the seven-injection treatment course, although one participant deviated from the administration schedule by 1 day for one injection. Of the 47 participants who were assigned to control injections, one did not complete the treatment course, withdrawing after the second injection because of work commitments, and another participant deviated from the administration schedule by 4 days because of an unrelated upper respiratory tract infection that necessitated postponement of the injection. There was a high rate of diary card data collection for the primary outcome: 99% of participants supplied > 50% of data for all days; 96% of participants supplied > 75% of daily data; and 94% of participants supplied > 90% of daily data. One patient holidayed outside continental Europe for 52% of the data collection period, so was excluded from the per-protocol analysis, in accordance with the predefined statistical analysis plan. Five participants, all in the control arm, significantly deviated from use of the rescue medications that were specified in the trial protocol according to criteria specified prior to unblinding. Participants were unable to identify if they had received active intradermal allergen treatment or histamine control (*Table 2*).

Clinical outcomes

All 93 randomised participants were evaluated for the primary outcome and were included in the ITT analysis. The CSMS showed a clear correlation with daily pollen counts in London (*Figure 3*), which peaked at levels in the above-average range. However, intradermal immunotherapy did not significantly affect the primary end point, that is, the CSMS over the whole grass pollen season (difference in median AUC = 14; 95% CI -172.5 to 215.1; $p = 0.80$) (see *Figure 3* and *Table 3*). Furthermore, significant differences were not seen between the trial arms in the secondary end points of overall symptom scores or rescue medication use ($p = 0.44$) during the whole season, or the CSMSs during peak season (12 June to 26 July 2013) ($p = 0.99$; see *Table 3*).

Among other prespecified secondary end points, allergic rhinitis symptoms, measured by daily nasal symptom scores, were significantly higher in the intradermal allergen immunotherapy group than in the histamine control group, with a difference in median AUC of 35 (95% CI 4.0 to 67.5; $p = 0.03$) (see *Table 3* and *Figure 4*). Furthermore, there was a trend for rhinitis symptoms measured by VAS to be higher in the arm that received intradermal immunotherapy group, with a difference in median AUC of 53 (95% CI -11.6 to 125.2; $p = 0.05$) (see *Table 3* and *Figure 4*). No significant differences were seen between groups in daily eye or lung symptoms (see *Table 3*), although there was a trend for mouth symptoms to be higher in the intradermal allergen group (difference in median AUC 10.0; 95% CI -3.8 to 24; $p = 0.05$). No significant group differences were observed in eye symptoms measured by VASs, Mini-RQLQ scores, EQ-5D-5L scores, global evaluation of symptoms scores, numbers of symptom-free or medication-free days or number of days during which prednisolone was used as rescue medication (see *Table 3*). Analysis of the ITT population after imputation of missing data values gave results that were consistent with the main ITT analysis (see *Appendix 7*). The per-protocol analysis included 45 participants who received intradermal allergen immunotherapy and 39 who received the histamine control treatment (see *Appendix 8*). In this population, daily individual nasal ($p = 0.05$) and mouth symptoms ($p = 0.02$) were also higher in the actively treated group, with a trend for worse lung symptoms ($p = 0.05$). Participants in

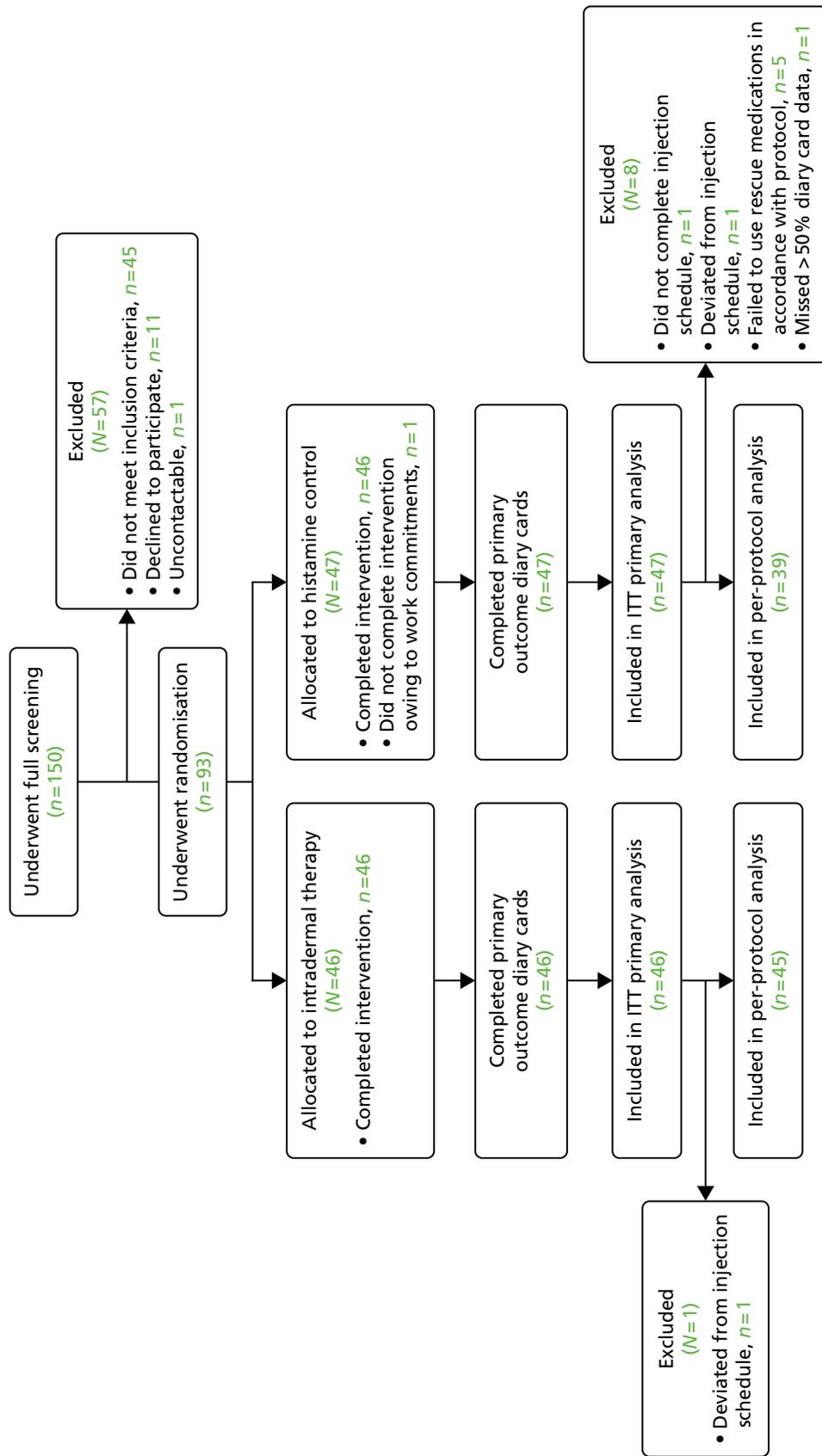


FIGURE 2 Consolidated Standards of Reporting Trials diagram. All randomised participants were included in the ITT analysis. Only participants who adequately adhered to treatment and rescue medications were included in the per-protocol analysis.

TABLE 1 Baseline characteristics of study participants

Baseline characteristic	Intradermal immunotherapy (N = 46)	Control (N = 47)
Age at screening (years), mean (SD)	32 (9.9)	35 (10.8)
Female sex, n (%)	19 (41)	12 (26)
Ethnicity, n (%)		
White	37 (80)	37 (79)
Mixed	3 (7)	2 (4)
Asian	4 (9)	3 (6)
Black	0 (0)	3 (6)
Other	2 (4)	2 (4)
Allergy symptoms outside grass pollen season, n (%)	16 (35)	18 (38)
Total IgE (kU _L /l), median (IQR)	160 (80–263)	121 (64–255)
<i>P. pratense</i> -specific IgE (kU _L /l), median (IQR)	22 (9–49)	27 (10–54)
<i>P. pratense</i> SPT wheal diameter (mm), mean (SD)	11 (5.0)	12 (4.2)
SPT positive, n (%)		
Timothy grass	46 (100)	47 (100)
Mixed grass	46 (100)	47 (100)
Silver birch	24 (52)	19 (40)
Mugwort	9 (20)	11 (23)
House dust mite	24 (52)	28 (60)
Cat	18 (39)	24 (51)
Dog	36 (78)	41 (87)
Horse	6 (13)	4 (9)
<i>Aspergillus</i>	2 (4)	1 (2)
<i>Alternaria</i>	7 (15)	6 (13)
<i>Cladosporium</i>	2 (4)	2 (4)
Vital signs		
Pulse rate (b.p.m.), mean (SD)	72 (10.9)	69 (9.6)
Blood pressure – systolic (mmHg), mean (SD)	133 (15.5)	137 (12.5)
Blood pressure – diastolic (mmHg), mean (SD)	80 (9.6)	81 (9.4)
Spirometry		
FEV ₁ (l), mean (SD)	4 (0.9)	4 (0.7)
FVC (l), mean (SD)	5 (1.2)	5 (1.0)
FEV ₁ % predicted spirometry, mean (SD)	101 (10.8)	101 (11.2)
Allergy history, n (%)		
Asthma (controlled with salbutamol)	15 (33)	17 (36)
Urticaria	13 (28)	16 (34)
Eczema	14 (30)	7 (15)
Food allergy	6 (13)	5 (11)
Drug allergy	5 (11)	5 (11)
Insect allergy	2 (4)	3 (6)

continued

TABLE 1 Baseline characteristics of study participants (*continued*)

Baseline characteristic	Intradermal immunotherapy (N = 46)	Control (N = 47)
Medical history, n (%)		
Respiratory	10 (22)	10 (21)
Dermatology	9 (20)	11 (23)
Musculoskeletal	3 (7)	9 (19)
Gastrointestinal	6 (13)	3 (6)
Genitourinary	5 (11)	4 (9)
Neurological	1 (2)	6 (13)
ENT	4 (9)	3 (6)
Psychiatric	3 (7)	2 (4)
Haematological	1 (2)	3 (6)
Cardiovascular	2 (4)	1 (2)
Hepatic	1 (2)	1 (2)
Endocrine	1 (2)	1 (2)
Neoplasia	2 (4)	0 (0)
Immunological	1 (2)	0 (0)
Infection	1 (2)	0 (0)
Other	3 (7)	2 (4)

b.p.m., beats per minute; ENT, ear, nose and throat; FVC, forced vital capacity.

TABLE 2 Verification of participant blinding

Patient guess trial arm	Trial arm	
	Intradermal immunotherapy (N = 44)	Control (N = 43)
Intradermal immunotherapy	22	22
Control	22	21

Note

At the end of the pollen season, participants verified blinding by guessing if they had received active or control treatment.

the intradermal allergen group in this population also had significantly worse nasal symptoms measured by VAS ($p = 0.01$) and recorded fewer symptom-free days than subjects in the control group ($p = 0.04$).

Given the unexpected observation that allergic rhinitis nasal symptom scores were higher in participants who had received intradermal allergen immunotherapy, a post hoc analysis was performed to compare the daily data for each individual allergic symptom in the two trial arms (see *Appendix 9*). Sneezing ($p = 0.01$) and cough scores ($p = 0.03$) were both significantly higher in the intradermal immunotherapy group, with non-significant trends for greater chest tightness ($p = 0.08$) and mouth itching ($p = 0.06$). In contrast, eye swelling was lower in the intradermal immunotherapy group ($p = 0.03$). Further post hoc analysis of individual nasal symptoms measured by VAS also revealed higher scores after intradermal immunotherapy for running ($p = 0.006$), sneezing ($p = 0.006$) and itching ($p = 0.003$) (see *Appendix 10*).

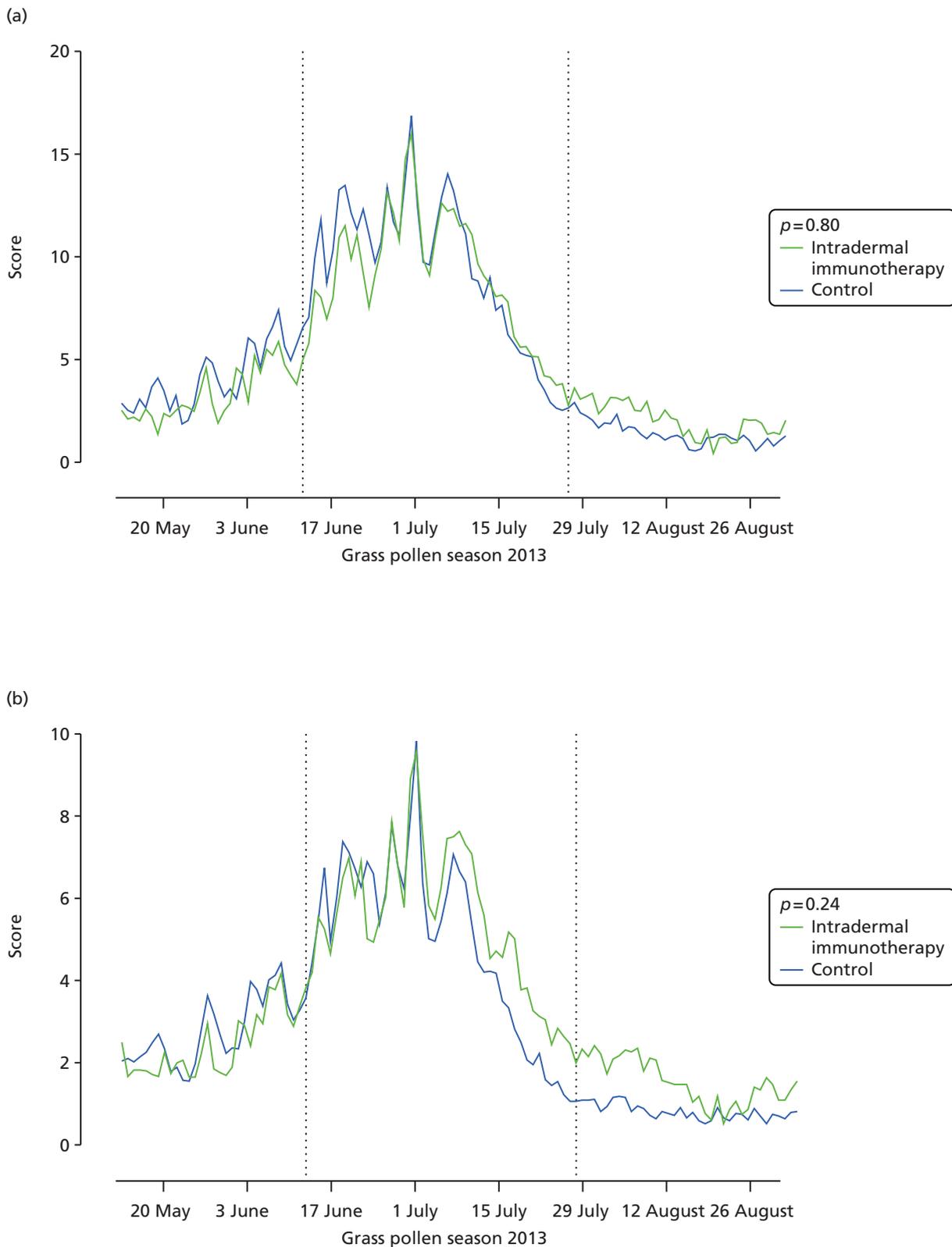


FIGURE 3 Primary outcome and daily symptom and daily medication scores in the primary intention-to-treat analysis. (a) Mean daily combined symptom and medication; and (b) median daily symptom scores (sum of scores for nose, eyes, lungs, mouth according to treatment arm). The p -values are based on (c) mean medication scores; and (d) daily grass pollen counts in central London over the 2013 grass pollen season. AUC values for each participant were compared Mann–Whitney U -tests. Broken vertical lines indicate the beginning and end of the peak pollen season (12 June to 26 July 2013). (*continued*)

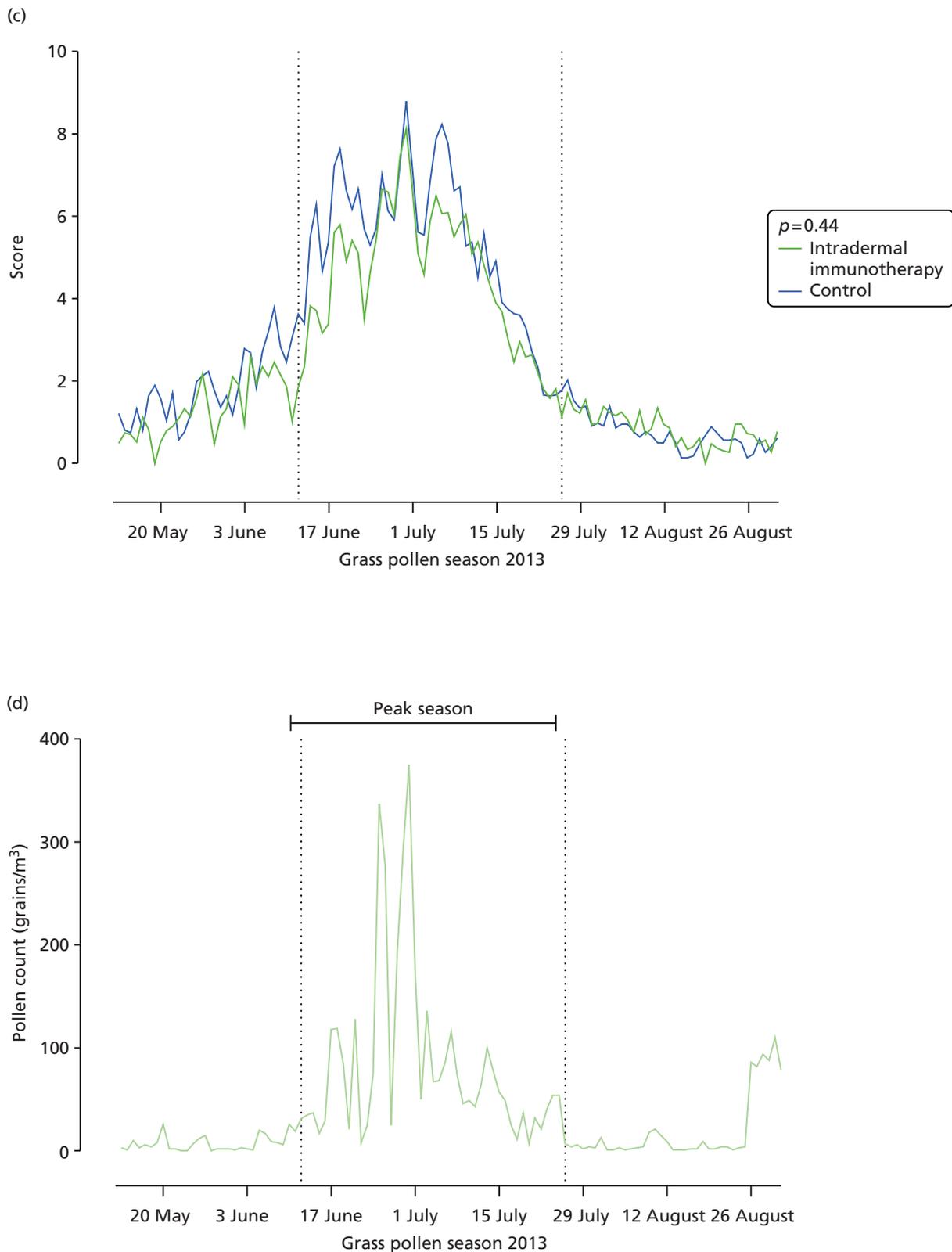


FIGURE 3 Primary outcome and daily symptom and daily medication scores in the primary intention-to-treat analysis. (a) Mean daily combined symptom and medication; and (b) median daily symptom scores (sum of scores for nose, eyes, lungs, mouth according to treatment arm). The p -values are based on (c) mean medication scores; and (d) daily grass pollen counts in central London over the 2013 grass pollen season. AUC values for each participant were compared Mann–Whitney U -tests. Broken vertical lines indicate the beginning and end of the peak pollen season (12 June to 26 July 2013).

TABLE 3 Effect of intradermal immunotherapy on primary and secondary outcomes (ITT)

Trial outcomes	Intradermal immunotherapy (<i>n</i> = 46), median (IQR)	Control (<i>n</i> = 47), median (IQR)	Difference (95% CI)	<i>p</i> -value
Primary outcome				
CSMS during entire season	502 (333–841)	487 (365–717)	14 (–172.5 to 215.1)	0.80
Secondary outcomes				
Symptom score during entire season	335 (183–503)	264 (156–398)	59 (–1.3 to 110.9)	0.24
Medication score during entire season	242 (116–405)	263 (129–482)	–19 (–153.0 to 100.2)	0.44
CSMS score during peak season	356 (232–521)	365 (278–508)	–8 (–75.8 to 66.3)	0.90
Nasal symptom score during entire season	174 (120–207)	121 (81–200)	35 (4.0 to 67.5)	0.03
Mouth symptom score during entire season	34 (8–90)	14 (5–45)	10 (3.8 to 24)	0.05
Eye symptom score during entire season	79 (41–153)	78 (52–180)	–7 (–18.5 to 2.9)	0.54
Lung symptom score during entire season	17 (3–32)	12 (0–34)	4 (–1 to 15)	0.17
Nasal allergic symptoms measured by VAS	156 (104–275)	122 (54–184)	53 (–11.6 to 125.2)	0.05
Eye allergic symptoms measured by VAS	84 (32–197)	144 (41–176)	–3 (–46.0 to 35.8)	0.40
Global evaluation of symptom scores	3 (2–4)	3 (1–4)	0 (0 to 1)	0.48
Symptom-free days	35 (19–53)	41 (23–61)	–6 (–17 to 3)	0.15
Number of days prednisolone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	0.36
Medication-free days	81 (65–93)	76 (65–94)	4 (–11 to 21)	0.22
Mini-RQLQ	16 (13–23)	18 (10–25)	–0.3 (–4.2 to 3.7)	0.89
EQ-5D-5L	87 (83–94)	88 (81–94)	9 (–24.8 to 43.6)	0.59

Notes

Data for primary outcome and all symptom scores represent AUC values.

Median difference between groups is calculated by stratified Hodges–Lehmann method.

The *p*-values are based on a stratified Mann–Whitney *U*-test (van Elteren's test), adjusted for stratification factors.

The *p*-values for Mini-RQLQ and EQ-5D-5L are based on a linear mixed model, adjusted for stratification factors.

Entire grass pollen season 13 May to 3 August 2013; peak season 12 June to 26 July 2013.

Safety

There was a low rate of AEs that were related to treatment (*Table 4*). There were three serious AEs, although all were unrelated to treatment. One participant in the intradermal allergen immunotherapy group was hospitalised for severe tonsillitis. One control arm participant was admitted for overnight polysomnography, and another control participant required treatment to remove an infected dental plate. There were no deaths during the study. Three participants in the intradermal immunotherapy group and six in the control group were recorded with treatment-related AEs – all mild grade 1 systematic reactions. These reactions manifested as generalised pruritus without wheals, except for one intradermal allergen participant who developed erythema, which tracked from the injection site in a lymphatic distribution (IgE-mediated lymphangitis) approximately 20 minutes after every intradermal injection.

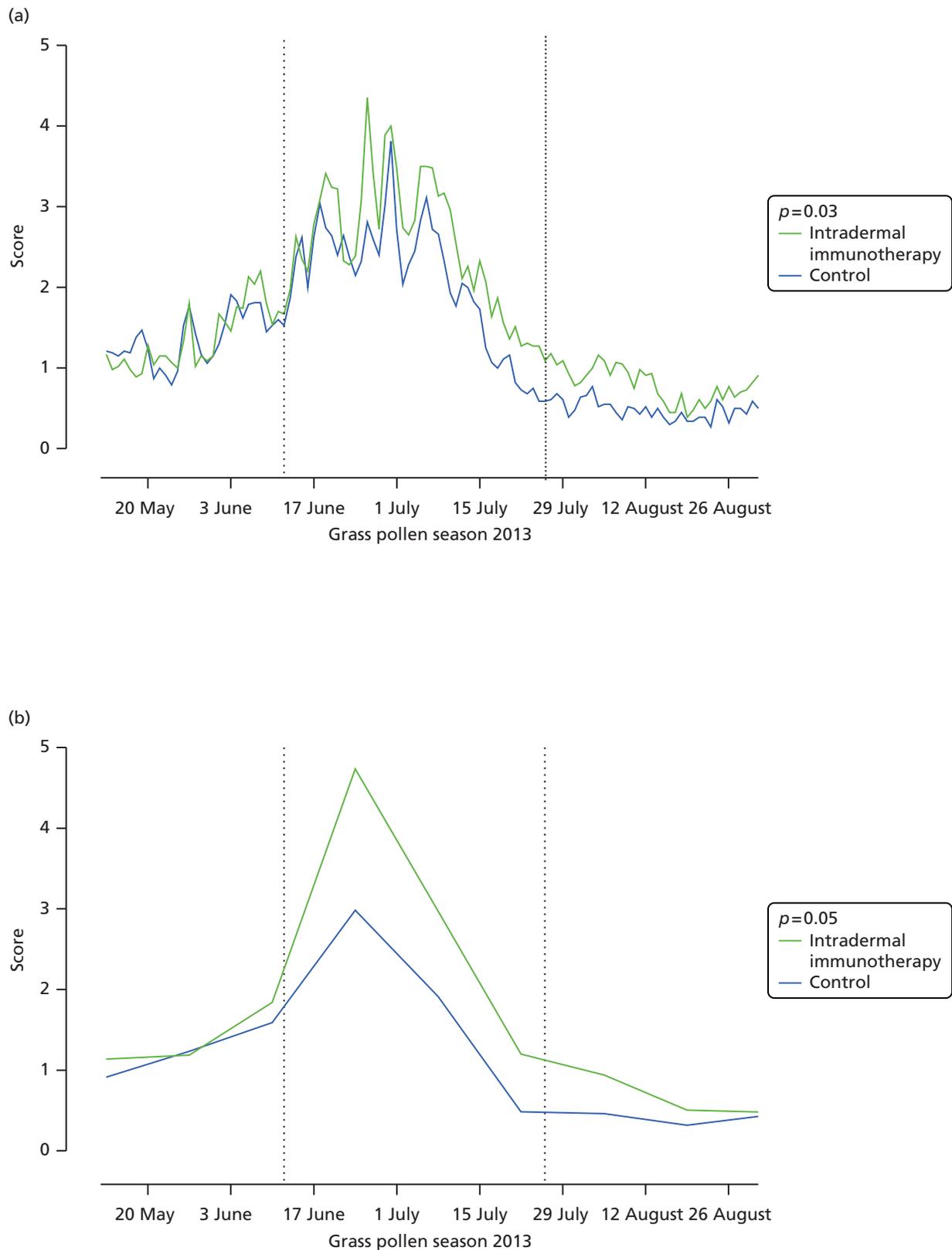


FIGURE 4 Nasal symptoms. (a) Mean daily nasal symptom scores (sum of scores for sneezing, blockage and running); and (b) mean nasal symptoms measured by VAS (total of blockage, running, itching and sneezing). AUC values for each participant were compared according to treatment arm. The p -values are based on Mann-Whitney U -tests.

TABLE 4 Frequency of AEs reported from first intradermal allergen immunotherapy or control injection until end of pollen season

Adverse events/reactions	Intradermal immunotherapy (n = 46)				Control (n = 47)				p-value	
	Number of participants with ≥ 1 AEs	% participants	Number of events	% event rate	Number of participants with ≥ 1 AEs	% participants	Number of events	% event rate		
Any AEs	40	87.0	148		42	89.4	145		0.76	
Serious AEs	1	2.2	1	0.7	2	4.3	2	1.4	1.00	0.62
Tonsillitis	1	2.2	1	0.7	0	0.0	0	0.0	0.49	1.00
Overnight stay – polysomnography	0	0.0	0	0.0	1	2.1	1	0.7	1.00	0.49
Extraction of infected dental plate	0	0.0	0	0.0	1	2.1	1	0.7	1.00	0.49
Relation of AE to treatment										
Definite/probable	3	6.5	15	10	6	12.8	14	9.7	0.49	0.89
Possible	0	0.0	0	0.0	0	0.0	0	0.0	1.00	1.00
Remote	30	65.2	68	46	34	72.3	70	48.3	0.46	0.69
None	32	69.6	65	44	34	72.3	61	42.1	0.77	0.75
AE withdrawals	0	0.0	0	0.0	0	0.0	0	0.0	1.00	1.00
Systemic adverse reactions	3	6.5	15	10.0	6	12.8	13	9.0	0.49	0.73
Generalised pruritus	2	4.3	8	5.4	4	8.5	9	6.2	0.68	0.77
IgE-mediated lymphangitis	1	2.2	7	4.7	0	0.0	0	0.0	0.49	0.01
Light-headedness	0	0.0	0	0.0	2	4.3	2	1.4	0.49	0.24
Facial flushing/feeling hot	0	0.0	0	0.0	2	4.3	3	2.1	0.49	0.12
Systemic adverse reactions^a										
Grade 1	3	6.5	15	10	6	12.8	12	8.3	0.49	0.58
Grade 2	0	0.0	0	0.0	0	0.0	0	0.0	1.00	1.00
Grade 3	0	0.0	0	0.0	0	0.0	0	0.0	1.00	1.00
Grade 4	0	0.0	0	0.0	0	0.0	0	0.0	1.00	1.00

^a Classified using the WAO grading system for systemic reactions to subcutaneous immunotherapy.²⁷

Note

Statistical comparison was by Fisher's exact test for five or more events and chi-squared test for fewer than five events.

Immunological end points

Serum immunoglobulins specific for whole *P. pratense* (Timothy grass) and major Timothy grass allergens Phl p 1 and Phl p 5 were compared before (between October 2012 and January 2013) and after (May 2013) intradermal allergen or control injection therapy. In the histamine control arm, there was a typical small seasonal decline in allergen-specific IgE antibodies (all $p < 0.001$; Figure 5). This seasonal decline in IgE was, however, significantly less in the intradermal allergen immunotherapy group than the control group (all $p = 0.001$), indicating that intradermal allergen treatment stimulated allergen-specific IgE production. A treatment effect was also seen on *P. pratense*-specific IgG titres, which fell in the control but not the intradermal allergen group over the same period ($p = 0.03$; Figure 6), although no effect was seen on IgG4 responses.

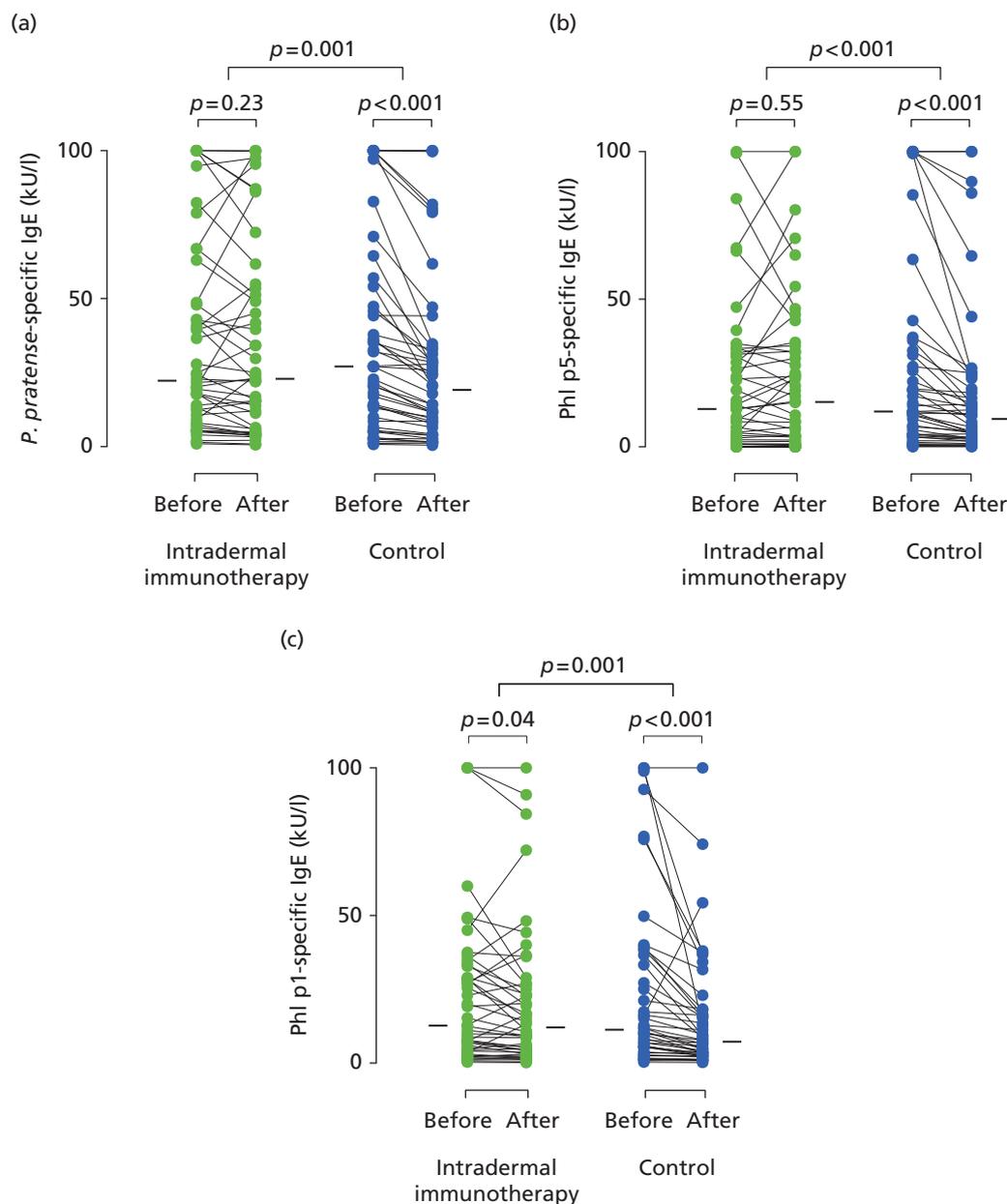


FIGURE 5 Immunological outcomes. Levels of (a) *P. pratense*-specific IgE; (b) Phl p5-specific IgE; and (c) Phl p1-specific IgE before and after completion of seven intradermal allergen or histamine control injections. Solid bars represent median values. The p -values for pre- and post-treatment serology comparisons are based on the Wilcoxon signed-rank test. The p -values for between-group IgE comparisons are based on ANCOVA.

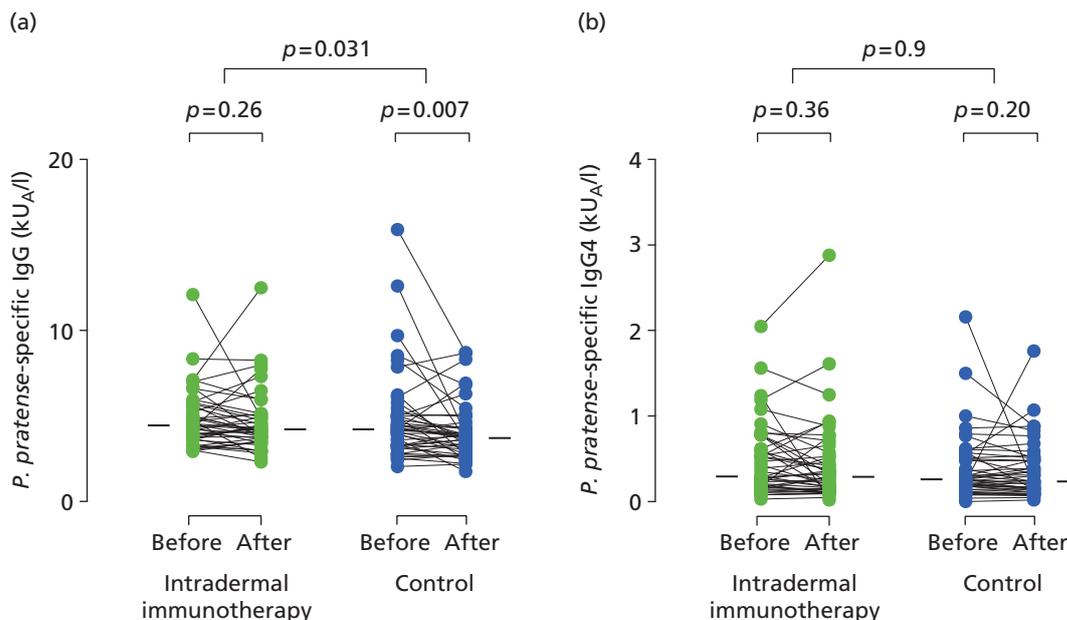


FIGURE 6 Immunological outcomes. Levels of (a) *P. pratense*-specific IgG; and (b) *P. pratense*-specific IgG4 before and after completion of seven intradermal allergen or histamine control injections. The p -values for pre- and post-treatment serology comparisons are based on the Wilcoxon signed-rank test. The p -values for between-group IgG comparisons are based on ANCOVA.

For surface phenotype analysis, CD4⁺ T cells were successfully expanded from 19 of 20 skin biopsies (10 from the intradermal immunotherapy group and nine from the control group) collected 24 hours after an intradermal grass pollen challenge, at the end of the 2013 grass pollen season. Cutaneous CD4⁺ T cells derived from grass pollen challenged sites showed higher expression of Th2 surface marker CRTH2 in the intradermal allergen immunotherapy group (median 13.4%, IQR 6.3–25.4) than the control group (median 6.3%, IQR 1.9–7.6; $p = 0.04$), whereas expression of the T helper type 1 cell (Th1) marker CXCR3 was lower in the intradermal allergen immunotherapy group [median 33.5 (IQR 24.7–47.3) vs. median 56 (IQR 45.8–63.8); $p = 0.01$] (Figure 7). No differences were seen in the expression of T helper type 17 cell marker CCR6 or the interleukin 25 receptor (not shown). Insufficient T cells could be expanded from diluent-challenged skin biopsies for analysis. Microarray transcriptional profiling was performed on cultured T cells that were derived from 15 allergen-challenged skin biopsies (seven intradermal allergen treatment and eight control arm subjects). Only 14 genes were significantly overexpressed by skin T cells in the intradermal allergen immunotherapy group [defined as > 1.5-fold higher expression than in control group and $p < 0.05$ using a three-way analysis of variance (ANOVA) model] including the Th2 cytokine interleukin 5 (IL-5) ($p = 0.03$) (Table 5; Microarray Gene Expression Omnibus accession number GSE72324).

Immunohistochemistry performed on the entire 40 diluent- and 40 allergen-challenged skin biopsies (20 intradermal allergen treatment and 20 control arm subjects) showed grass pollen-induced recruitment of eosinophils, neutrophils, CD3⁺ T cells and CD4⁺ T cells, but no significant treatment effect (Figure 8). Furthermore, no significant treatment effect was seen on surface expression of peripheral blood basophil activation markers (Figure 9).

Intradermal skin challenge responses

Early- (15 minutes) and late-phase (24 hours) skin responses could be measured in 86 participants 4 months after the final treatment injection in September 2013, and the measurements were repeated at either 7, 10 or 13 months (Figure 10a). The size of late-phase responses in the control group was consistent with that previously reported under the same conditions⁹ (shown for comparison in Figure 10). Late-phase responses

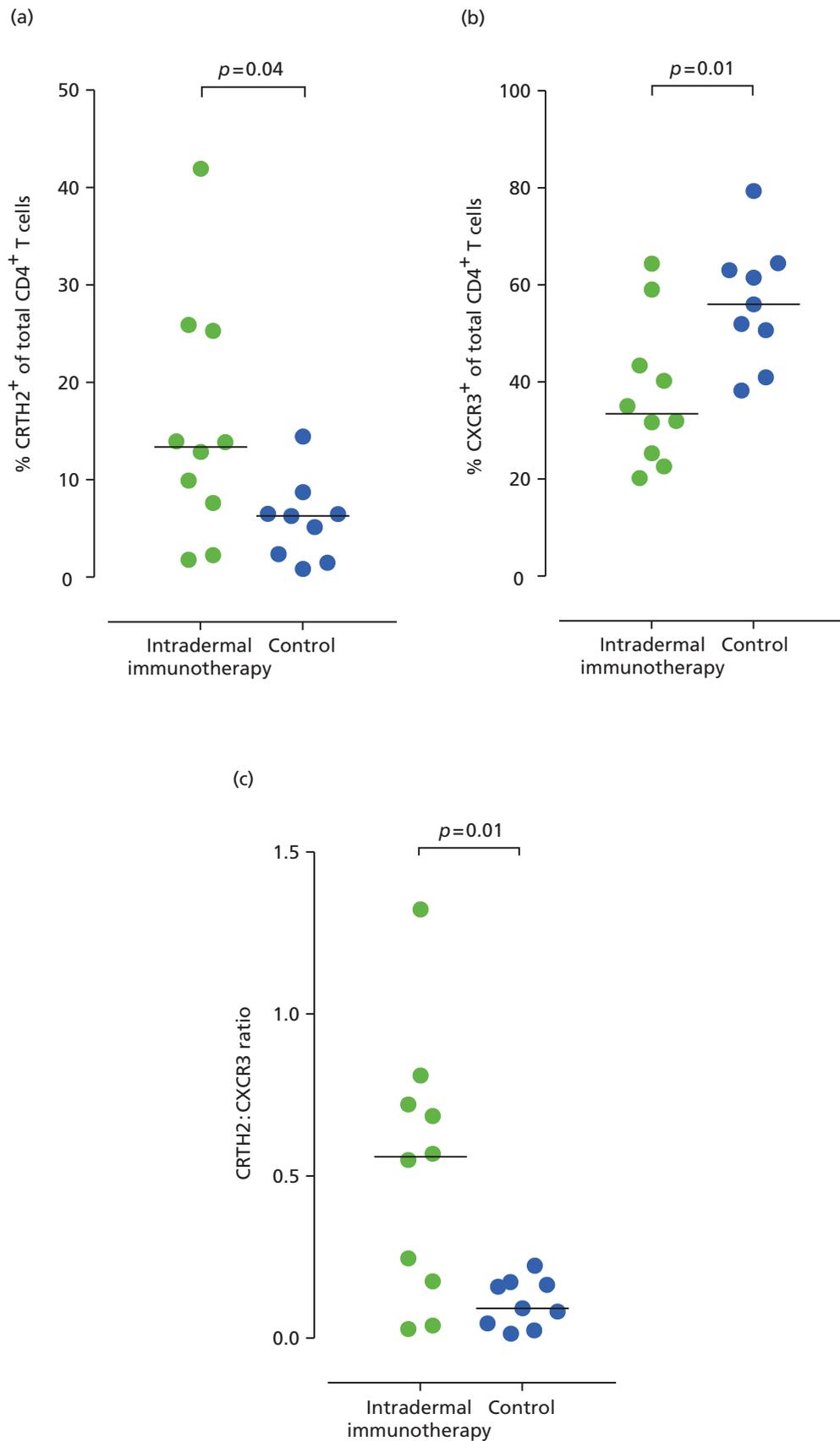


FIGURE 7 Expression of (a) CRTH2 (Th2 marker); (b) CXCR3 (Th1 marker); and (c) ratio of CRTH2 to CXCR3 expression on CD4⁺ cells expanded from skin biopsies (24 hours post-skin challenge). The *p*-values are based on Mann-Whitney *U*-tests.

TABLE 5 Microarray gene expression profiles of activated CD4⁺ T cells derived from skin biopsy explants

Gene	p-value	Fold difference
Intradermal immunotherapy 'down' vs. control group		
LOC100133042	0.02	-1.80
CEP55	0.03	-1.78
GFOD1	0.00	-1.77
HIST2H2AB	0.04	-1.62
H2AFZ	0.02	-1.61
LOC730534	0.01	-1.57
HSD17B4	0.02	-1.57
HIST1H2AD	0.03	-1.56
HDAC1	0.01	-1.55
CCL3L1	0.03	-1.53
CALR	0.02	-1.52
CDCA5	0.01	-1.52
PRDX5	0.01	-1.51
FEN1	0.02	-1.50
Intradermal immunotherapy 'up' vs. control group		
EPS15	0.02	1.51
MYB	0.01	1.52
GK	0.03	1.53
RNASET2	0.03	1.55
LOC729383	0.02	1.56
GPR171	0.00	1.59
LOC729387	0.04	1.60
SLC11A2	0.02	1.60
HS.508682	0.04	1.68
IL5	0.03	1.71
GBP5	0.05	1.79
TNFSF8	0.01	1.79
TNIP3	0.03	1.87
CENTA1	0.05	2.11
Notes		
T cells were cultured from skin biopsies that were taken 24 hours after an intradermal <i>P. pratense</i> skin challenge. Cells were activated with phorbol myristate acetate (PMA)/ionomycin prior to RNA isolation and microarray analysis. Data were analysed by a three-way ANOVA model.		

remained significantly suppressed in the group that had received intradermal immunotherapy at both 4 and 7 months (both $p = 0.03$), although the degree of suppression at these time points was clearly less than that which we previously reported immediately after completion of six injections. Late responses were not suppressed at 10 or 13 months. These data suggest that the suppressive effect of intradermal immunotherapy on late-phase responses was wearing off within 4 months. In contrast with the late-phase response, no significant differences between treatment arms were seen in early-phase responses at 4-, 7-, 10- or 13-month time points (see *Figure 10b*).

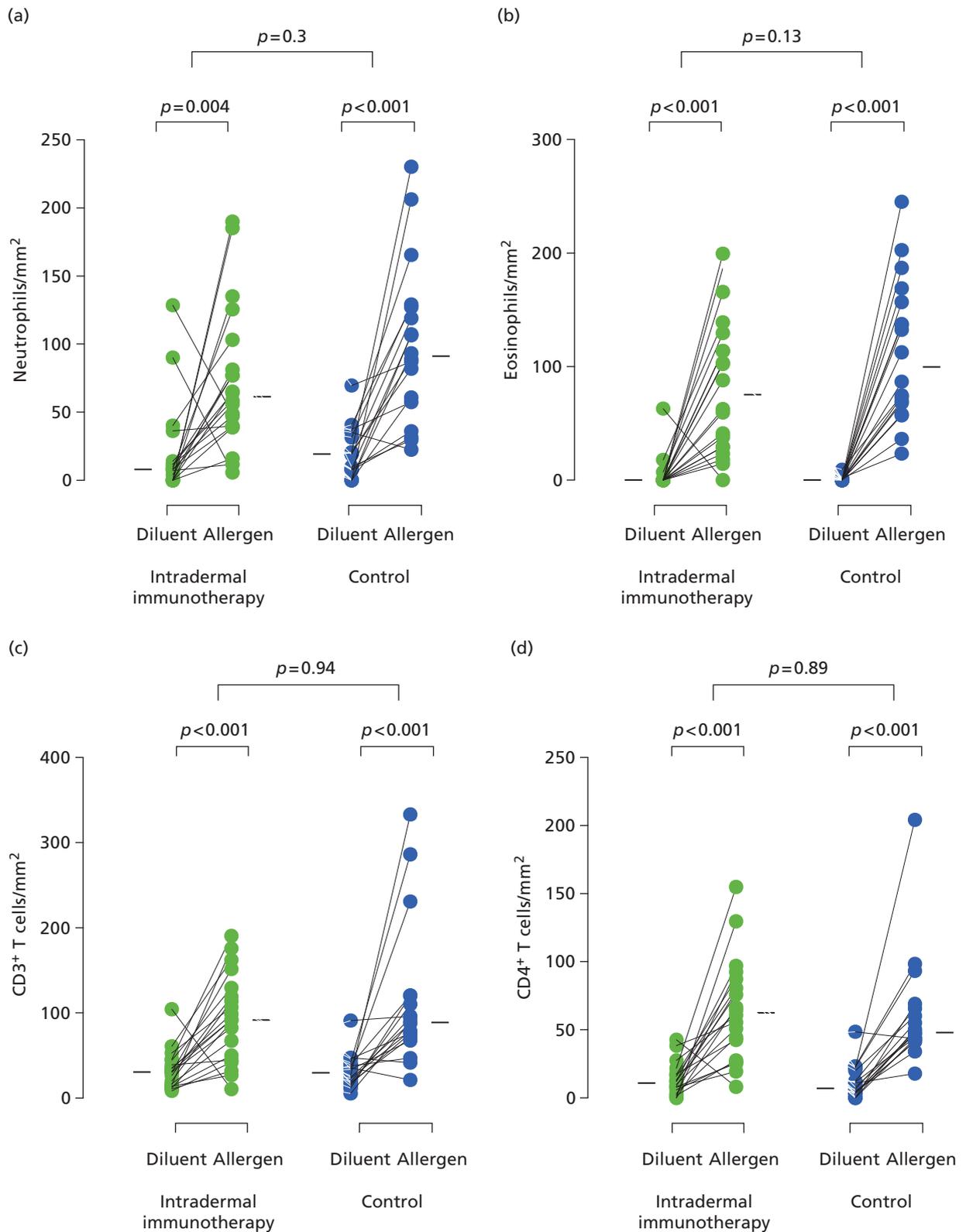


FIGURE 8 Immunohistochemistry analysis of skin biopsies. Comparison of allergen-induced inflammatory cell numbers in skin biopsies from intradermal immunotherapy and control arm participants. Data shown indicate numbers of (a) neutrophils; (b) eosinophils; (c) CD3⁺ T cells; and (d) CD4⁺ T cells in skin biopsies taken after diluent and *P. pratense* intradermal skin challenges in September 2013. Cells were stained using the APAAP method. Solid bars represent median values. The p -values comparing diluent- and allergen-challenged biopsies are based on the Wilcoxon signed-rank test. The p -values for between-group comparisons are based on ANCOVA.

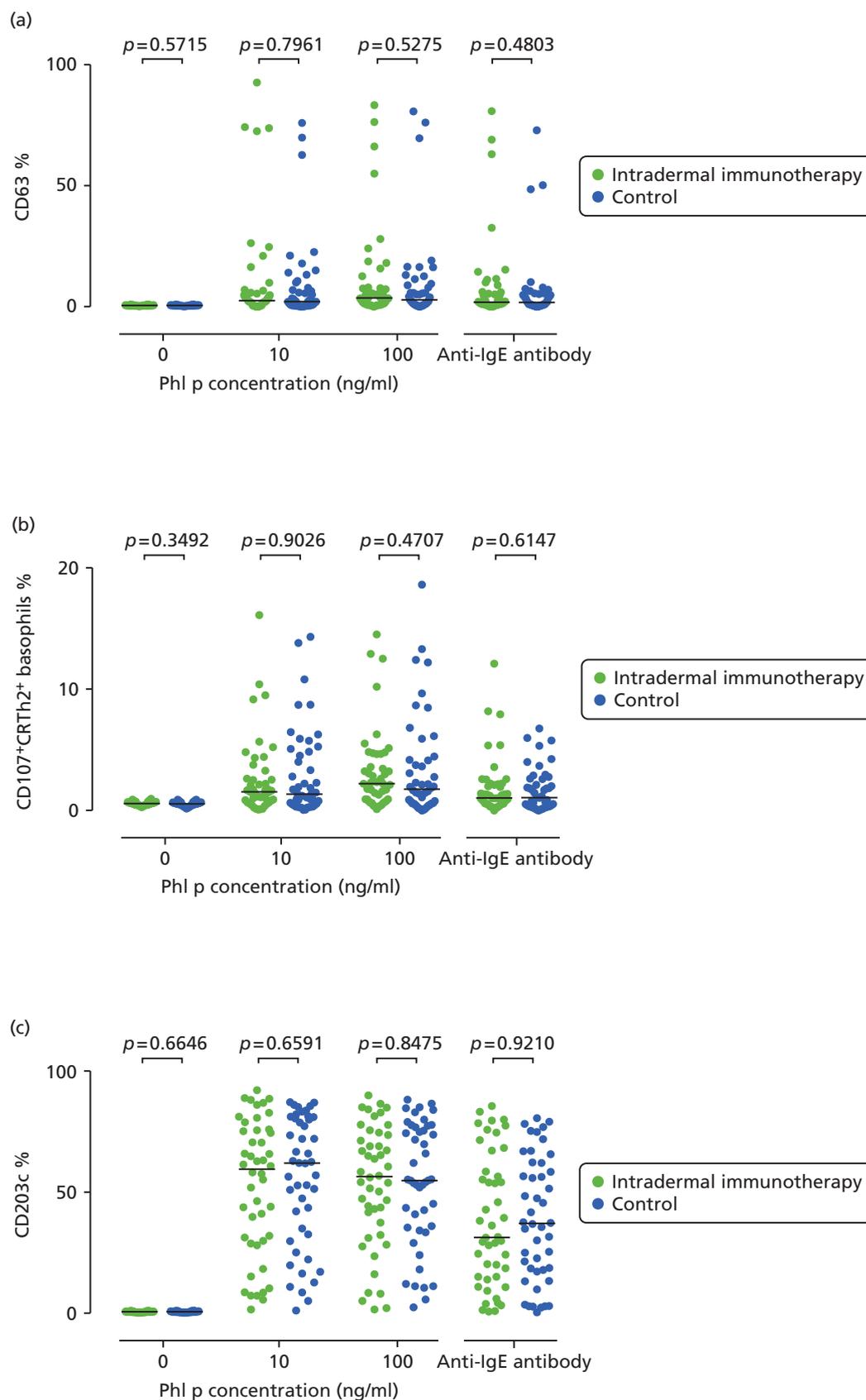


FIGURE 9 Basophil activation tests. Percentage of basophils staining positive for activation markers. (a) CD63; (b) CD107a; and (c) CD203c. Whole blood was stimulated under the conditions described. The *p*-values are based on Mann–Whitney *U*-tests.

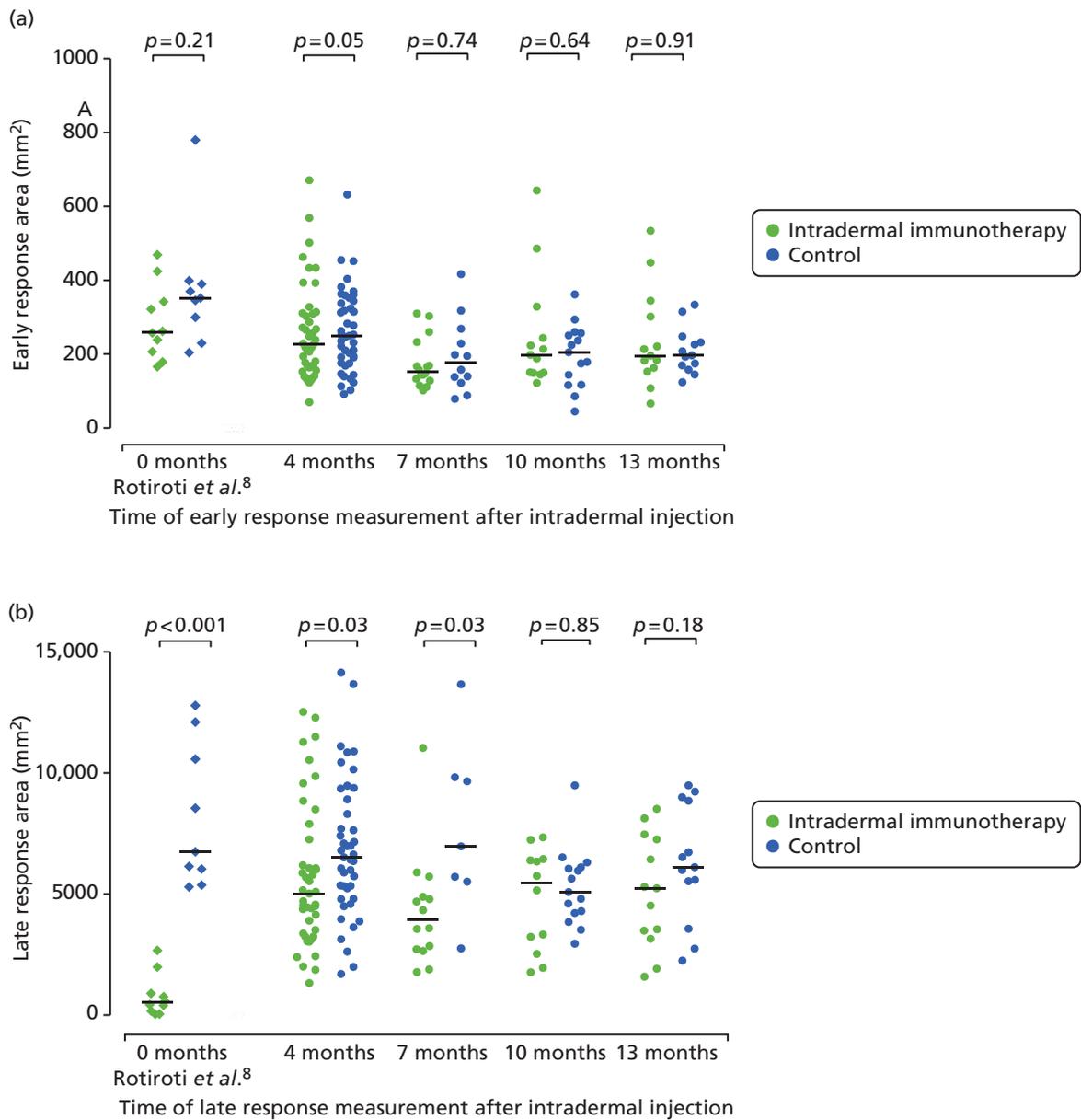


FIGURE 10 Late-phase skin responses. Areas of cutaneous (a) early-; and (b) late-phase responses (15 minutes and 24 hours after intradermal skin challenge, respectively), performed 4 months and either 7, 10 or 13 months post treatment (September 2013). Late response suppression is shown from our previous study (Rotiroti *et al.*⁶) immediately after six 2-weekly intradermal injections. Solid bars represent median values. The p -values are based on Mann–Whitney U -tests.

Chapter 4 Discussion and conclusions

In this RCT, we have conclusively demonstrated that preseasonal treatment with seven intradermal grass pollen injections containing 7 ng of major allergen Phl p 5 is not a clinically effective treatment for seasonal allergic rhinitis. Furthermore, no benefit of this treatment approach was evident from analysis of secondary end points. In contrast, analysis of certain prespecified secondary end points showed that intradermal allergen immunotherapy was associated with a modest and unexpected worsening of allergic rhinitis nasal symptoms, as measured by daily symptom scores and 2-weekly VAS scores. Furthermore, in a per-protocol analysis we also found evidence for worsening of both lung and mouth symptoms in the group that received intradermal allergen treatment, together with fewer symptom-free days. In mechanistic studies we also observed evidence for a degree of immunological priming to allergen, manifest as a small relative increase in allergen-specific IgE responses and skewing of skin CD4⁺ T-cell surface markers in favour of a Th2 response. The study also confirmed our earlier observations that repeated intradermal allergen injections inhibits allergen-induced late-phase skin responses. However, this effect appeared to have dissipated when assessed 10 months after stopping intradermal immunotherapy, suggesting that the immunological effect of this intervention was transient.

No serious AEs occurred that were attributable to grass pollen intradermal allergen immunotherapy, and 92 of the 93 participants completed the seven-injection course. The one participant who withdrew during the treatment period did so for unrelated reasons. Five participants deviated significantly from the protocol in use of rescue medication, mainly in excessive use of antihistamines or topical nasal steroid or eye drops. Two participants also used prednisolone without reference to a study physician. We were unable to identify an explanation for why these five participants were all in the histamine control arm, but their exclusion in the per-protocol analysis did not affect the main outcome of the study.

Strengths of this study include the stringent selection of participants in accordance with clinical criteria specified in guidelines for grass pollen immunotherapy,⁴ the high rate of daily diary card data collection and the successful blinding of the active treatment. In addition, we used the clinical end point recommended by the WAO for trials of immunotherapy in allergic rhinitis. A possible limitation of this study is that the dose of grass pollen was not increased during the treatment course. We did not do this because of our previous observation that repeating the same dose was sufficient to achieve almost complete suppression of the late-phase response. In addition, our goal was to develop a treatment regimen that had the potential to be widely adopted, and dose escalations would probably increase the risk of systemic reactions. Another possible weakness is that injections were not continued throughout the grass pollen season, although previous RCTs of subcutaneous grass pollen immunotherapy have demonstrated efficacy for preseasonal regimens.²⁸ An allergen dose equivalent to 7 ng of the major Timothy grass pollen allergen Phl p 5 was selected for several reasons. First, we previously reported in our proof-of-concept study conducted in a similar population that six 2-weekly injections at this dose led to almost complete inhibition of the cutaneous allergen-induced late-phase response induced by these injections. This is similar to the effect of clinically effective cutaneous late-phase responses seen following high-dose subcutaneous immunotherapy¹⁰ and markedly exceeds that following treatment with sublingual grass pollen vaccines.¹¹ Second, the late-phase response induced by this dose corresponds to an average diameter of approximately 10 cm, a size that we considered to be at the limits of tolerability for patients for a routine treatment, especially as the response can be much larger in a proportion of subjects. Although it is impossible to equate this dosage precisely with that used in the uncontrolled historical studies of Phillips,^{12,13} it is notable that he described his treatment as inducing 'a local reaction about the size of the patient's palm',¹² which is similar to the response size we induced.

In this study, we measured only late-phase skin responses at the end of the 2013 grass pollen season, by which time some 4 months had passed since the completion of the intradermal allergen/control immunotherapy injection regimen. We did not perform these measurements earlier for two reasons. First, this would have necessitated giving an intradermal allergen challenge to the control arm participants,

and we were concerned that this, in itself, might exert a biological effect and alter clinical outcomes in this group during the pollen season. Second, measuring the late-phase response sizes could have compromised blinding before collection of the clinical outcome data, as our previous data suggested that these responses would be > 90% suppressed at this time. Therefore, the first late-phase response measurements were obtained 4 months after the final preseasonal injection. Late-phase responses were still significantly lower in the intradermal allergen immunotherapy group than in the control group at this time point, and also at the subsequent 7-month time point. This difference was, however, significantly less than we previously observed immediately after six intradermal injections in the proof-of-concept study, suggesting that late-phase response suppression is transient and mostly reversed within 4 months, and completely reversed by 10 months. The lack of clinical benefit and potential worsening of allergic symptoms despite suppression of the late skin response in this study may indicate that the late-phase skin response is not relevant to expression of grass pollen allergic disease. An alternative explanation, which is more likely in our view, is that the consistent suppression of the late-phase response following subcutaneous and sublingual immunotherapy^{10,11} may be necessary, but not sufficient alone, to account for associated clinical improvement.

Allergen-specific IgE concentrations were measured in serum samples that were collected at initial screening, that is, between October 2012 and January 2013, and again immediately after completion of the intradermal allergen or control injections (May 2013). In the control arm, there was a consistent but small decline in the IgE levels that were specific for whole grass allergen and the Phl p 1 and Phl p 5 major allergens over this period. This seasonal variation in IgE levels is well described²⁹ and can be explained by the proximity of the first time point to the 2012 grass pollen season, with recent environmental grass pollen exposure presumed to have stimulated memory B-cell responses. However, the ensuing fall in allergen-specific IgE was not observed in the active arm, indicating that intradermal allergen immunotherapy continued to stimulate IgE synthesis. This 'priming' effect on IgE responses also occurs with subcutaneous immunotherapy¹⁰ but is further evidence that the intradermal allergen injections were biologically active and exerted a systemic immunological effect. Similarly, like conventional subcutaneous and sublingual grass pollen immunotherapy,^{11,29,30} intradermal allergen treatment also stimulated allergen-specific IgG responses. Allergen-specific IgG responses to grass pollen immunotherapy block IgE-dependent histamine release from basophils and IgE-mediated facilitated antigen presentation to T cells.^{10,31} Persistence of this effect has been associated with long-term efficacy.³² In this study, we did not observe a treatment effect on basophil activation in response to allergen stimulation *in vitro*. It is therefore possible that the lack of efficacy of intradermal allergen immunotherapy stemmed from the failure of the dermal route to sufficiently stimulate a protective allergen-specific IgG response.

Immunohistochemistry was performed on skin punch biopsies from 20 active and 20 control participants. Biopsies were collected immediately after the late-phase response was measured, that is, at the 4-month time point and 24 hours after intradermal allergen challenge. Although late-phase responses were still partially inhibited at this time point, we observed no significant inhibition of allergen-induced infiltration of eosinophils, neutrophils, CD3⁺ T cells or CD4⁺ T cells by intradermal allergen immunotherapy. Biopsies were also examined for Fox p3⁺ Tregs, but no immunostaining could be observed despite successful staining of positive control nasal polyp tissue. In half of the participants who underwent biopsy, the biopsy was divided into two fragments, and one piece was immediately cultured for T-cell expansion. Only T cells from allergen-challenged skin (not diluent-challenged skin) could be expanded in sufficient numbers for analysis. This is consistent with the immunohistochemistry findings showing that only small numbers of T cells were present within diluent-challenged skin but that these numbers increased significantly after intradermal allergen challenge. Cultured skin CD4⁺ T cells in the active arm showed higher surface expression of the prostaglandin-D2 receptor CRTH2, a specific marker of Th2 cells.³³ Conversely, in the active treatment arm these T cells showed lower levels of surface Th1 marker CXCR3. In samples where sufficient cells were expanded, T cells were also stimulated and subjected to transcriptional profiling by microarray. Only 13 genes were found to be significantly overexpressed in the active intradermal immunotherapy group compared with the control arm. This relatively small number probably reflects a high degree of biological variability, but, significantly, one of the overexpressed genes encoded the Th2 cytokine IL-5. Collectively, these findings therefore suggest that intradermal allergen immunotherapy resulted in local priming of cutaneous Th2 responses, and suggest a mechanism for how this intervention

may have facilitated IgE synthesis. This priming effect could also account for why intradermal immunotherapy may have acted to potentiate certain symptoms when participants were subsequently exposed to grass pollen naturally during the 2013 season.

Previous non-interventional human studies have linked cutaneous allergen exposure to IgE responses and development or exacerbation of allergy, albeit in the context of atopic eczema when skin barrier function is compromised. For example, in children with atopic eczema, exposure to peanut protein via the dermal route has been associated with development of peanut allergy.^{34,35} It is plausible that grass pollen intradermal allergen injections may have acted similarly to target the dermis. Our findings also raise the possibility that repeated intracutaneous exposure to aeroallergens, for example in patients with eczema who have disrupted skin barrier function, could have the potential to exacerbate respiratory allergic disease.

There is considerable current interest in the concept of administering immunotherapy as allergen applied epicutaneously in patches to non-eczematous skin.³⁶ Preliminary clinical trials have provided evidence that this may be effective for treatment of grass pollen allergy and similar patches are also under investigation for peanut allergy.^{37,38} Unlike the intradermal allergen immunotherapy tested in this study, epicutaneous treatment targets the epidermis rather than the dermis directly. However, recent studies have investigated methods that enhance keratinocyte activation and skin penetration by epicutaneous allergen, such as use of microneedles³⁹ or skin stripping with tape.⁴⁰ Such methods are likely to promote dermal allergen exposure⁴⁰ and in at least one animal model the application of allergen to stripped skin potentiated systemic Th2 responses and the in vivo response to allergen.⁴¹ The findings from our trial provide the first human evidence that novel immunotherapy approaches that facilitate exposure of the dermis to allergen also have the potential to worsen symptoms, even if local macroscopic responses appear to be suppressed by the vaccine.

Conclusions

The results of this study provide evidence that low-dose intradermal allergen injection immunotherapy is not clinically effective, even if it is able to suppress late-phase skin responses. Furthermore, we found evidence that this intervention resulted in immunological priming in certain assays in parallel with worsening of certain symptoms during the grass pollen season. These findings support the concept that dermal allergen exposure has the potential to exacerbate, rather than ameliorate, allergic responses. We conclude that novel immunotherapy strategies that promote dermal allergen exposure have the potential to be deleterious, even if local macroscopic responses appear to be suppressed by this approach.

Acknowledgements

This work was supported by a Medical Research Council (MRC) and NIHR partnership. The King's Health Partnership Challenge Fund provided additional research funding for this project. This research was also supported by the NIHR Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and KCL. Dr Till was supported a Higher Education Funding Council for England (HEFCE) Clinical Senior Lectureship Award. Dr Lam was funded by a MRC-Asthma UK-funded PhD studentship. Dr Slovick received funding from Athena SWAN and the Royal College of Surgeons (England). Professor Cousins acknowledges support from NIHR Leicester Respiratory Biomedical Research Unit.

We are indebted to the members of the public who provided PPI input to project: Bernard Chan for assistance with data entry; James Dobbyn, John Brooks, Sharon Jones and Gerry Trillana of the NIHR Clinical Research Facility at Guy's Hospital; Dr Alina Dumitru for assistance in setting up the recruitment campaign; Dr Elena Ortiz-Zapater for assistance with mechanistic studies; Paul Tunstell of Guy's Hospital Pharmacy for GMP manufacture of grass pollen and histamine solutions for use in the trial; the UK Meteorological Office for managing the UK pollen network; and Bhopal Pandey, Kris Chan, Natalia Acero Martinez, Dr Trevor Blackall and Dr Robert Francis for collection and provision of pollen count data. The authors also gratefully acknowledge the contributions of the Trial Steering Committee (chairperson: Dr Samantha Walker, Asthma UK) and the Data Monitoring and Ethics Committee (chairperson: Professor Peter Burney, Imperial College London).

Contributions of authors

Ms Anna Slovick (Clinical Fellow, KCL, and Ear, Nose and Throat trainee) was overall trial co-ordinator and participated in the set-up of the trial, recruitment, administration of intradermal injections and collection of clinical outcome data; performed mechanistic assays; and participated in preparation of the first draft of this manuscript.

Dr Abdel Douiri (Senior Lecturer in Medical Statistics, KCL) participated in the design of the trial, preparation of the manuscript and was the trial statistician.

Dr Rachel Muir (Research Matron) participated in the set-up of the trial, recruitment, administration of intradermal injections and collection of clinical outcome data.

Dr Andrea Guerra (Clinical Fellow, KCL) participated in the set-up of the trial, recruitment, administration of intradermal injections and collection of clinical outcome data, and performed mechanistic assays.

Mr Konstantinos Tsioulos (Clinical Fellow, KCL) participated in the set-up of the trial, recruitment, and administration of intradermal injections.

Ms Evie Hays (Research technician) performed mechanistic assays.

Dr Emily PS Lam (PhD student, degree now awarded) performed mechanistic assays.

Dr Ms Joanna Kelly (Strategic Data Management Lead, KCTU) participated in the design and set-up of the trial.

Professor Janet L Peacock (Professor of Medical Statistics, KCL) participated in the statistical analysis and data interpretation.

Dr Sun Ying (Reader in Allergy, KCL) participated in design, supervision and co-ordination of mechanistic assays.

Dr Mohamed H Shamji (Research Fellow, Imperial College London) participated in design, supervision and coordination of mechanistic assays.

Professor David J Cousins (Professor Respiratory Science, University of Leicester) supervised mechanistic assays.

Professor Stephen R Durham (Professor of Allergy & Respiratory Medicine, Imperial College London; Consultant Allergist, Royal Brompton & Harefield NHS Trust) participated in the study conception, design, interpretation of the results and manuscript preparation.

Dr Stephen J Till (Reader in Allergy, KCL; Consultant Allergist, Guy's and St Thomas' NHS Foundation Trust) was chief investigator of the PollenLITE trial and conceived of the study, participated in its design and co-ordination, and prepared the first draft of this manuscript with the assistance of AS.

Publications

Slovick A, Douiri A, Muir R, Guerra A, Tsioulos K, Hay E, *et al.* Intradermal grass pollen immunotherapy increases T_H2 and IgE responses and worsens respiratory allergic symptoms [published online ahead of print October 20 2016]. *J Allergy Clin Immunol* 2016.

Data sharing statement

All available data can be obtained by contacting the corresponding author.

References

1. Slovic A, Douiri A, Muir R, Guerra A, Tsioulos K, Hay E, *et al.* Intradermal grass pollen immunotherapy increases T_H2 and IgE responses and worsens respiratory allergic symptoms [published online ahead of print October 20 2016]. *J Allergy Clin Immunol* 2016. <http://dx.doi.org/10.1016/j.jaci.2016.09.024>
2. Bauchau V, Durham SR. Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J* 2004;**24**:758–64. <http://dx.doi.org/10.1183/09031936.04.00013904>
3. Bauchau V, Durham SR. Epidemiological characterization of the intermittent and persistent types of allergic rhinitis. *Allergy* 2005;**60**:350–3. <http://dx.doi.org/10.1111/j.1398-9995.2005.00751.x>
4. Walker SM, Durham SR, Till SJ, Roberts G, Corrigan CJ, Leech SC, *et al.* Immunotherapy for allergic rhinitis. *Clin Exp Allergy* 2011;**41**:1177–200. <http://dx.doi.org/10.1111/j.1365-2222.2011.03794.x>
5. Noon L. Prophylactic inoculation against hay fever (historical document). *Ann Allergy* 1955;**13**:713–16.
6. Calderon MA, Alves B, Jacobson M, Hurwitz B, Sheikh A, Durham S. Allergen injection immunotherapy for seasonal allergic rhinitis. *Cochrane Database Syst Rev* 2007;**1**:CD001936. <http://dx.doi.org/10.1002/14651858.cd001936.pub2>
7. Radulovic S, Calderon MA, Wilson D, Durham S. Sublingual immunotherapy for allergic rhinitis. *Cochrane Database Syst Rev* 2010;**12**:CD002893. <http://dx.doi.org/10.1002/14651858.cd002893.pub2>
8. Kay AB, Ying S, Varney V, Gaga M, Durham SR, Moqbel R, *et al.* Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Exp Med* 1991;**173**:775–8. <http://dx.doi.org/10.1084/jem.173.3.775>
9. Rotiroti G, Shamji M, Durham SR, Till SJ. Repeated low-dose intradermal allergen injection suppresses allergen-induced cutaneous late responses. *J Allergy Clin Immunol* 2012;**130**:918–24. <http://dx.doi.org/10.1016/j.jaci.2012.06.052>
10. Francis JN, James LK, Paraskevopoulos G, Wong C, Calderon MA, Durham SR, *et al.* Grass pollen immunotherapy: IL-10 induction and suppression of late responses precedes IgG4 inhibitory antibody activity. *J Allergy Clin Immunol* 2008;**121**:1120–5. <http://dx.doi.org/10.1016/j.jaci.2008.01.072>
11. Lima MT, Wilson D, Pitkin L, Roberts A, Nouri-Aria K, Jacobson M, *et al.* Grass pollen sublingual immunotherapy for seasonal rhinoconjunctivitis: a randomized controlled trial. *Clin Exp Allergy* 2002;**32**:507–14. <http://dx.doi.org/10.1046/j.0954-7894.2002.01327.x>
12. Phillips E. Relief of hay-fever by intradermal injections of pollen extract. *JAMA* 1926;**86**:182–4. <http://dx.doi.org/10.1001/jama.1926.02670290022008>
13. Phillips E. Intradermal pollen therapy during the attack. *J Allergy* 1933;**5**:29–36. [http://dx.doi.org/10.1016/S0021-8707\(33\)90167-7](http://dx.doi.org/10.1016/S0021-8707(33)90167-7)
14. Radulovic S, Jacobson MR, Durham SR, Nouri-Aria KT. Grass pollen immunotherapy induces Foxp3-expressing CD4⁺ CD25⁺ cells in the nasal mucosa. *J Allergy Clin Immunol* 2008;**121**:1467–72. <http://dx.doi.org/10.1016/j.jaci.2008.03.013>
15. Kersey TW, Van Eyk J, Lannin DR, Chua AN, Tafra L. Comparison of intradermal and subcutaneous injections in lymphatic mapping. *J Surg Res* 2001;**96**:255–9. <http://dx.doi.org/10.1006/jsre.2000.6075>
16. Francis JN, Till SJ, Durham SR. Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;**111**:1255–61. <http://dx.doi.org/10.1067/mai.2003.1570>

17. Meiler F, Klunker S, Zimmermann M, Akdis CA, Akdis M. Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors. *Allergy* 2008;**63**:1455–63. <http://dx.doi.org/10.1111/j.1398-9995.2008.01774.x>
18. Senti G, von Moos S, Kündig TM. Epicutaneous allergen administration: is this the future of allergen-specific immunotherapy? *Allergy* 2011;**66**:798–809. <http://dx.doi.org/10.1111/j.1398-9995.2011.02560.x>
19. Romani N, Flacher V, Tripp CH, Sparber F, Ebner S, Stoitzner P. Targeting skin dendritic cells to improve intradermal vaccination. *Curr Top Microbiol Immunol* 2012;**351**:113–38. http://dx.doi.org/10.1007/82_2010_118
20. Slovick A, Douiri A, Kelly J, Guerra A, Muir R, Tsioulos K, et al. Protocol for a double-blind randomised controlled trial of low dose intradermal grass pollen immunotherapy versus a histamine control on symptoms and medication use in adults with seasonal allergic rhinitis (PollenLITE). *Clin Transl Allergy* 2013;**3**:27. <http://dx.doi.org/10.1186/2045-7022-3-27>
21. Canonica GW, Baena-Cagnani CE, Bousquet J, Bousquet PJ, Lockey RF, Malling HJ, et al. Recommendations for standardization of clinical trials with Allergen Specific Immunotherapy for respiratory allergy. A statement of a World Allergy Organization (WAO) taskforce. *Allergy* 2007;**62**:317–24. <http://dx.doi.org/10.1111/j.1398-9995.2006.01312.x>
22. Juniper EF, Thompson AK, Ferrie PJ, Roberts JN. Development and validation of the mini Rhinoconjunctivitis Quality of Life Questionnaire. *Clin Exp Allergy* 2000;**30**:132–40. <http://dx.doi.org/10.1046/j.1365-2222.2000.00668.x>
23. Herdman M, Gudex C, Lloyd A, Janssen M, Kind P, Parkin D, et al. Development and preliminary testing of the new five-level version of EQ-5D (EQ-5D-5L). *Qual Life Res* 2011;**20**:1727–36. <http://dx.doi.org/10.1007/s11136-011-9903-x>
24. Frew AJ, Kay AB. The relationship between infiltrating CD4+ lymphocytes, activated eosinophils, and the magnitude of the allergen-induced late phase cutaneous reaction in man. *J Immunol* 1988;**141**:4158–64.
25. Gaga M, Frew AJ, Varney VA, Kay AB. Eosinophil activation and T lymphocyte infiltration in allergen-induced late phase skin reactions and classical delayed-type hypersensitivity. *J Immunol* 1991;**147**:816–22.
26. Varney VA, Gaga M, Frew AJ, Aber VR, Kay AB, Durham SR. Usefulness of immunotherapy in patients with severe summer hay fever uncontrolled by antiallergic drugs. *BMJ* 1991;**302**:265–9. <http://dx.doi.org/10.1136/bmj.302.6771.265>
27. Cox L, Larenas-Linnemann D, Lockey RF, Passalacqua G. Speaking the same language: The World Allergy Organization Subcutaneous Immunotherapy Systemic Reaction Grading System. *J Allergy Clin Immunol* 2010;**125**:569–74.
28. Corrigan CJ, Kettner J, Doemer C, Cromwell O, Narkus A, Study Group. Efficacy and safety of preseasonal-specific immunotherapy with an aluminium-adsorbed six-grass pollen allergoid. *Allergy* 2005;**60**:801–7. <http://dx.doi.org/10.1111/j.1398-9995.2005.00790.x>
29. Durham SR, Emminger W, Kapp A, de Monchy JG, Rak S, Scadding GK, et al. SQ-standardized sublingual grass immunotherapy: confirmation of disease modification 2 years after 3 years of treatment in a randomized trial. *J Allergy Clin Immunol* 2012;**129**:717–25.e5. <http://dx.doi.org/10.1016/j.jaci.2011.12.973>
30. Gleich GJ, Zimmermann EM, Henderson LL, Yunginger JW. Effect of immunotherapy on immunoglobulin E and immunoglobulin G antibodies to ragweed antigens: a six-year prospective study. *J Allergy Clin Immunol* 1982;**70**:261–71. [http://dx.doi.org/10.1016/0091-6749\(82\)90062-8](http://dx.doi.org/10.1016/0091-6749(82)90062-8)

31. Shamji MH, Ljørring C, Francis JN, Calderon MA, Larché M, Kimber I, *et al.* Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. *Allergy* 2012;**67**:217–26. <http://dx.doi.org/10.1111/j.1398-9995.2011.02745.x>
32. James LK, Shamji MH, Walker SM, Wilson DR, Wachholz PA, Francis JN, *et al.* Long-term tolerance after allergen immunotherapy is accompanied by selective persistence of blocking antibodies. *J Allergy Clin Immunol* 2011;**127**:509–16.e1–5.
33. Cosmi L, Annunziato F, Galli MIG, Maggi RME, Nagata K, Romagnani S. CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. *Eur J Immunol* 2000;**30**:2972–9. [http://dx.doi.org/10.1002/1521-4141\(200010\)30:10%3C2972::AID-IMMU2972%3E3.0.CO;2-%23](http://dx.doi.org/10.1002/1521-4141(200010)30:10%3C2972::AID-IMMU2972%3E3.0.CO;2-%23)
34. Brough HA, Liu AH, Sicherer S, Makinson K, Douiri A, Brown SJ, *et al.* Atopic dermatitis increases the effect of exposure to peanut antigen in dust on peanut sensitization and likely peanut allergy. *J Allergy Clin Immunol* 2015;**135**:164–70. <http://dx.doi.org/10.1016/j.jaci.2014.10.007>
35. Brough HA, Santos AF, Makinson K, Penagos M, Stephens AC, Douiri A, *et al.* Peanut protein in household dust is related to household peanut consumption and is biologically active. *J Allergy Clin Immunol* 2013;**132**:630–8. <http://dx.doi.org/10.1016/j.jaci.2013.02.034>
36. Senti G, von Moos S, Tay F, Graf N, Sonderegger T, Johansen P, *et al.* Epicutaneous allergen-specific immunotherapy ameliorates grass pollen-induced rhinoconjunctivitis: a double-blind, placebo-controlled dose escalation study. *J Allergy Clin Immunol* 2012;**129**:128–35. <http://dx.doi.org/10.1016/j.jaci.2011.08.036>
37. Agbotounou W, Martin L, Dupont B, Pascal I, Vauléon C, Benhamou PH. Epicutaneous immunotherapy (EPIT) is safe for the treatment of peanut allergy in allergic patients. *J Allergy Clin Immunol* 2013;**2**:AB91. <http://dx.doi.org/10.1016/j.jaci.2012.12.992>
38. Dupont C. *Peanut Epicutaneous Immunotherapy (EPIT) in Peanut-allergic Children: 18 months Treatment in the ARACHILD Study.* AAAAI Annual Meeting, 28 February to 4 March 2014, San Diego, CA.
39. Spina L, Weisskopf M, von Moos S, Graf N, Kündig TM, Senti G. Comparison of microneedles and adhesive-tape stripping in skin preparation for epicutaneous allergen delivery. *Int Arch Allergy Immunol* 2015;**167**:103–9. <http://dx.doi.org/10.1159/000434681>
40. von Moos S, Johansen P, Tay F, Graf N, Kündig TM, Senti G. Comparing safety of abrasion and tape-stripping as skin preparation in allergen-specific epicutaneous immunotherapy. *J Allergy Clin Immunol* 2014;**134**:965–7.e4. <http://dx.doi.org/10.1016/j.jaci.2014.07.037>
41. Mondoulet L, Dioszeghy V, Puteaux E, Ligouis M, Dhelft V, Letourneur F, *et al.* Intact skin and not stripped skin is crucial for the safety and efficacy of peanut epicutaneous immunotherapy (EPIT) in mice. *Clin Transl Allergy* 2012;**2**:22. <http://dx.doi.org/10.1186/2045-7022-2-22>

Appendix 1 PollenLITE trial website with prescreening questions

PollenLITE

Home About The Trial Where We Are

Do you long for a summer free from hay fever?

We're inviting hay fever sufferers aged 18 - 65 to take part in the **PollenLITE** clinical research trial and test a new kind of vaccine that could actually 'switch off' the allergic reaction to grass pollen.

Hay fever medications will be supplied free of charge during the summer of 2013. Travel expenses will be reimbursed.

Do something positive, with PollenLITE.

Thank you for your interest in the PollenLITE study. Registration has now closed. Everyone who registered on the website will be contacted over the next month.

* This trial is supported by the Medical Research Council and the National Institute for Health Research. In this trial, half of participants will receive the active vaccine and half will receive a dummy vaccine.

MRC & Asthma UK Centre in Allergic Mechanisms of Asthma
 Imperial College London
 Guy's and St Thomas' NHS Foundation Trust

About The Trial ? Where We Are + Guy's Hospital +

Home About The Trial Where We Are

Register Here

1) About you

Is your age between 18-65 years? Y N

Name*

Date of Birth* DD/MM/YY Sex M/F

Email*

Confirm Email*

Telephone

2) About your health

Do you have poorly controlled asthma? Y N

Have you ever had a life-threatening allergic reaction (anaphylaxis)?* Y N

Do you currently smoke cigarettes or other tobacco products? Y N

Are you pregnant, nursing, or planning pregnancy? (Males click no)* Y N

3) About your hay fever

Do you suffer from hay fever in May, June or July? Y N

Have your hay fever symptoms interfered with your daily activities or sleep? Y N

Do you use allergy medications (tablets or nasal sprays) for your hay fever? Y N

How did you hear about us?

Data entered will only be used internally as part of the PollenLITE trial and will not be disclosed to third parties for other purposes.

Apply Now

MRC & Asthma UK Centre in Allergic Mechanisms of Asthma
 Imperial College London
 Guy's and St Thomas' NHS Foundation Trust

Appendix 2 PollenLITE recruitment advertisement panel used on public transport



PollenLITE™
Hay Fever Study

Feel as if your nose should have won a gold medal for running?

This trial is supported by the Medical Research Council and the National Institute for Health Research. In this trial, half of participants will receive the active vaccine and half will receive a dummy vaccine.

To register or find out more visit: www.pollenLITE.co.uk

If you long for a summer without the misery that hay fever brings, and you're aged 18-65 we invite you to take part in the PollenLITE clinical research trial.

The study will test a completely new type of vaccine that we hope could 'switch off' the allergy to grass pollen.

You will undergo allergy testing for the causes of hay fever and hay fever medications will be supplied free of charge during the summer of 2013. Your travel expenses will also be reimbursed.

MRC & Asthma UK Centre in Allergic Mechanisms of Asthma
MRC Asthma UK Centre in Allergic Mechanisms of Asthma
Imperial College London
Guy's and St Thomas' NHS Foundation Trust

Appendix 3 Example of daily symptom and medication-use diary card

Symptom Scores: For each part of the body, please fill the boxes as per below: 0=no symptoms, 1=mild, 2=moderate, 3=severe

July 2013	M	T	W	T	F	S	S	M	T	W	T	F	S	S	M	T	W	T	F	S	S	M	T	W	T	F	S	S	M	T	W
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Lungs																															
Breathlessness																															
Cough																															
Wheeze																															
Tightness																															
Nose																															
Sneezing																															
Blockage																															
Running																															
Mouth & Throat																															
Itching																															
Drying																															
Eyes																															
Itching																															
Redness/Sore																															
Streaming																															
Swelling																															

Medication Scores: Please specify how many tablets/squirts of nasal spray/eye drops you have required per day. For example,
 1 antihistamine tablet per day=1, 2squirts of nasal spray per nostril per day=4, 1 eye drop for each eye twice a day=4 0=not used,
 1= 1 tablet/nasal spray squirt/eye drop, 2= 2tablets/nasal spray squirts/eye drops etc.

Medications & Doses																														
Antihistamine																														
Nasal Spray																														
Eye Drops																														
Prednisolone																														

Please mark an * if you forget to fill the form;
 H if you are on holiday, S if you were near to the sea.

Holiday Destination:
Seaside Destination:

Appendix 4 Visual analogue scale

Completed every 2 weeks during Summer 2013.

Please place a vertical mark along the line where you feel the severity of your symptoms lie. So, if you were to place a mark on the far left of the line, it would mean that you are completely symptom free. However, if you marked the far right of the line, your symptoms are as bad as they possibly could be.

Nasal Symptoms:

Nasal Blockage/Congestion

Runny nose

Itchy nose

Sneezing

Eye Symptoms:

Itchy eyes

Watery eyes

Appendix 5 Global Evaluation scores (completed September 2013)

Global Evaluation No. 1

Sept 2013 visit

The subject should be asked: "How do you assess the severity of your rhinoconjunctivitis symptoms when they were the most severe during this grass pollen season (Tick each single symptom)?"

Rhinoconjunctivitis/ Hayfever symptom		Symptoms			
		0 (None)	1 (Mild)	2 (Moderate)	3 (Severe)
Nasal Symptoms					
1.	Runny nose				
2.	Blocked nose				
3.	Sneezing				
4.	Itchy nose				
Eye symptoms					
1.	Itchy eyes				
2.	Watery eyes				

Global Evaluation No. 2

Sept 2013 visit

The subject should be asked: "How was your hayfever this year compared with years before you started immunotherapy treatment (Tick only one)?"

Assessment						
Much better (+3)	Better (+2)	A little better (+1)	The same (0)	A little worse (-1)	Worse (-2)	Much worse (-3)

Appendix 6 Statistical analysis plan

PollenLite Statistical Analysis Plan

PollenLite Trial

A Randomised, Double-blind, Single-centre,
Controlled Trial of Low Dose Intradermal
Allergen Immunotherapy in Adults with
Seasonal Allergic Rhinitis

Statistical Analysis Plan

Version 2.

5th September 2014

Version 1.0 started: 02/04/2012

ISRCTN: 78413121

Statistical approver: Dr Abdel Douiri & Prof. Janet Peacock &
Principal investigator: Dr Stephen Till

Signatures:

Date: 05-09-2014

PollenLite Statistical Analysis Plan

CONTENTS

A) QUANTITATIVE ANALYSIS PLAN	
Abbreviations	
1. Description of the trial	
1.1 Principal research objectives to be addressed	
Primary objectives.....	
Secondary objectives	
1.2 Trial design and flowchart	
1.3 Populations and Study Sample	
Target Population	
Trial Population	
Trial Sample	
Inclusion criteria	
Exclusion criteria	
Safety analysis population.....	
1.4 Method of allocation of groups	
1.5 Description of interventions	
Standard treatment	
Control group	
Intervention group.....	
1.6 Duration of the treatment period	
1.7 Frequency of follow-up and duration of the trial	
1.8 Trial efficacy end point	
Efficacy assessments.....	
Efficacy assessments.....	
Primary efficacy end point	
Secondary efficacy end points	
Assessment of safety	
1.9 Sample size estimation (including clinical significance)	
1.9 Brief description of proposed analyses	
2. Data analysis plan – Data description	
2.1 Recruitment and representativeness of recruited patients	
2.2 Baseline comparability of randomised groups	
2.3 Loss to follow-up and other missing data	
2.5 Adverse event reporting	
2.6 Assessment of outcome measures (unblinding)	
2.7 Descriptive statistics for main outcome measures	
3. Data analysis plan – Inferential analysis	
3.1 Main analysis of treatment differences	
3.1.1 Analysis of primary outcomes	
3.1.2 Analysis of secondary outcomes	
3.1.3 Responder analysis	
3.1.4 Model assumption checks	
3.2 Exploratory analyses	
3.4 Interim analysis	
4. Reporting conventions	
5. Software	
B) ECONOMIC ANALYSIS PLAN	
Health economic objectives.....	

PollenLite Statistical Analysis Plan

Economic measures
Statistics
C) SCHEDULE OF ASSESSMENTS AND MEASURES
Amendments to versions
Reference list.....

PollenLite Statistical Analysis Plan

A) QUANTITATIVE ANALYSIS PLAN**Principal investigator**

Dr Stephen Till

Clinical Senior Lecturer and Consultant (Hon) in Adult Allergy
Asthma, Allergy & Lung Biology,

[REDACTED]
 [REDACTED]
 [REDACTED]
 [REDACTED]
 [REDACTED]

Trial Investigators:

Dr Stephen Till

Dr Abdel Douiri

Dr Sun Ying

Dr Mohamed Shamji

Professor Stephen Durham

Ms Joanna Kelly

Ms Caroline Murphy

Professor Janet Peacock

Trial manager

Ms Caroline Murphy

King's Clinical Trials Unit [REDACTED]

[REDACTED]
 [REDACTED]
 [REDACTED]

Trial data manager

Ms Joanna Kelly

King's Clinical Trials Unit [REDACTED]

[REDACTED]
 [REDACTED]
 [REDACTED]
 [REDACTED]

Trial statisticians

Dr Abdel Douiri and Professor Janet Peacock

Department Of Primary Care And Public Health Sciences,

[REDACTED]
 [REDACTED]
 [REDACTED]
 [REDACTED]

PollenLite Statistical Analysis Plan

Abbreviations

AE	Adverse Event
AR	Adverse Reaction
CI	Chief Investigator
DMC	Data Monitoring Committee
DSUR	Development Safety Update Reports
eCRF	Electronic Case Record Form
eSMS	Emergency Scientific & Medical Services
EudraCT	European Union Drug Regulating Authorities Clinical Trials
FEV1	Forced Expiratory Volume
GCP	Good Clinical Practice
IgE	Immunoglobulin E
IMP	Investigational Medicinal Product
ISRCTN	International Standardised Randomised Controlled Trial Number
KCL	King's College London
KCTU	King's Clinical Trials Unit, King's College London (UKCRC registered KCTU)
KHP-CTO	Kings Health Partners Clinical Trials Office (function of the sponsor)
MHRA	Medicines & Healthcare products Regulatory Agency
NIHR	National Institute for Health Research
NRES	National Research Ethics Service
PEF	Peak Expiratory Flow
PollenLITE	Pollen Low dose Intradermal Therapy Evaluation
REC	Research Ethics Committee
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
SOP	Standard Operating Procedure
SPC / SmPC	Summary of Product Characteristics
SUSAR	Suspected Unexpected Serious Adverse Reaction
TSC	Trial Steering Committee
UKCRN	UK Clinical Research Network

PollenLite Statistical Analysis Plan

1. Description of the trial

Subcutaneous immunotherapy with high dose grass pollen was first described over 100 years ago. This treatment suppresses allergen-induced cutaneous late responses, with lesser effects on early responses. In contrast, low dose subcutaneous immunotherapy has failed to show clinical benefit. Uncontrolled reports from the early 20th century describe low dose allergen inoculation directly into the dermis, an immunologically active area containing abundant dendritic cells and lymphatics. We previously reported that repeated 2-weekly intradermal injections of grass pollen - each containing approximately 7 ng of major allergen Phl p 5 – led to a progressive suppression of the allergen-induced cutaneous response, and that by the sixth injection, this was inhibited by over 90%.

The purpose of this trial is to investigate the clinical efficacy of intradermal desensitisation with low doses of grass pollen allergen for seasonal allergic rhinitis.

1.1 Principal research objectives to be addressed

We hypothesise that low dose intradermal grass pollen allergen immunotherapy is an effective treatment for seasonal allergic rhinitis ('hay fever'), reducing symptoms and rescue medication requirements, and improving quality of life for hay fever sufferers.

Primary objectives

The primary objective is to determine if pre-seasonal low dose intradermal grass pollen allergen immunotherapy (either 7 or 8 two-weekly injections of 10 Biological Units (33.333 SQ-U)) reduces symptoms and requirements for anti-allergic drugs in seasonal allergic rhinitis during the 2013 grass pollen season compared to the control intervention (histamine only).

Secondary objectives

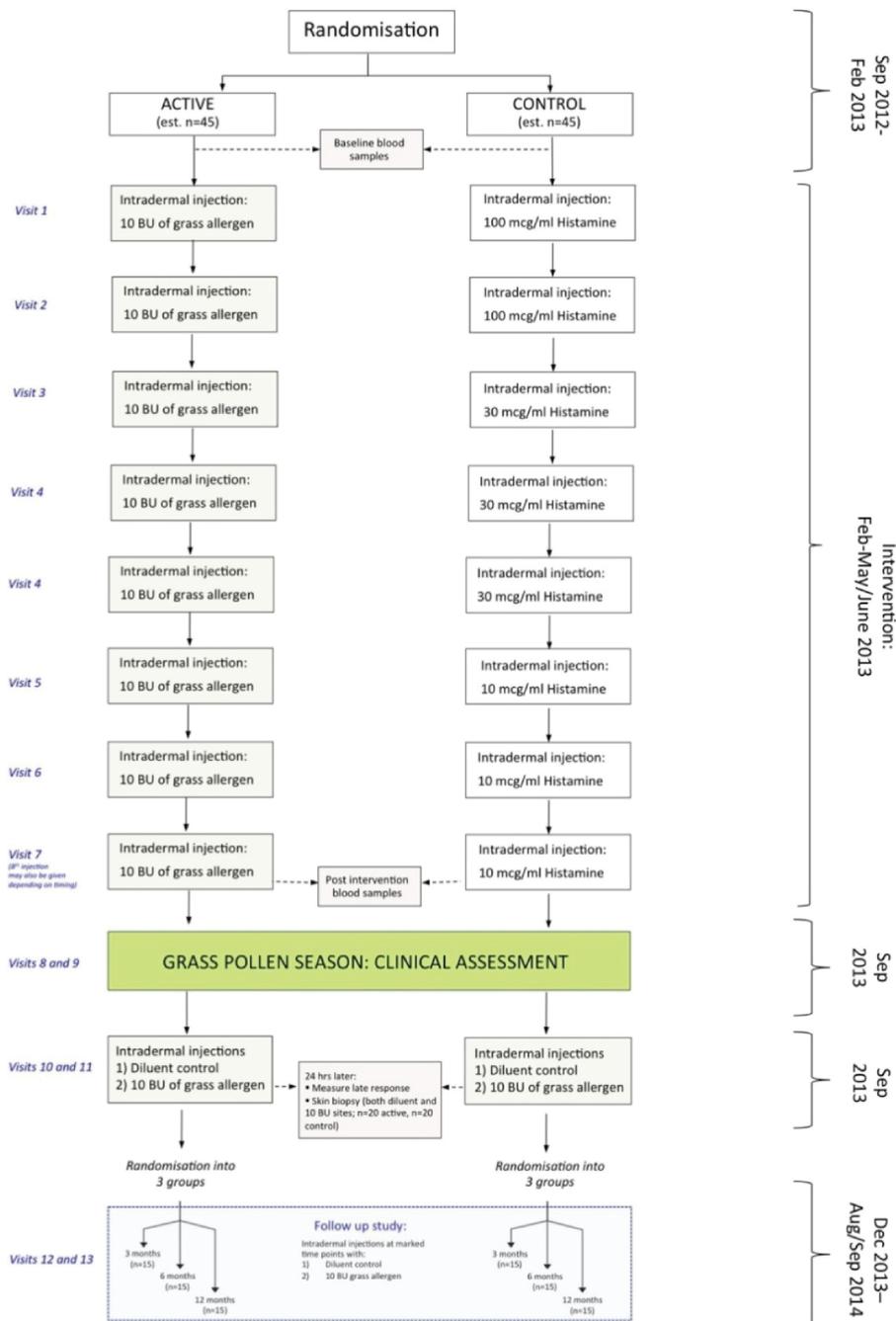
- 1) Determine if this intervention is associated with improvement in quality of life compared to the control intervention, as assessed during the 2013 grass pollen season.
- 2) Evaluate if this is a safe and well-tolerated form of treatment.
- 3) Investigate immunological mechanisms associated with this form of treatment, by examining humoral and cellular responses, both in peripheral blood and in tissue.
- 4) Explore if the intradermal desensitisation effect is long-lived i.e. persists following cessation of intradermal injections.

1.2 Trial design and flowchart

Single centre double-blind randomised parallel group controlled trial

Figure 1. Trial flowchart

PollenLite Statistical Analysis Plan



1.3 Populations and Study Sample

Target Population

PollenLite Statistical Analysis Plan

The target population, to which inferences from the end of the PollenLite trial are intended to generalise, is the population of patients with history of allergic rhinoconjunctivitis.

Trial Population

The trial population, from which the study sample is drawn, is further defined to be patients aged 18-65 years at commencement of pollen low dose intradermal therapy, who are screened at Guy's Hospital, King's College London, and who have history of moderate-severe persistent rhinoconjunctivitis.

Trial Sample

The achieved trial sample comprises those patients who consent to participate and are actually randomised into the PollenLite trial. These patients are the study subjects. This randomised trial sample is also the trial Intention To Treat (ITT) population. Subjects will be analysed according to the treatment group to which they are randomised. The trial ITT population comprises all randomised participants, regardless of eligibility (inclusion/exclusion) error, post-randomisation withdrawal, and whether the correct study treatments were received, or other interventions received.

Inclusion criteria

- 1) Adults aged 18 to 65 years.
- 2) A clinical history of grass pollen-induced allergic rhinoconjunctivitis for at least 2 years with peak symptoms in May, June, or July.
- 3) A clinical history of moderate-severe persistent rhinoconjunctivitis symptoms interfering with usual daily activities or with sleep.
- 4) A clinical history of rhinoconjunctivitis that remains troublesome despite treatment with either antihistamines or nasal corticosteroids during the grass pollen season.
- 5) Positive skin prick test response, defined as wheal diameter greater than or equal to 3 mm, to *Phleum pratense*.
- 6) Positive specific IgE, defined as greater than or equal to IgE class 2, against *Phleum pratense*.
- 7) For women of childbearing age, a willingness to use an effective form of contraception for the duration of intradermal injections.
- 8) The ability to give informed consent and comply with study procedures.

Exclusion criteria

- 1) Pre-bronchodilator FEV1 less than 70% of predicted value at screening visit.
- 2) A history of seasonal grass pollen-induced asthma requiring regular treatment with salbutamol or inhaled corticosteroids. Patients with mild seasonal grass pollen-induced asthma may be included, provided symptoms are satisfactorily controlled with occasional salbutamol only.

PollenLite Statistical Analysis Plan

- 3) A clinical history of symptomatic seasonal allergic rhinitis and/or asthma due to tree pollen or weed pollen near or overlapping the grass pollen season, although patients with mild intermittent symptoms requiring only occasional antihistamines may be included.
- 4) A clinical history of symptomatic allergic rhinitis and/or asthma caused by a perennial allergen to which the participant is regularly exposed, although patients with mild intermittent symptoms requiring only occasional antihistamines may be included.
- 5) Emergency department visit or hospital admission for asthma in the previous 12 months.
- 6) History of chronic obstructive pulmonary disease.
- 7) History of significant recurrent acute sinusitis, defined as 2 episodes per year for the last 2 years, all of which required antibiotic treatment.
- 8) History of chronic sinusitis, defined as a sinus symptoms lasting greater than 12 weeks outside the grass pollen season, which includes 2 or more major factors or 1 major factor and 2 minor factors. Major factors are defined as facial pain or pressure, nasal obstruction or blockage, nasal discharge or purulence or discoloured postnasal discharge, purulence in nasal cavity, or impaired or loss of smell. Minor factors are defined as headache, fever, halitosis, fatigue, dental pain, cough, and ear pain, pressure, or fullness.
- 9) At randomisation, current symptoms of, or treatment for, upper respiratory tract infection, acute sinusitis, acute otitis media, or other relevant infectious process; serous otitis media is not an exclusion criterion. Participants may be re-evaluated for eligibility after symptoms resolve.
- 10) Current smokers or a history of greater than or equal to 5 pack years.
- 11) Previous treatment by immunotherapy with grass pollen allergen within the previous 5 years.
- 12) History of life-threatening anaphylaxis or angioedema.
- 13) Ongoing systemic immunosuppressive treatment.
- 14) History of intolerance of grass pollen immunotherapy, rescue medications or their excipients.
- 15) For females of childbearing age a positive serum or urine pregnancy test with sensitivity of less than 50 mIU/mL within 72 hours of first administration of study therapy.
- 16) Lactating females.
- 17) The use of any investigational drug within 30 days of the screening visit.
- 18) Ongoing treatment with leukotriene receptor antagonists, beta-blockers, calcium channel blockers, tricyclic antidepressants, monoamine oxidase inhibitors or anti-IgE monoclonal antibody.
- 19) The presence of any medical condition that the investigator deems incompatible with participation in the trial.
- 20) Individuals with insufficient understanding of the trial.

Safety analysis population

The safety analysis population is comprised of those randomised subjects who receive at least one treatment with pre-seasonal intradermal injections of *Phleum pratense* grass pollen extract and/or Histamine.

PollenLite Statistical Analysis Plan

1.4 Method of allocation of groups

Once baseline assessments are complete (Screening visit), the individuals will be randomised to one of the treatment arms.

Randomisation will be done in a 1:1 ratio. Participants will be stratified into 2 equal groups according to i) size of skin test response to grass pollen at screening visit, and ii) presence or absence of rhinitis symptoms outside the grass pollen season and block randomised.

Females of childbearing age will be required to undergo a urine pregnancy test with sensitivity of less than 50 mIU/mL within 72 hours of randomisation and first administration of study therapy at Visit 1.

Pre-randomisation allocation concealment will be achieved through the blinding of the study medication. In addition, to minimise unconscious bias through unintentional unblinding, the control intervention will consist of a reducing dose of histamine.

24hr Emergency Code Break and Medical Information will be provided by Guy's & St Thomas' NHS Foundation Trust Emergency Scientific Medical Services (eSMS). Each randomised subject will be provided with a card detailing code break telephone numbers and emergency contact details. Subjects will be requested to carry this card with them at all times whilst participating in the trial.

1.5 Description of interventions

Intradermal grass pollen injections plus rescue medications (intervention) group will be compared to a histamine injections plus rescue medications (control) group in adults with moderate-severe grass pollen-induced allergic rhinitis ('summer hay fever')

Rescue medications

Rescue medications will be provided to all participants in both trial arms before and throughout the pollen season. These will include: desloratadine (5 mg, up to 1 tablet daily), (olopatadine eye drops, 1.0 mg/mL, up to 1 drop per eye twice daily), fluticasone propionate nasal spray 50 mcg per spray, up to 2 sprays per nostril once daily), and prednisone (for use at 30 mg per day for up to 5 days). Participants will be asked to use only these medications to treat their hay fever symptoms on an as required basis. However, participants who are not getting hay fever symptoms will be encouraged to try not to use these medications. Participants will be asked to use only these medications. A short course of prednisolone will be available if symptoms are particularly severe. Participants will be instructed to contact a trial physician prior to taking any prednisolone. The doctor will then provide instructions on dose and duration of treatment. Concurrent treatment with beta-blockers, calcium channel blockers, tricyclic antidepressants, monoamine oxidase inhibitors or anti-IgE monoclonal antibody will not be permitted.

Control group

PollenLite Statistical Analysis Plan

Intradermal injection of histamine, administered at a concentration of 100 mcg/ml (histamine dose validated by Sherer et al., Clin Exp Allergy. 2007;37:39-46).

Intervention group

Intradermal injections of *Phleum pratense* grass pollen extract, each containing estimated 7 ng of major allergen Phl p 5.

1.6 Duration of the treatment period

Intervention consists of maximum of 8 injections, given at approximately 2-weekly intervals over 3 months. Two further open label injections of grass pollen (10 BO) will be given over a 3 to 12 month follow up period for mechanistic assays.

1.7 Frequency of follow-up and duration of the trial

Frequency of follow-ups is summarised in trial diagram (Figure 1), including screening and 13 visits. The duration of the trial is 2 years. The trial will end when the last subject makes the last visit to determine the late response following the final open label follow up intradermal injection at the Aug 2014 time point.

1.8 Trial efficacy end point

Pollen counts

The peak of grass pollen season will be defined as starting on the first 3 consecutive days between 13 May and 31 August 2013 when grass pollen counts in central London are >30 grains/cm³, using counts supplied by the UK Met Office. The end of the peak season will be defined as the first of 3 consecutive days when grass pollen counts are <30 grains/cm³. In the event of 2 or more peaks during the 2013 season, these individual peak periods will be analysed separately.

Efficacy assessments

Using diaries patients recorded their individual symptoms scores (reflecting the preceding 24 hours) on a daily basis from mid-May through to the end of August. The symptom scoring systems have been adapted from previous trials of grass pollen immunotherapy. The symptom score will be based on individual symptoms in the nose (sneezing, blockage, and running), eyes (itching, redness, tears, and swelling), mouth and throat (itching and dryness), and chest (breathlessness, cough, wheezing, and tightness), recorded on a scale of 0 to 3 (with a score of 0 indicating no symptoms and 1, 2, and 3 indicating mild, moderate, and severe symptoms, respectively). The maximum daily symptoms score will therefore be 39.

PollenLite Statistical Analysis Plan

All possible rescue medications will be provided to each participant approximately 2 weeks before and throughout the pollen season. Each drug was given according to the recommendation of the manufacturer. No other medication was allowed. Daily medication use will also be recorded in diary cards by participants and a medication score calculated based on use according to need of the following medications: desloratadine, 5 mg, up to 1 tablet daily (6 points per day); olopatadine eye drops, 1.0 mg/mL, up to 1 drop per eye twice daily (1.5 points per drop, up to 6 points per day); fluticasone nasal spray, 50 mcg per spray, up to 2 sprays per nostril once daily (2 point per spray, up to 8 points per day); and prednisone, 5 mg per tablet, up to 6 tablets per day (2 points per tablet, up to 12 points per day). The maximum daily medication score will therefore be 32.

Since scores for symptoms and medications are different in magnitude these parameters will be normalised in accordance with World Allergy Organization guidance on immunotherapy trials. In order to make the range of the outcome measure invariant over the number of symptoms scored, we divide by the number of individual symptoms evaluated, so that the score has a range from 0 to 3. Medication scores will be then normalised to the symptoms scores so that it is given equal range 0 to 3.

Primary efficacy end point

The primary outcome measure will be combined symptom and medication score (SMS) defined as the area under curve (AUC) of the sum of the normalised daily rescue medication score and the daily symptom score for all days of the pollen season.

Efficacy will then be assessed by comparison of this combined score in active and control groups and estimate of the treatment effect will be expressed in means of median differences with confidence intervals, with a significance level of $p = 0.05$.

Secondary efficacy end points

- 1) Symptom scores (AUC) calculated as above.
- 2) Medication scores (AUC), calculated as above.
- 3) Rhinoconjunctivitis Quality of Life: mini Rhinitis Quality of Life Scores (RQLQ) scores (overall score and domain scores) will be recorded three times during the pollen season (June 12, June 26 and July 10) and once after the season on 4 September 2013. These values will be compared in active and control groups. The mini RQLQ covers five dimensions of health including sleep, non-nose/eye symptoms, practical problems, nasal symptoms, eye symptoms.
- 4) Health related quality of life: This will be evaluated using the EQ-5D-5L questionnaire three times during the pollen season (June 12, June 26 and July 10) and once after the season on 4 September 2013.
- 5) Visual Analogue Scores (see Additional file). These will be recorded every 2 weeks during the pollen season and AUC values calculated.
- 6) Global evaluation scores (see Additional file).
- 7) The number of primary care (i.e. general practitioner) visits for hay fever during summer 2013.

PollenLite Statistical Analysis Plan

- 8) Combined symptom and medication scores during the peak of the 2013 grass pollen season.
- 9) Number of medication free days covering the grass pollen season period of 13th May-end August 2013 will be compared in active and control groups.
- 10) Number of symptom free days covering the grass pollen season period of 13th May-end August 2013 will be compared in active and control groups.
- 11) Individual symptoms scores (AUC) for each organ: nose, mouth, eyes and lungs.
- 12) Total number of days during which prednisolone used between 13th May-end August 2013.

Assessment of safety

Adverse events were documented throughout the study. Systemic reactions were graded according to the EAACI classification. Details on AE are described in the protocol

1.9 Sample size estimation (including clinical significance)

Power calculations for the primary outcome (combined symptom and medication score) were performed based on a previous clinical trial of subcutaneous grass pollen immunotherapy conducted by Varney et al. The power calculation has been conservatively based on the detection of a clinical effect size 80% of that reported in the Varney trial. Since subcutaneous grass pollen immunotherapy is the gold standard treatment such an effect size would be viewed as clinically meaningful. This power calculation has been performed after readjustment to medication scores such that the combined symptom and medication score endpoint gives equal weighting to both parameters. Using this method, group sample sizes of 35 and 35 achieve 90% power to detect a difference of 80% in combined symptom and medication scores between the null hypothesis that both arms means are 638.0 with estimated group standard deviations of 271.0 and the alternative hypothesis that the mean of the intervention arm is 419.0 at a significance level of 0.05, using a two-sided Mann-Whitney test assuming that the actual distribution is normal. To adjust for the unknown distribution of the primary outcome and based on the lower bound for the asymptotic relative efficiency (ARE) of the Mann-Whitney U test. We have increased the sample size by a further 15% to 40 in each arm. Further accounting for a post-randomisation dropout rate of up to 10% consistent with previous trials of grass pollen immunotherapy, a total sample size of 90 (45 each arm) is required. Recruitment will take place several months before visit 1. At visit 1 randomisation will be performed and the first injection administered. To ensure that a minimum of 90 participants is randomised, up to 100 screened participants will be booked for visit 1, allowing for a 10% drop-out rate between screening and randomisation. In the event that more than 90 eligible participants attend for visit 1, all will be included in the study and randomised up to a maximum of 100.

1.9 Brief description of proposed analyses

PollenLite Statistical Analysis Plan

Analyses will be carried out by the trial statistician. In the first instance data will be analysed under intention-to-treat assumptions (i.e. analyse all those with data in groups as randomised irrespective of treatment received).

2. Data analysis plan – Data description

2.1 Recruitment and representativeness of recruited patients

Recruitment, randomisation and follow-up for PollenLite will be summarised by arm in a CONSORT flow-diagram.

This will include the main reasons for there being missing data (withdrawal, lost to follow up) by stages of the trial, and will also include the numbers for whom this occurs per arm.

Also included will be the number randomised, who comprise the intention to treat trial population, and the numbers followed-up to be in the analyses of the primary outcome.

2.2 Baseline comparability of randomised groups

Summary measures for the baseline characteristics of each group will be presented as mean and standard deviation for continuous (approximate) normally distributed variables, medians and interquartile ranges for non-normally distributed variables, and frequencies and percentages for categorical variables. No significance testing.

The characteristics will include socio-demographic descriptors (including sex and age), randomisation stratifiers, allergy history, symptoms, rhinoconjunctivitis severity (severe/moderate), and other baseline (screening) clinical measures.

This will allow a visual assessment of whether the randomisation procedure succeeded in producing comparable arms, and will not include the improper use of p-values from statistical hypothesis testing between arms at baseline. This will also show baseline characteristics of the trial sample for description in the main paper.

2.3 Loss to follow-up and other missing data

At least 50% of daily SMS scores has to be complete in order for a diary to be acceptable for evaluation. Data from subjects who do not submit valid diary data for at least 2 of the 4 peak pollen weeks will be considered as Missing data.

The proportions of participants missing each variable will be summarised in each arm and at each time point.

The baseline characteristics of those missing follow up will be compared to those with complete follow up with p-values from univariate statistical tests.

The reasons for withdrawal from the trial will be summarised.

Sample size estimation assumed 10% of patients would not provide evaluable end of study information. If this rate is observed, data for some patients will be only partially observed. Efforts were planned to reduce missing data by reminding participant to their 24 hour diary at the beginning, midway and at

PollenLite Statistical Analysis Plan

the end of pollen season. If data from one assessment point are missing, the mean value of the two adjacent ones will be used. Another alternative, the daily SMS could be determined by calculating a 3-day (or up to one week maximum) rolling average (previous, current and following days). For patients with missing data and for patients who withdrew or dropped two weeks before the peak pollen period end, multiple imputations method will be used in order to provide an overall treatment effect estimate with a standard error that is properly inflated to incorporate uncertainty associated with imputing values (i.e. between-imputation variability in the estimated treatment effect). Since this may introduce a bias if the main reason for drop-out was deterioration, sensitivity analysis will be examined to explore departures from the missing at random assumption using White et al intention to treat strategy.

2.5 Adverse event reporting

Adverse events (AE), adverse reactions (AR), serious adverse events (SAE) and serious adverse reactions (SAR) will be summarised.

2.6 Assessment of outcome measures (unblinding)

Evidence for unblinding of treatment to interviewers will be studied.

2.7 Descriptive statistics for main outcome measures

The Area under the Curves (AUC) of the individual and combined symptom and medication scores for the period corresponding to the grass pollen season (mid May-Aug) will be plotted against time as a summary measure of the primary outcome. This will provide each patient's longitudinal outcome as a single quantity, which will be calculated for Symptom and Medication scores.

3. Data analysis plan – Inferential analysis

3.1 Main analysis of treatment differences

The main statistical analyses will estimate the difference in mean outcomes between patients randomised to 45 and 45 by intention to treat at the various post-treatment observation time points. Group difference estimates and associated confidence intervals will be reported.

3.1.1 Analysis of primary outcomes

The planned primary efficacy analyses, difference between the two arms in AUC of the combined symptom and medication scores, will be analyzed on randomized patients using non-parametric approach, (stratified) Mann-Whitney U test (Van Elteren test statistic), adjusted for the baseline stratification factors size of the skin test to grass pollen and presence or absence of rhinitis symptoms. And the (stratified) Hodges-Lehmann estimation to calculate median differences with confidence intervals, with a significance level of $P = 0.05$.

PollenLite Statistical Analysis Plan

If the data distribution is normal or log-normal, analysis of covariance (ANCOVA), adjusted for the baseline stratification factors size of the skin test to grass pollen and presence or absence of rhinitis symptoms will replace the non-parametric analysis.

3.1.2 Analysis of secondary outcomes

Similar analyses as for the primary outcome measure will be conducted for secondary (symptom scores, medication scores and individual symptoms) and mechanistic outcomes. Subgroup analysis by holiday's destination will also be investigated. All patients who were on holiday in continental Europe will be included in the per protocol analysis. Those who holidayed outside of Europe are to also be in per protocol analysis but data for days where they are abroad are to be counted as missing data and >50% missing data threshold will be applied (See page 14, paragraph "Loss to follow-up and other missing data"). Extensive sensitivity analysis on all holiday destinations will be conducted.

Regression models will be also used to evaluate the change in RQLQ scores to isolate the effect of the intervention on each arm after adjusting for stratification factors.

In analysing the recovery of the cutaneous late response at each 3, 6 and 12 month time point, the size of late response in the group that originally received active therapy will be compared with the group that originally received the control intervention. As a further sensitivity analysis, all key outcomes will be re-analysed adjusting for any observed differences at baseline that are judged to be of clinical importance. Differences between the groups will be estimated with 95% confidence intervals.

3.1.3 Responder analysis

Responder analysis will be performed. Because we do not have a baseline year for comparison, the median AUC for the placebo group will be defined as the comparator, and responders defined as those subjects with AUC less than this value, using different cut-offs (20%, 25%, 30% etc.). The optimal value for distinguishing actively treated from placebo groups will be selected using receiver-operator curves, and numbers thus defined as responders and non-responders in each group were compared by chi-squared analysis. Clinical and laboratory characteristics of these groups will be also investigated.

3.1.4 Model assumption checks

If a model assume normally distributed outcomes; this will be checked when describing the data and if substantial departures from normality occur, transformations will be considered. Residuals will be plotted to check for normality and inspected for outliers.

3.2 Exploratory analyses

PollenLite Statistical Analysis Plan

Any examination of subgroups, not specifically identified in the protocol, will be considered exploratory in nature and will be clearly identified.

3.4 Interim analysis

No interim analysis is planned although pre-defined stopping criteria will be discussed by the TSC and the Independent DMEC and agreed if appropriate.

4. Reporting conventions

Reporting conventions will adhere when possible to the International Conference on Harmonization (ICH) Guidance document E3, "Structure and Content of Clinical Study Reports". Some specific conventions are outlined below:

1. All tables and listings will be in landscape format.
2. All statistical analysis software output for tables and listings will be distributed in PDF files.

5. Software

Data management: An online data collection system for clinical trials (MACRO; InferMed Ltd) will be used. This is hosted on a dedicated server at KCL and managed by the MH&N CTU. The MH&N CTU Data Manager will extract data periodically as needed and provide these in comma separated (.csv) format.

Statistical analysis: The principal software package will be STATA, with verification of results from syntax for selected analyses in SAS.

PollenLite Statistical Analysis Plan

B) ECONOMIC ANALYSIS PLAN

Health economic objectives

To assess the cost-effectiveness of low dose intradermal grass pollen allergen immunotherapy in adult patients with moderate-severe persistent rhinoconjunctivitis.

Economic measures

Economic measures will include cost of the intervention, volume of resource use for health services and related unit costs, and EQ-5D scores. Economic analyses will conform to NICE's preferred methodology. Outcomes will be reported as quality-adjusted life years (QALYs) and symptom-free days. Results will be subjected to simple and probabilistic sensitivity analysis.

Statistics

Because of the skewed nature of medication use and QoL data will be analysed using a (stratified) nonparametric test (Mann–Whitney) to compare resource use and QALYs.

PollenLite Statistical Analysis Plan

C) SCHEDULE OF ASSESSMENTS AND MEASURES***By Visit***

Visit -1 (Screening visit; Sep 2012-Jan 2013):

- Informed consent
- Medical history
- Allergy history
- Skin prick testing
- Recording of concomitant medications
- Limited Physical Examination
- Vital signs
- Spirometry
- Blood sample (5 ml) for total IgE and specific IgE (hospital lab)
- Blood sample (10 ml) for mechanistic assays (baseline sample)

Visit 1 (first intervention visit; 18th Feb-1st Mar 2013)

- Urine pregnancy test
- Recording of concomitant medications
- Intradermal injection with active or control drug
- Clinical observation for one hour
- Recording of adverse events (adverse events before randomisation at Visit 1 will not be recorded)

Visits 2-6 (Mar-May 2013)

- Recording of concomitant medications
- Intradermal injection with active or control drug
- Clinical observation for 30 minute
- Recording of adverse events

Visit 7 (May 2013)

- Recording of concomitant medications
- Blood sample (10 ml) for mechanistic assays (baseline sample)
- Intradermal injection with active or control drug (if visit 7 falls before 13 May, this injection will be repeated 12-16 days later)
- Clinical observation for 30 minutes
- Recording of adverse events

Visit 8 (early Jul 2013)

- Recording of concomitant medications
- Collection of May and June symptom/medication use diary cards
- Collection of Visual Analogue Scores for May/June
- Collection of Mini RQLQ and EQ-5D-5L forms for 12 and 26 June
- Recording of adverse events

Visit 9 (early Aug 2013)

- Recording of concomitant medications
- Collection of July symptom/medication use diary cards
- Collection of Visual Analogue Scores for July
- Collection of Mini RQLQ and EQ-5D-5L forms for 10 July

PollenLite Statistical Analysis Plan

- Recording of adverse events

Visit 10 (Sep 2013)

- Recording of concomitant medications
- Collection of Aug symptom/medication use diary cards
- Collection of Visual Analogue Scores for Aug
- Collection of Mini RQLQ and EQ-5D-5L forms for 4 September
- Global assessment score (1) and (2) completion
- Record number of GP visits over summer for hay fever
- Verify blinding: All participants to guess if received active or control intervention
- Additional informed consent – skin biopsy specific form (n=40)
- Intradermal injection with diluent (negative control) and 10 BU (33.333 SQ-U) grass pollen allergen (open label)
- Measurement of skin early response size (after 15 mins)
- Clinical observation for 30 minutes
- Recording of adverse events

Visit 11 (24 hrs after Visit 10 in Sep 2013)

- Recording of concomitant medications
- Measurement of skin late response size (all participants)
- Skin biopsy of diluent and allergen intradermal injection sites (40 random participants only)
- Recording of adverse events

Visit 12 (randomised to either Dec 2013, Mar 2013 or Aug 2014)

- Recording of concomitant medications
- Intradermal injection with diluent (negative control) and 10 BU (33.333 SQ-U) grass pollen allergen (open label)
- Measurement of skin early response size (after 15 mins)
- Recording of serious adverse events

Visit 13 (24 hrs after Visit 12)

- Recording of concomitant medications
- Measurement of skin late response size
- Recording of serious adverse events

Laboratory Tests

Visit -1 (Screening visit):

A sample of venous blood (5 ml) will be collected for total IgE and specific IgE, which will be analysed routinely by the Immunology department of Guy's and St Thomas' NHS Foundation Trust. A sample of blood (10 ml baseline sample) will also be collected at the same time for mechanistic studies in academic laboratories. This sample will be centrifuged and serum aliquoted and stored at -20°C in the Chief Investigator's KCL laboratory pending analysis in the laboratory of Professor Durham (co-investigator) at Imperial College. All identifying data will be in anonymised form. Study participants will be asked to provide informed consent storage of their samples for a minimum

PollenLite Statistical Analysis Plan

of ten years for future studies as novel serum-based assays of immune tolerance become available.

Visit 7:

A further sample of blood (15 ml post-intervention sample) will be collected for mechanistic studies in academic laboratories. As previously, a 10 ml sample will be centrifuged and serum aliquoted and stored at -20°C in the Chief Investigator's KCL laboratory pending analysis in the laboratory of Professor Durham (co-investigator) at Imperial College. Study participants will again be asked to provide informed consent storage of their samples for a minimum of ten years for future studies as novel serum-based assays of immune tolerance become available. The additional 5 ml will be collected into a heparinised tube for basophil activation studies in fresh whole blood.

Visit 11:

Two 3-mm skin punch biopsies will be collected 24 hours after diluent and allergen intradermal injections. The biopsy will be taken from the injection site under local anaesthesia. This will only be performed in a sub-group of 40 participants identified at random by the King's Clinical Trials Unit (who are performing randomisation for the whole trial). Biopsies will be fixed in paraformaldehyde, processed, and stored at -80°C in the Chief Investigator's KCL laboratory prior to analysis by immunochemistry. In addition, the first 20 biopsies will be divided into 2 equal pieces using a sterile scalpel: one piece will be processed as above, and the second piece will be cultured in vitro for T cell analysis in the Chief Investigator's KCL laboratory.

PollenLite Statistical Analysis Plan

- Figure 1. Trial flowchart

	Screening		Intervention period												2013-4			
	2012-3		2013												2013-4			
	Year	Month	2-weekly intervals (range: 12-16 days)*												Start July	Start Aug	Sep	Sep
Year	Month	18 Feb-1 Mar	4-15 Mar	18-29 Mar	1 Apr- 12 Apr	15-26 Apr	29 Apr- 10 May	13-24 May	(if visit 7 falls before May 13, further infection approx 2 wks later	8	9	10	11	12	13			
General Assessments																		
Informed consent		X																
Informed consent – skin biopsy specific form																		
Medical history		X																
Allergy history		X																
Limited physical exam		X																
Vital signs		X																
Spirometry		X																
Adverse events			X	X	X	X	X	X	X	X	X	X	X	X	X	SAE only		
Concomitant medications		X	X	X	X	X	X	X	X	X	X	X	X	X	X	SAE only		
Randomization		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
Re-randomisation for skin biopsy and follow-up intradermal injection		X																
Clinical Assessments																		
Skin prick tests		X																
Urine pregnancy test			X															
Local Laboratory Assessments																		
Total IgE		X																
Timothy grass RAST		X																
Intervention																		
Active or control intradermal injection		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
1 hour observation		X																
30 mins observation		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
Clinical outcomes																		
Symptom score																		
Medication score																		
Visual Analogue Score																		
miniRQLQ																		
EO-5D-5L																		
Visit for diary/score card collection																		
Record number of GP visit for hay fever over summer																		
Global assessment (1)																		
Global assessment (2)																		
Verify blinding (participants to be asked if received active or control)																		
Mechanistic Laboratory Assessments																		
Serum for antibody assays		X																
Whole blood for basophil assays																		
Intradermal allergen challenge (diluent and 10 BU grass pollen)																X		
Measurement of skin early response (15 mins post challenge)																X		
Measurement of skin late response (24 hrs post challenge)																X		
Skin biopsy (diluent and allergen sites) (n=40 only)																X		

PollenLite Statistical Analysis Plan

Amendments to versions

Previous:

- Version 1.0: 02-04-2012 Developed from the EME-NIHR submission research protocol
- Version 1.2: 08-05-2013 Emails/Phones discussion with Steve Till and Janet Peacock
 - Addition of four additional secondary outcomes concurred with various immunotherapy and regulatory guidelines
 - Number of medication free days covering the grass pollen season period of 13th May-end August 2013 will be compared in active and control groups
 - Number of symptom free days (well days) covering the grass pollen season period of 13th May-end August 2013 will be compared in active and control groups
 - Individual symptoms scores (AUC) for each organ: nose, mouth, eyes and lungs
 - Total number of days during which prednisolone used between 13th May-end August 2013
 - Addition of responder analysis in planned statistics as recommended by various immunotherapy and regulatory guidelines
- Version 1.3: 08-07-2014 with Steering committee
 - Page 14: lower the percentage of permissible data to 50%, previously this was 75%.
 - Page 16: To support the primary outcome finding, a subgroup analysis by holiday's destination will also be investigated.

Current:

Principal amendments from previous version:

- Version 2.0: 05-09-2014:
 - Page 13: the sentence "Investigators' terms of adverse events were coded using the Medical Dictionary for Regulatory Activities (MedDRA)" was removed from "Assessment of safety" paragraph.
 - Page 16: the sentence "All patients who were on holiday in continental Europe will be included in the per protocol analysis. Those who holidayed outside of Europe are to also be in per protocol analysis but data for days where they are abroad are to be counted as missing data and >50% missing data threshold will be applied (See page 14, paragraph "Loss to follow-up and other missing data"). Extensive sensitivity analysis on all holiday's destination will be conducted." was added in "Analysis of secondary outcomes" paragraph

PollenLite Statistical Analysis Plan

Reference list

Reference List

Protocol for a double-blind randomised controlled trial of low dose intradermal grass pollen immunotherapy versus a histamine control on symptoms and medication use in adults with seasonal allergic rhinitis (PollenLITE). Protocol paper version 2.8 date: 08-05-2012

Canonica GW, Baena-Cagnani CE, Bousquet J, et al. Recommendations for standardization of clinical trials with allergen specific immunotherapy for respiratory allergy. A statement of a World Allergy Organization (WAO) taskforce. *Allergy* 63:317–324, 2007

Moher D, Hopewell S, Schulz KF, Montori V, Gotzsche PC, Devereaux PJ, Elbourne D, Egger M, Altman DG. Consort 2010 explanation and elaboration: Updated guidelines for reporting parallel group randomised trials. *Bmj*. 2010;340:c869

Varney VA, Hamid QA, Gaga M, Ying S, Jacobson M, Frew AJ, et al. Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *The Journal of clinical investigation*. 1993;92(2):644-51

White IR, Horton NJ, Carpenter J, Pocock SJ. (2011) Strategy for intention to treat analysis in randomised trials with missing outcome data. *BMJ* 342:d40

Lehmann EL. *Nonparametrics: Statistical methods based on ranks*. 1975. San Francisco: Holden-Day. 457 pp.

Appendix 7 Effect of intradermal immunotherapy on primary and secondary outcomes (intention to treat): missing data imputed

Trial outcomes	Intradermal immunotherapy (n = 46), median (IQR)	Control (n = 47), median (IQR)	Difference (95% CI)	p-value
Primary outcome				
CSMS during entire season	502 (333–841)	509 (365–738)	8 (–174.7 to 210.9)	0.91
Secondary outcomes (median (IQR))				
Symptom score during entire season	335 (183–525)	264 (156–434)	61 (–7.8 to 123.2)	0.22
Medication score during entire season	242 (116–405)	263 (129–482)	–24 (–173.1 to 107.5)	0.39
CSMS score during peak season	363 (232–570)	370 (292–573)	–11 (–95.8 to 77.5)	0.80
Nasal symptom score during entire season	178 (120–218)	131 (80–200)	33 (0.3 to 68.5)	0.03
Mouth symptom score during entire season	39 (8–90)	14 (6–45)	11 (3.1 to 26.1)	0.05
Eye symptom score during entire season	79 (41–158)	78 (52–180)	–7 (–20.0 to 3.0)	0.51
Lung symptom score during entire season	20 (3–32)	12 (0–40)	4 (–1.0 to 15.3)	0.17
Nasal allergic symptoms measured by VAS	162 (107–275)	124 (66–166)	59 (–3.7 to 133.2)	0.02
Eye allergic symptoms measured by VAS	97 (37–197)	112 (42–169)	2 (–45.6 to 49.0)	0.56
Global Evaluation of symptom scores	3 (2–4)	3 (1–3)	0 (0.0 to 1.0)	0.43
Symptom-free days	35 (19–53)	41 (23–61)	–6 (–17.0 to 3.0)	0.15
Number of days prednisolone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	0.36
Medication-free days	81 (65–93)	76 (56–94)	4 (–11.0 to 21.0)	0.22
Mini-RQLQ	16 (13–23)	18 (10–25)	–0.3 (–4.2 to 3.7)	0.89
EQ-5D-5L	87 (83–94)	88 (81–94)	9 (–24.8 to 43.6)	0.59

Note

Data for primary outcome and all symptom scores represent AUC values.

Median difference between groups calculated by stratified Hodges–Lehmann method.

The p-values are based on a stratified Mann–Whitney U-test (van Elteren's test), adjusted for stratification factors.

The p-values for the Mini-RQLQ and EQ-5D-5L are based on a linear mixed model, adjusted for stratification factors.

Entire grass pollen season 13 May to 3 August 2013; peak season 12 June to 26 July 2013.

Appendix 8 Effect of intradermal immunotherapy on primary and secondary outcomes (per-protocol analysis)

Trial outcomes	Intradermal immunotherapy (n = 45), median (IQR)	Control (n = 39), median (IQR)	Difference (95% CI)	p-value
Primary outcome				
CSMS during entire season	517 (344–841)	453 (279–685)	82 (–121.8 to 280.1)	0.23
Secondary outcomes				
Symptom score during entire season	340 (189–503)	241 (150–398)	76 (25.9 to 133.5)	0.09
Medication score during entire season	255 (119–405)	254 (113–358)	21 (–125.0 to 157.0)	0.83
CSMS score during peak season	363 (242–546)	342 (242–476)	18 (–73.2 to 127.5)	0.51
Nasal symptom score during entire season	173 (123–207)	119 (80–205)	40 (13.3 to 71.5)	0.02
Mouth symptom score during entire season	38 (8–90)	14 (4–43)	14 (4.9 to 32.0)	0.02
Eye symptom score during entire season	80 (41–153)	72 (48–145)	0 (–16.0 to 17.6)	0.85
Lung symptom score during entire season	17 (3–32)	11 (0–21)	9 (1.0 to 17.0)	0.05
Nasal allergic symptoms measured by VAS	162 (105–275)	118 (50–154)	68 (8.3 to 134.6)	0.01
Eye allergic symptoms measured by VAS	90 (32–197)	114 (42–159)	1 (–52.8 to 62.0)	0.49
Global Evaluation of symptom scores	3 (2–4)	3 (1–3)	1 (0.0 to 1.0)	0.25
Symptom-free days	34 (19–47)	44 (25–67)	–12 (–22.0 to –2.0)	0.04
Number days prednisolone used during entire season	0 (0–0)	0 (0–0)	0 (0 to 0)	0.33
Medication-free days	80 (65–92)	78 (66–98)	–1 (–20.0 to 17.0)	0.87
Mini-RQLQ	16 (13–23)	17 (10–22)	–2.0 (–5.89 to 1.88)	0.31
EQ-5D-5L	88 (83–94)	88 (84–94)	3 (–28.4 to 35.2)	0.83

Note

Data for primary outcome and all symptom scores represent AUC values.

Median difference between groups calculated by stratified Hodges–Lehmann method.

The p-values are based on a stratified Mann–Whitney U-test (van Elteren's test), adjusted for stratification factors.

The p-values for the Mini-RQLQ and EQ-5D-5L are based on a linear mixed model, adjusted for stratification factors.

Entire grass pollen season 13 May to 3 August 2013; peak season 12 June to 26 July 2013.

Appendix 9 Effect of intradermal immunotherapy on daily organ symptom scores (intention to treat)

Trial outcomes	Intradermal immunotherapy (n = 46), median (IQR)	Control (n = 47), median (IQR)	Difference (95% CI)	p-value
Nose				
Sneezing	76 (43.3–103.0)	55 (35.0–71.0)	21 (7.0 to 34.0)	0.01
Blockage	41 (14.0–74.5)	36 (12.5–61.0)	6 (–2.5 to 13.5)	0.33
Running	51 (30.0–81.5)	46 (22.5–65.4)	10 (–3.0 to 22.8)	0.17
Mouth				
Itching	19 (4.0–52.3)	8 (1.0–25.0)	4 (1.8 to 6.8)	0.06
Drying	7 (0.0–40.0)	3 (0.0–15.0)	3 (0.0 to 9.6)	0.18
Eyes				
Itching	48 (21.0–68.0)	44 (26.0–72.5)	–1 (–5.0 to 2.0)	0.99
Redness/sore	17 (4.0–42.0)	14 (7.0–45.0)	–1 (–6.0 to 3.0)	0.55
Streaming	11 (2.0–19.0)	14 (2.0–24.0)	0 (–4.0 to 3.0)	0.69
Swelling	2 (0.0–9.0)	5 (0.0–14.0)	–2 (–4.0 to 0.0)	0.03
Lungs				
Breathlessness	0 (0.0–4.0)	0 (0.0–8.1)	0 (0.0 to 2.0)	0.27
Cough	8 (1.0–23.3)	1 (0.0–12.1)	2 (0.0 to 6.0)	0.02
Wheezing	3 (0.0–7.0)	0 (0.0–8.0)	0 (0.0 to 2.0)	0.25
Tightness	2 (0.0–4.0)	0 (0.0–4.0)	0 (0.0 to 2.0)	0.08

Note

Data shown represent AUC values.

Median difference between groups calculated by stratified Hodges–Lehmann method.

The p-values are based on a stratified Mann–Whitney U-test (van Elteren's test), adjusted for baseline stratification factors.

Appendix 10 Effect of intradermal immunotherapy on individual visual analogue scale scores (intention to treat)

Trial outcomes	Intradermal immunotherapy (n = 46), median (IQR)	Control (n = 47), median (IQR)	Difference (95% CI)	p-value
Nose				
Blockage	152 (71.4–238.7)	118 (39.1–178.8)	39 (1.6 to 82.8)	0.12
Running	169 (96.0–265.6)	117 (62.0–162.7)	58 (–8.2 to 124.5)	0.006
Itching	138 (93.2–281.7)	81 (41.9–141.6)	64 (–16.3 to 165.4)	0.003
Sneezing	187 (133.1–295.3)	125 (46.1–182.4)	77 (–1.6 to 150.9)	0.006
Eyes				
Itching	120 (53.7–248.3)	135 (41.9–217.8)	4 (–35.3 to 46.1)	0.97
Watering	69 (21.0–129.5)	71 (33.6–119.4)	1 (–40.5 to 55.5)	0.792

Note

Data shown represent AUC values.

Median difference between groups calculated by stratified Hodges–Lehmann method.

The p-values are based on a stratified Mann–Whitney U-test (van Elteren's test), adjusted for baseline stratification factors.

A decorative graphic consisting of numerous thin, parallel green lines that curve from the left side of the page towards the right, creating a sense of movement and depth.

**EME
HS&DR
HTA
PGfAR
PHR**

Part of the NIHR Journals Library
www.journalslibrary.nihr.ac.uk

This report presents independent research funded by the National Institute for Health Research (NIHR). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health

Published by the NIHR Journals Library