Study Protocol

Nasal Challenge with Microbial Constituents and Allergen

Development of Human Nasal Challenge Models with Microbial Constituents and Grass Pollen:

- **monophosphoryl lipid A (MPLA)**
- **poly-inosine-cytosine (poly-IC)**
- **poly-inosine-cytosine stabilised with poly-L-lysine and carboxymethylcellulose (poly-ICLC)**
- **resiquimod**
- **tuberculin**
- **Timothy grass pollen and mechanistic study with Vitamin D**

Study Number: 13SM1837

CHIEF INVESTIGATOR: Dr Trevor Hansel

CO-INVESTIGATORS

- Prof. Peter Openshaw and Prof Robin Shattock: TLR-agonists (MPLA, poly-IC, poly-ICLC, resiquimod)
- Prof Ajit Lalvani, Dr Onn Min Kon, Dr Robert Davidson: tuberculin
- Prof Sebastian Johnston: allergen
- Dr Zoltan Takats: mass spectrometry and metabolomics
- Prof Kasia Hawrylowicz on vitamin D mechanistic study with grass pollen

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NRES reference: 13/LO/1899

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Name & Role	Date	Signature

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- Prof. Robin Shattock & Peter Openshaw: TLR-agonists
- Prof Ajit Lalvani, Dr Onn Min Kon & Dr Robert Davidson: tuberculin
- Prof Sebastian Johnston: allergen
- Prof Kasia Hawrylowicz on vitamin D mechanistic study with grass pollen

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Sponsor

Imperial College London is the main research Sponsor for this study. For further information regarding the sponsorship conditions, please contact the Head of Regulatory Compliance at:

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National Institute of Health Research (NIHR) Imperial College and Imperial NHS Trust Biomedical Research Centre (BRC)

This protocol describes the "Nasal challenge with microbial constituents and allergen" study and provides information about procedures for entering participants. Every care was taken in writing this protocol and this has been reviewed by the co-investigators. Problems relating to this study should be referred, in the first instance, to the Chief Investigator.
This study will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (2nd edition). It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

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GLOSSARY OF ABBREVIATIONS

ACQ	asthma control questionnaire
AE	adverse events
BRC	Biomedical Research Centre
CC	chemokine of cysteine-cysteine pattern
CXC	chemokine of cysteine-X-cysteine pattern
CRF	case report form
FEV_1	forced expiratory volume in 1st second
IGRA	interferon-γ release assay
IL	interleukin
LPS	lipopolysaccharide
LTBI	latent tuberculosis infection
MLF	mucosal lining fluid
MPLA	monophosphoryl lipid A
NIHR	National Institute of Healthcare Research
NAC	nasal allergen challenge
NC	nasal challenge
PBMC	peripheral blood mononuclear cell
PNIF	peak nasal inspiratory flow
Poly-IC	poly inosine-cytosine
Poly-ICLC	poly-IC stabilised with poly-L-lysine and carboxymethycellulose
QFT-it	Quantiferon TB Gold-in-Tube
SAM	synthetic absorptive matrix
SAE	serious adverse event
SSP	special sampling probe
TB	tuberculosis
TNSS	total nasal symptom scores
TU	tuberculin unit
ViDAR	vitamin D nasal allergen challenge study

KEYWORDS

human challenge study,
allergy, asthma, hay fever, tuberculosis, respiratory tract infection, nasal, respiratory, lipopolysaccharide, virus, grass pollen, inflammation, innate immune response biomarkers, mucosal lining fluid, nasosorption
cytokines, chemokines, gene expression, eosinophil, T cells

STUDY SUMMARY

TITLES

Development of Human Nasal Challenge Models with Microbial Constituents and Grass Pollen:

- monophosphoryl lipid A (MPLA)
- poly-inosine-cytosine (poly-IC)
- poly-inosine-cytosine stabilised with poly-L-lysine and carboxymethylcelluylose (poly-ICLC)
- resiquimod
- tuberculin
- Timothy grass pollen

ViDAR: A mechanistic study of Vitamin D supplementation in allergic rhinitis

DESIGN Nasal challenges with a topical spray to the nostrils, with serial mucosal samples taken before and after the challenge. Open design, incremental ascending dose with strict safety precautions.

AIMS To characterise the molecular and cellular basis of the nasal mucosal immune response to a variety of challenges

OUTCOME MEASURES

Tolerability and clinical symptoms

Levels of nasal mucosal lining fluid (MLF) cytokines and chemokines

Metabolites assessed on mass spectrometry

Transcriptomics and flow cytometry on nasal curettage specimens

POPULATIONS

Healthy volunteers (non-atopic)

Atopic subjects with allergic rhinitis

Atopic subjects with mild allergic asthma

Subjects with latent tuberculosis (TB) :

Control healthy volunteers with negative interferon-gamma release assay (IGRA)

Vitamin D insufficient atopic subjects

ELIGIBILITY Adults (aged 18 to 60 years) will need to meet strict inclusion and exclusion criteria. Participants in this study will be obtained from clinics at Imperial College Healthcare Trust and other Northwest London Hospitals and by newspaper advertising

DURATION 3 years

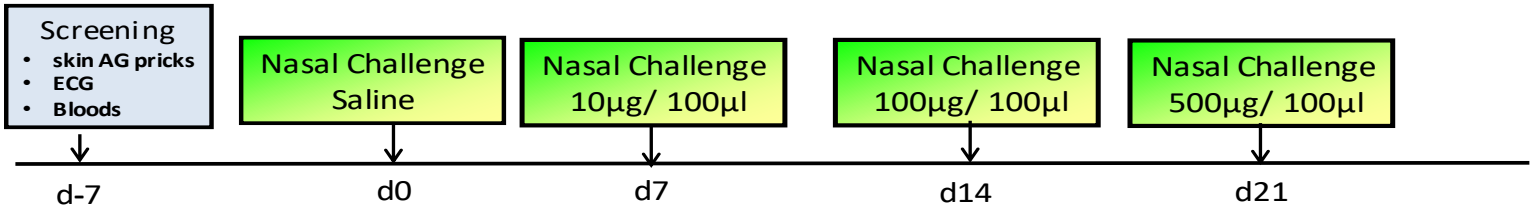
REFERENCE DIAGRAMS
APPENDIX 2A

Appendix 2A

Nasal Challenge with TLR Agonists:
Poly-IC or poly-ICLC (TLR3) or Resiquimod (TLR7/8) or MPLA (TLR4)
Part A: Incremental Ascending Dose Study based on Tolerability and SAM

4 Cohorts of 8 Healthy Volunteers (4 atopic; 4 non-atopic) *One cohort per agonist*
Each subject given saline control and 3 ascending doses

Safety & Dosing Tolerability
Nasal mucosa examination, nasal & systemic symptoms checked at all timepoints
- 1st subject challenged (observed for 8h)
- If dose well tolerated, 2nd & 3rd subjects challenged (observed until 8h)
- If dose well tolerated, then subjects 4-8 challenged (observed until 8h)



Nasal Challenge Procedures
- Initial bilateral lavage at -20m
- PNIF & nasal symptoms: -30m, 15m, 30m, 45m, 1-8h
- Nasal SAM: -30m,-10m, 15m, 30m, 45m, 1-8h

Nasal SAM, neat:
- Mass Spectrometry
- Lipidomics
- Prostanoids

Nasal SAM, eluate:
- Cytokines & chemokines

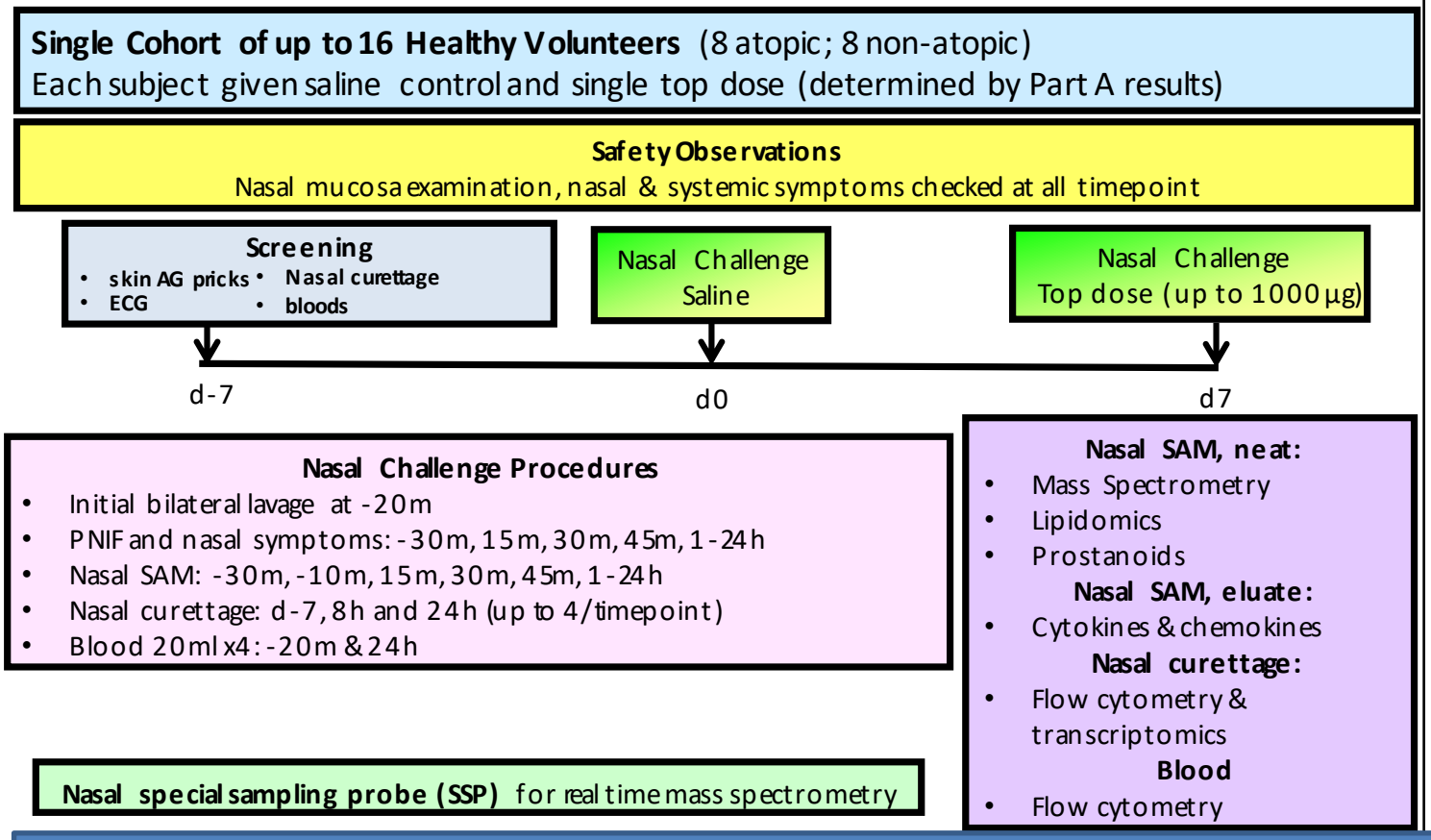
Nasosorption analysis will be undertaken after completing Part A of study to ensure viability of dosing regime

UPDATE FEB 2016: RESIQUIMOD WILL NOT BE GIVEN AT A HIGHER DOSE THAN 10µg/100µl PER NOSTRIL

APPENDIX 2B

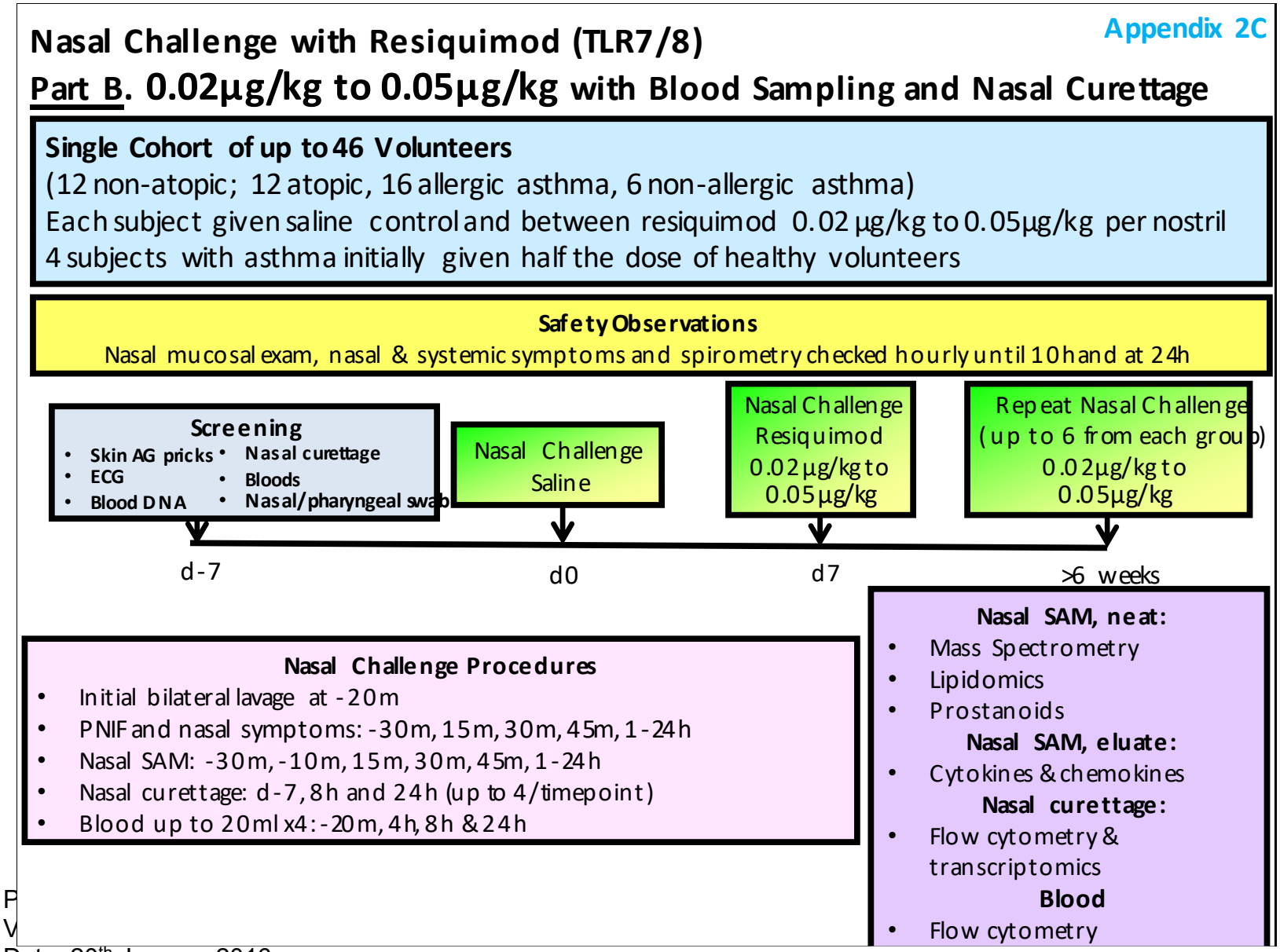
Appendix 2B

Nasal Challenge with TLR Agonists:
Poly-IC or poly-ICLC (TLR3) or Resiquimod (TLR7/8) or MPLA (TLR4)
Part B. Single Top Dose with Additional Special Sampling Probe (SSP) and Curettage

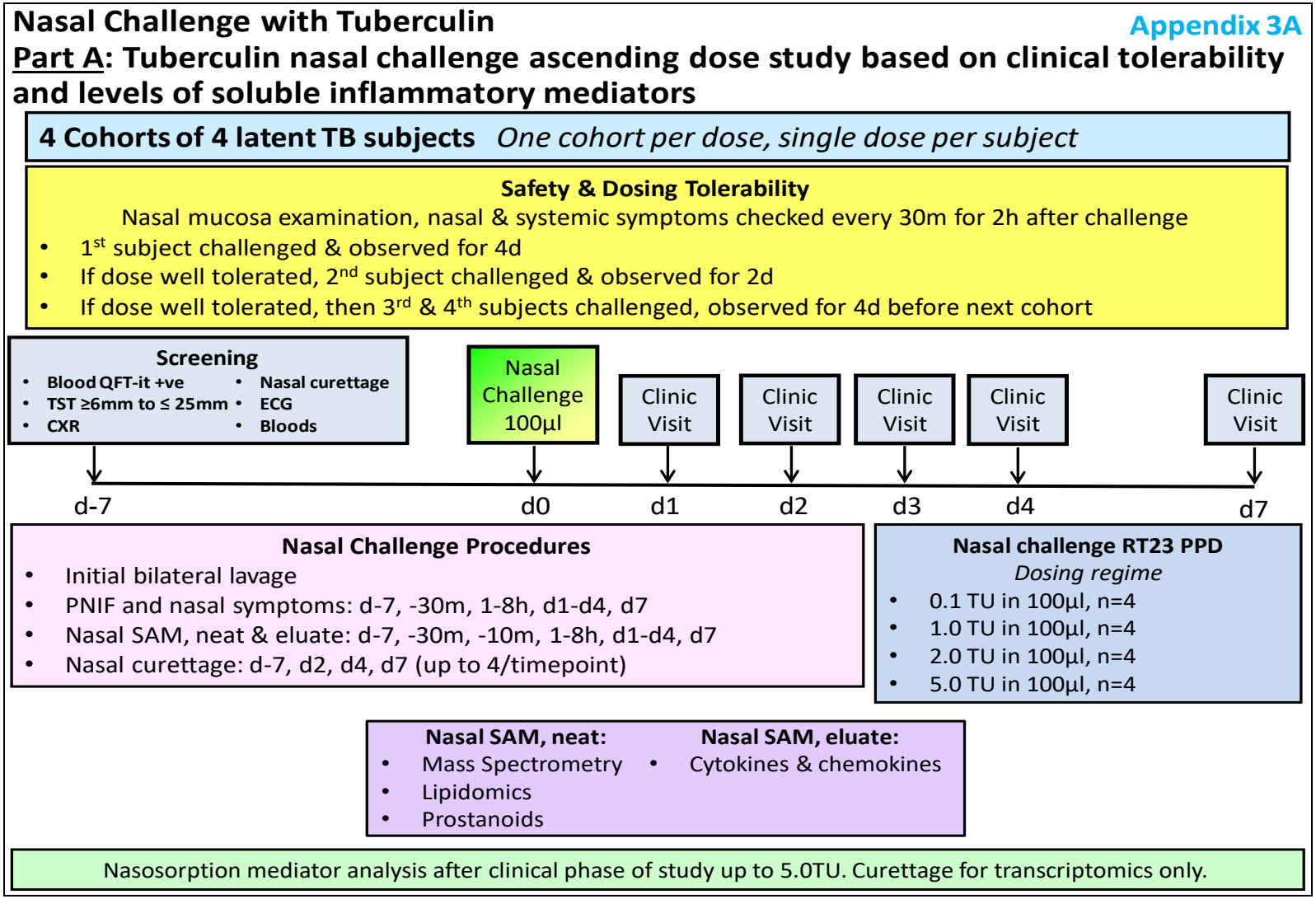


UPDATE FEB 2016: RESIQUIMOD WILL NOT BE GIVEN AT A HIGHER DOSE THAN 10µg/100µl PER NOSTRIL

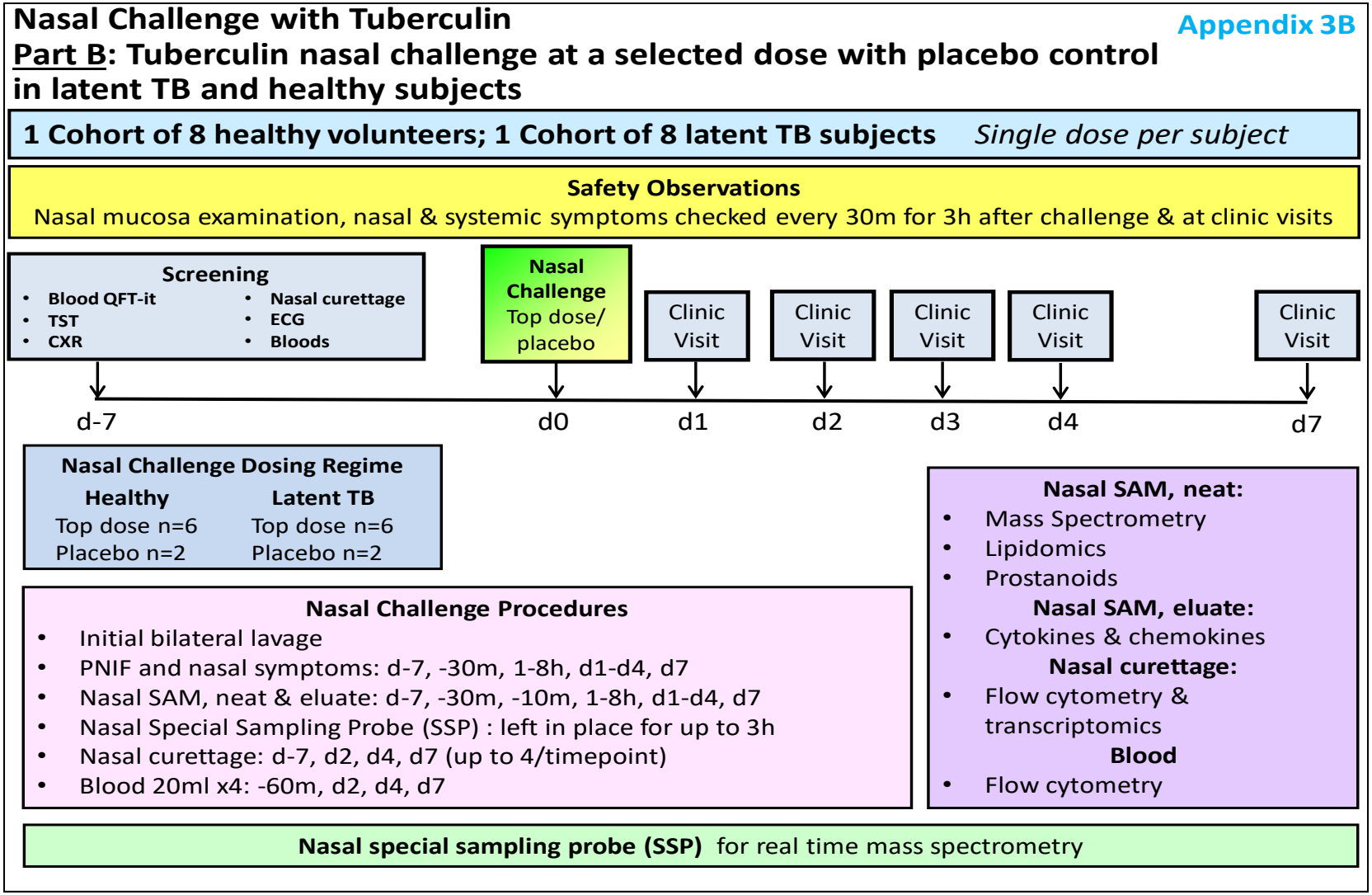
APPENDIX 2C


Nasal Challenge with Resiquimod (TLR7/8)
Appendix 2C
Part B. 0.02µg/kg to 0.05µg/kg with Blood Sampling and Nasal Curettage
Single Cohort of up to 46 Volunteers
(12 non-atopic; 12 atopic, 16 allergic asthma, 6 non-allergic asthma)
Each subject given saline control and between resiquimod 0.02 µg/kg to 0.05µg/kg per nostril
4 subjects with asthma initially given half the dose of healthy volunteers
Safety Observations
Nasal mucosal exam, nasal & systemic symptoms and spirometry checked hourly until 10h and at 24h
Screening
Skin AG pricks
ECG
Blood DNA
Nasal curettage
Bloods
Nasal/pharyngeal swab
Nasal Challenge Saline
Nasal Challenge Resiquimod 0.02µg/kg to 0.05µg/kg
Repeat Nasal Challenge (up to 6 from each group) 0.02µg/kg to 0.05µg/kg
d-7
d0
d7
>6 weeks
Nasal Challenge Procedures
Initial bilateral lavage at -20m
PNIF and nasal symptoms: -30m, 15m, 30m, 45m, 1-24h
Nasal SAM: -30m, -10m, 15m, 30m, 45m, 1-24h
Nasal curettage: d-7, 8h and 24h (up to 4/timepoint)
Blood up to 20ml x4: -20m, 4h, 8h & 24h
Nasal SAM, neat:
Mass Spectrometry
Lipidomics
Prostanoids
Nasal SAM, eluate:
Cytokines & chemokines
Nasal curettage:
Flow cytometry & transcriptomics
Blood
Flow cytometry

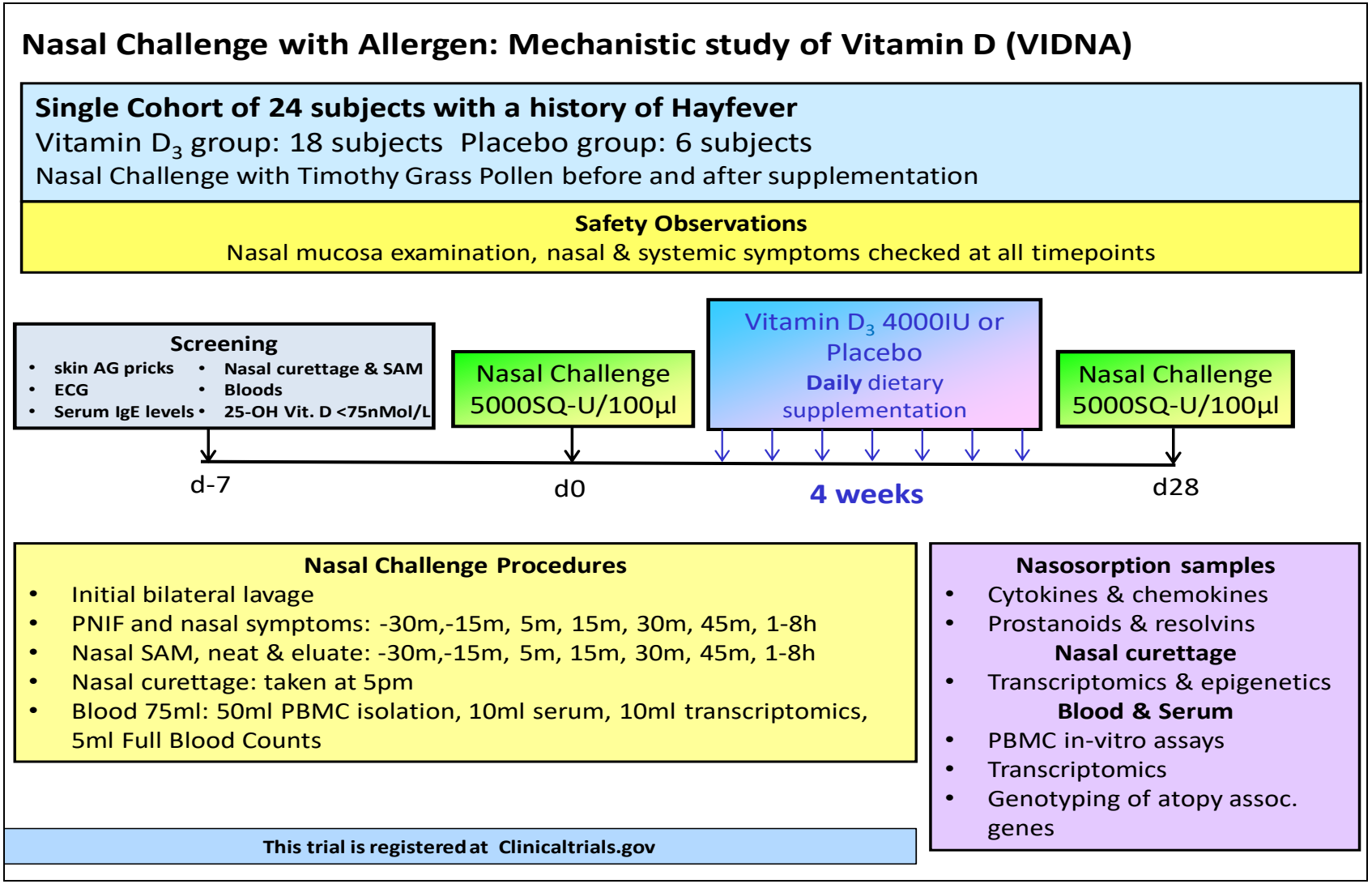
APPENDIX 3A


Nasal Challenge with Tuberculin
Appendix 3A
Part A: Tuberculin nasal challenge ascending dose study based on clinical tolerability and levels of soluble inflammatory mediators
4 Cohorts of 4 latent TB subjects One cohort per dose, single dose per subject
Safety & Dosing Tolerability
Nasal mucosa examination, nasal & systemic symptoms checked every 30m for 2h after challenge
• 1st subject challenged & observed for 4d
• If dose well tolerated, 2nd subject challenged & observed for 2d
• If dose well tolerated, then 3rd & 4th subjects challenged, observed for 4d before next cohort
Screening
• Blood QFT-it +ve
• TST ≥6mm to ≤ 25mm
• CXR
• Nasal curettage
• ECG
• Bloods
Nasal Challenge 100µl
Clinic Visit
Clinic Visit
Clinic Visit
Clinic Visit
Clinic Visit
d-7
d0
d1
d2
d3
d4
d7
Nasal Challenge Procedures
• Initial bilateral lavage
• PNIF and nasal symptoms: d-7, -30m, 1-8h, d1-d4, d7
• Nasal SAM, neat & eluate: d-7, -30m, -10m, 1-8h, d1-d4, d7
• Nasal curettage: d-7, d2, d4, d7 (up to 4/timepoint)
Nasal challenge RT23 PPD
Dosing regime
• 0.1 TU in 100µl, n=4
• 1.0 TU in 100µl, n=4
• 2.0 TU in 100µl, n=4
• 5.0 TU in 100µl, n=4
Nasal SAM, neat:
• Mass Spectrometry
• Lipidomics
• Prostanoids
Nasal SAM, eluate:
• Cytokines & chemokines
Nasosorption mediator analysis after clinical phase of study up to 5.0TU. Curettage for transcriptomics only.

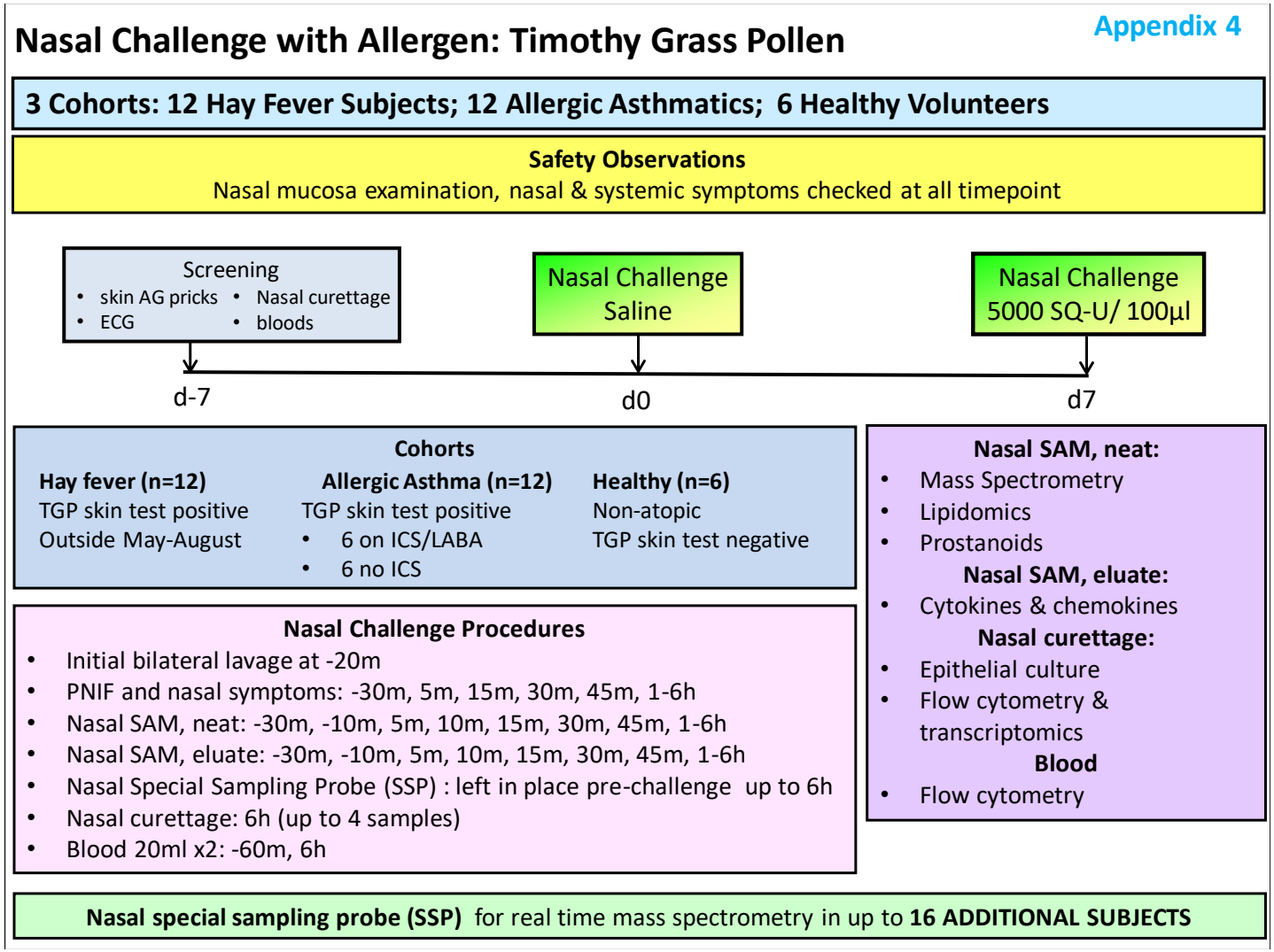
APPENDIX 3B


Nasal Challenge with Tuberculin
Appendix 3B
Part B: Tuberculin nasal challenge at a selected dose with placebo control in latent TB and healthy subjects
1 Cohort of 8 healthy volunteers; 1 Cohort of 8 latent TB subjects Single dose per subject
Safety Observations
Nasal mucosa examination, nasal & systemic symptoms checked every 30m for 3h after challenge & at clinic visits
Screening
Blood QFT-it
TST
CXR
Nasal curettage
ECG
Bloods
Nasal Challenge Top dose/ placebo
Clinic Visit
Clinic Visit
Clinic Visit
Clinic Visit
Clinic Visit
d-7
d0
d1
d2
d3
d4
d7
Nasal Challenge Dosing Regime
Healthy
Latent TB
Top dose n=6
Placebo n=2
Top dose n=6
Placebo n=2
Nasal Challenge Procedures
Initial bilateral lavage
PNIF and nasal symptoms: d-7, -30m, 1-8h, d1-d4, d7
Nasal SAM, neat & eluate: d-7, -30m, -10m, 1-8h, d1-d4, d7
Nasal Special Sampling Probe (SSP) : left in place for up to 3h
Nasal curettage: d-7, d2, d4, d7 (up to 4/timepoint)
Blood 20ml x4: -60m, d2, d4, d7
Nasal SAM, neat:
Mass Spectrometry
Lipidomics
Prostanoids
Nasal SAM, eluate:
Cytokines & chemokines
Nasal curettage:
Flow cytometry & transcriptomics
Blood
Flow cytometry
Nasal special sampling probe (SSP) for real time mass spectrometry

APPENDIX 4A


Nasal Challenge with Allergen: Mechanistic study of Vitamin D (VIDNA)
Single Cohort of 24 subjects with a history of Hayfever
Vitamin D3 group: 18 subjects Placebo group: 6 subjects
Nasal Challenge with Timothy Grass Pollen before and after supplementation
Safety Observations
Nasal mucosa examination, nasal & systemic symptoms checked at all timepoints
Screening
skin AG pricks
ECG
Serum IgE levels
Nasal curettage & SAM
Bloods
25-OH Vit. D <75nMol/L
Nasal Challenge 5000SQ-U/100µl
Vitamin D3 4000IU or Placebo
Daily dietary supplementation
Nasal Challenge 5000SQ-U/100µl
d-7
d0
4 weeks
d28
Nasal Challenge Procedures
Initial bilateral lavage
PNIF and nasal symptoms: -30m,-15m, 5m, 15m, 30m, 45m, 1-8h
Nasal SAM, neat & eluate: -30m,-15m, 5m, 15m, 30m, 45m, 1-8h
Nasal curettage: taken at 5pm
Blood 75ml: 50ml PBMC isolation, 10ml serum, 10ml transcriptomics, 5ml Full Blood Counts
Nasosorption samples
Cytokines & chemokines
Prostanoids & resolvins
Nasal curettage
Transcriptomics & epigenetics
Blood & Serum
PBMC in-vitro assays
Transcriptomics
Genotyping of atopy assoc. genes
This trial is registered at Clinicaltrials.gov

APPENDIX 4B


Nasal Challenge with Allergen: Timothy Grass Pollen
Appendix 4
3 Cohorts: 12 Hay Fever Subjects; 12 Allergic Asthmatics; 6 Healthy Volunteers
Safety Observations
Nasal mucosa examination, nasal & systemic symptoms checked at all timepoint
Screening
• skin AG pricks • Nasal curettage
• ECG • bloods
Nasal Challenge Saline
Nasal Challenge 5000 SQ-U/ 100µl
d-7
d0
d7
Cohorts
Hay fever (n=12)
TGP skin test positive
Outside May-August
Allergic Asthma (n=12)
TGP skin test positive
• 6 on ICS/LABA
• 6 no ICS
Healthy (n=6)
Non-atopic
TGP skin test negative
Nasal SAM, neat:
• Mass Spectrometry
• Lipidomics
• Prostanoids
Nasal SAM, eluate:
• Cytokines & chemokines
Nasal curettage:
• Epithelial culture
• Flow cytometry & transcriptomics
Blood
• Flow cytometry
Nasal Challenge Procedures
• Initial bilateral lavage at -20m
• PNIF and nasal symptoms: -30m, 5m, 15m, 30m, 45m, 1-6h
• Nasal SAM, neat: -30m, -10m, 5m, 10m, 15m, 30m, 45m, 1-6h
• Nasal SAM, eluate: -30m, -10m, 5m, 10m, 15m, 30m, 45m, 1-6h
• Nasal Special Sampling Probe (SSP) : left in place pre-challenge up to 6h
• Nasal curettage: 6h (up to 4 samples)
• Blood 20ml x2: -60m, 6h
Nasal special sampling probe (SSP) for real time mass spectrometry in up to 16 ADDITIONAL SUBJECTS

INTRODUCTION

1.1 BACKGROUND

Summary

- Respiratory diseases such as asthma and COPD involve inflammation of the airways, and microbes are increasingly recognised as being related to the pathogenesis of these diseases, with viruses the major cause of exacerbations (1). We propose the development of a range of topical nasal spray challenge models to study the upper respiratory tract mucosal immune response. We will develop nasal challenge with bacterial and viral pathogen associated molecular patterns (PAMPS) that stimulate Toll-Like Receptors (TLRs), tuberculin purified protein derivative (PPD), and allergen (AG: Timothy grass pollen). In this way we challenge the nasal upper respiratory tract mucosa with stimuli of the innate immune system (TLR agonists), as well as with a specific T helper type 1 (Th1) stimulus (tuberculin PPD) and a specific Th2 stimulus (AG).

- Following challenge we shall employ non-invasive methods of taking samples from the nasal mucosal surface. Synthetic absorptive matrix (SAM) strips are like blotting paper, and will be inserted onto the airway mucosal surfaces within a nostril to absorb concentrated nasal secretions, inflammatory mediators in the mucosal lining fluid (MLF) reflect underlying inflammation.

- Nasosorption is painless, and MLF can be obtained even from non-inflamed noses at frequent intervals, without the need for local anaesthetic. We have performed successful pilot studies with nasal strips in adults after nasal allergen challenge (NAC), and in babies and in young children. There is minimal protein binding to the SAM strip, and fluid can be eluted by spin filtration. High levels of mediators of inflammation can then be measured in the MLF: higher than detectable by nasal lavage.

- In addition, a novel probe will be placed on the surface of the nasal mucosa and we shall carry out real time mass spectrometry. We shall also perform nasal epithelial curettage with a Rhinoprobe device. This takes a pinhead of nasal tissue, again without requiring a local anaesthetic, and the sample can then be used for analysis of cells by flow cytometry and to determine gene expression (transcriptomics).

- Disease-specific signatures (including metabolomics and cytokines / chemokines) from the upper airways (nose) and lower airways (bronchi) of patients with asthma have been studied. Using nasosorption to obtain samples and identify molecular signatures of respiratory disease, we can achieve fundamental insights into airway diseases. This will refine the diagnosis, stratification and monitoring of a variety of airway diseases, and may provide targets and biomarkers for new therapy.

- Additionally we wish to study of the effects of vitamin D on nasal mucosal mediators of inflammation produced after nasal allergen challenge (NAC) with Timothy grass pollen. Vitamin D deficiency is a common condition and may be an important environmental factor in the development of allergy and asthma (2). In particular, vitamin D has been shown to have a range of anti-inflammatory effects on the immune system (3), and is thought to have some shared actions with corticosteroids (4). We shall study the actions of vitamin D on hay fever, since this is an allergic inflammatory disease of the upper airways that is caused by airborne

grass pollen during the British summer. We propose giving oral vitamin D supplementation for 4 weeks to selected subjects with vitamin D insufficiency in the British winter, and assessing whether there are effects on levels of mediators of inflammation produced after nasal allergen challenge (NAC) with a nasal spray consisting of grass pollen extract.

Challenge Models and Sampling Nasal Mucosal Lining Fluid (MLF) with Synthetic Absorptive Matrix (SAM) by Nasososorption

The inhaled allergen challenge model has been extensively employed as the classical means to study effects of anti-inflammatory agents on asthma (5) (6), while the allergen chamber is especially useful for immunotherapy (7). In the last 15 years a series of clinical studies have been carried out with specific monoclonal antibodies (mABs) in patients with asthma, generally beginning with inhaled allergen challenge studies, that have given insights into the heterogeneity of asthma phenotypes (8) (9) (10). These studies with mABs against soluble IgE and cytokines produced by Th2 cells (IL-4, IL-5, IL-13) have established the principle of personalised medicine: that a highly specific mAB tends to be more effective in selected stratified asthmatics that express higher levels of a relevant biomarker (11) (12).
Nasal challenge models have certain advantages over inhaled models, especially because the nose is much more accessible than the airways to obtain respiratory samples (13). Therefore repeat non-invasive sampling can be performed to study in detail the cellular and molecular basis of the mucosal upper respiratory tract (URT) immune response. Nasal and bronchial epithelial mucosal lining fluid (MLF) is a compartment that can be utilised to study inflammatory mediators in a variety of respiratory diseases.

We have developed a novel system that utilises strips of synthetic absorptive matrices (SAM) or synthetic sponge to sample MLF, before eluting the fluid and measuring levels of mediators that cause and are a feature of inflammation. Filter paper consisting of natural cellulose from the cotton plant has been widely used to absorb nasal secretions (14-18), and ophthalmic Weck-Cel sponges composed of natural cellulose have been used to sample saliva, cervical and vaginal secretions (19). Our group originally performed nasosorption with filter paper after nasal allergen challenge (NAC), but found in an initial study that detectable levels of cytokines such as IL-5 and IL-13 are generally low (20). Following this we found that different batches of filter paper vary in their degree of protein binding, and thus decided to identify a suitable SAM.

As a response to problems using filter paper of natural origin from the cotton plant, different SAMs have been developed: Accuwik Ultra (21) (22) (23) (24), Leukosorb (Pall Life Sciences), polyvinyl acetate (PVA) sponge (25), and polyurethane foam (26)[(27),(28)26) Different absorptive materials have been compared for sampling saliva prior to measuring antibodies (29). Recent studies have demonstrated that polyurethane foam performs well as a SAM for collecting nasal MLF (26;27).

Our experience with SAM for nasosorption has been published with regard to children with allergic rhinitis (21), infants with a family history of atopy (23), and in atopic adults after nasal allergen challenge (NAC) (24). We have also successfully used leukosorb after experimental human rhinovirus (HRV experimental challenge in asthma, when both nasosorption and bronchosorption were performed (Jackson D et al., manuscript in preparation).

Surface nasal sampling and real time mass spectrometry

Dr Zoltan Takats of Imperial College has developed special ways of sampling droplets or electrosurgical smoke of tissue for mass spectrometry (30-32). Using rapid evaporative

ionization mass spectrometry (REIMS) in conjunction with electrosurgery (the intelligent knife or *iknife*) enables real time feedback during surgery as to whether tissue contains cancerous cells (31). Building on this experience with molecular characterisation of many thousands of lower molecular weight metabolites, we hope to test a new surface probe on the nasal mucosa following the different challenges. This probe will bound minute volumes of water molecules against the mucosal surface lining fluid.

Role of host microbiome in innate immune response to resiquimod

Viral respiratory tract illness is often complicated by secondary bacterial infections, especially in those with compromised host defences. One mechanism may be due to the presence of virus in the nose altering epithelial cell receptor expression allowing any resident bacteria present in the associated mucosal biofilm to become pathogenic. Low bacterial diversity rather than bacterial load appear to be related to disease severity in those with chronic rhinosinusitis.

The nose has a greater biomass of commensal bacteria than other parts of the respiratory tract and likely affects the composition of the lower airways. Asthmatic bronchi have a disordered microbiome with a greater prevalence of *Haemophilus* species and in this population, viral and bacterial co-infection leads to greater chances of being hospitalized. In murine models, respiratory (and possibly gut) commensal bacteria play a role in regulating T cell function and other immune defences against acute respiratory viral infections (such as influenza). It has been demonstrated that lower respiratory tract immune responses to bacterial TLR ligands are attenuated for at least 6 weeks after infection. Our recent work with lipopolysaccharide (LPS, a component of the outer wall of Gram negative bacteria) given nasally suggests that baseline mucosal immune tone may be affected by environmental factors such as host microbiota.

To investigate how the host respiratory microbiome correlates with innate immune responses to viruses in asthmatic, allergic and healthy participants, we propose collecting anterior nasal and oropharyngeal swab samples from volunteers at their screening visit using sterile dry cotton-headed swabs and synthetic absorptive matrix strips (SAM) and analyse the bacterial profile using traditional laboratory culture techniques as well as microbiome analysis. The findings will be correlated with subsequent innate immune functional assays.

1.2 RATIONALE FOR CURRENT STUDY

There is now the need to extend nasal challenge from allergen to a range of other agents, utilising the novel SAM methodology to take serial nasosorption samples and then measure inflammatory mediators. Hence our aim in this proposal is to develop nasal challenge with a range of immunostimulatory agents. In parallel, we shall also develop challenges against specific/adaptive immune responses: tuberculin (Th1 and cell-mediated immune stimulus) nasal challenge will be developed in subjects with latent TB, and Timothy grass pollen will be employed as an allergen in subjects with hay fever outside the grass pollen season.

1. We shall begin by performing ascending dose studies with innate Toll-like receptor (TLR)-agonists. At St Mary's Hospital there is considerable experience under Professor Sebastian Johnston with the Human Rhinovirus (HRV, common cold virus) experimental challenge model in adults (33), while Professor Peter Openshaw is developing an adult human experimental challenge model with respiratory syncytial

virus (RSV) (34), (35). Instead of challenging with live viruses we shall give nasal topical challenge with key immunogenic viral components:

A. Monophosphoryl lipid A (MPLA) is an analogue of bacterial lipopolysaccharide (LPS) that is now licensed as an adjuvant and stimulates TLR4 and TLR2, but with much lower toxicity than LPS (36) (37) (38). In addition, a range of TLR4 antagonists are in development (39;40). Nasal challenge has been performed with LPS but without using nasosorption (41-44). In particular, it has recently been found that nasal allergen challenge in allergic subjects, when followed 24h later by LPS, causes an amplified cytokine response 6h later (44). Following nasal LPS challenge we have taken serial nasosorption samples and documented the innate immune response (Dhariwal J et al., manuscript in preparation).

   Nasal MPLA challenge followed by serial nasosorption sampling has not been performed to our knowledge. However MPLA has been extensively used as an adjuvant in human clinical trials (45), and as an adjuvant for subcutaneous and sublingual allergen immunotherapy (46;47).

B. Poly-inosine-cytosine (poly-IC) resembles double-stranded viral RNA and is an immunogenic agonist for TLR3 (48). Many of the major respiratory viral infections have an RNA genome: so this model is relevant to infection with HRV, RSV and influenza. We shall use a highly purified high molecular weight poly-IC from Invivogen. Ampligen is a poly-IC derivative with regular repeats of uridylic acid, and clinical trials have been carried out in man by Hemispherx (49-52). Recently it has also been given nasally in seven volunteers with allergy to birch pollen and tolerated well. The study group sampled nasal tissue using an interdental brush to measure mRNA expression. However, we plan to study both atopic and non-atopic volunteers and sample the mucosal lining fluid directly to measure the protein mediators that are secreted in addition to measuring RNA expression (53).

C. Poly-ICLC is poly-IC stabilised with poly-L-lysine and carboxymethylcellulose. It is more RNAse resistant than poly-IC, and better able to stimulate intracellular endosomal TLR3. Poly-ICLC has been developed by a Pharmaceutical Company (Oncovir of Washington DC, USA) and has been administered to man by injection in a range of clinical studies, including as a vaccine adjuvant (48;54).

D. Resiquimod stimulates TLR7/8 that is also stimulated by respiratory viruses (40). Resiquimod is an immune response modifier that has been successfully employed topically as a skin cream for actinic keratosis (55). Resiquimod is closely related to imiquimod, but is more potent (56), and can block airway remodelling in a rat asthma model (57). Resiquimod when given systemically to treat human hepatitis C infection causes cytokine release and a flu-like syndrome. We are unaware of studies with topical resiquimod given to the respiratory tract.

2. Tuberculin challenge employing purified protein derivative (PPD) has been studied in skin test PPD-positive subjects by Richard F. Silver and colleagues in Cleveland following bronchosegmental challenge (58) (59) (60) (61). Silver and colleagues performed BAL at a first bronchoscopy, before doing local broncho-segmental challenge with 0.5 tuberculin units of PPD in normal saline (10ml) instilled into a subsegment of the lingual. Repeat bronchoscopy and BALs were performed 48h later.

   - PPD challenge caused a BAL lymphocytosis with increased numbers of CD4+ T cells and IFN-γ producing cells (58).
   - The IFN-γ producing cells were then shown to be CD4+ Th1 cells of effector memory phenotype (CD45RO+, CCR7-) (59).

- The α4β1 integrin is involved in localization of these *M. tuberculosis*-specific Th1 cells (60).

We are also aware of a study that safely developed nasal challenge with leprosin A in leprosy (62), but are unaware of a nasal challenge study in man with TB components. In PPD-positive skin test individuals with latent TB, we hope to perform an ascending dose tolerability study with up to 2.0 tuberculin units in 100µg of saline nasal spray to each nostril. If local nasal inflammation is caused by the PPD spray challenge, the study will be discontinued, and the individual can be treated with local and systemic corticosteroids.

3. Allergen (Timothy grass pollen):
   A. Mechanistic study with Vitamin D: Our group has studied IL-5 and IL-13 nasal responses in nasal MLF after NAC (24). We have also taken nasal epithelial curette samples and looked at transcriptomics (Trevor Hansel with Merck Pharmaceuticals, manuscript in preparation) and developed a method of flow cytometry for assessment of mechanically-dispersed leukocytes from the nasal epithelial curette sample (Ross Walton, Jaideep Dhariwal and colleagues). We now wish to investigate the mechanistic processes that underlie vitamin D supplementation in allergic disorders and in particular after nasal challenge with grass pollen. A number of studies have shown a correlation between serum vitamin D levels and the severity of asthma (63) (64) (3). However, vitamin D supplementation failed to show clinical benefit in a recently published randomized trial of vitamin D supplementation in adults with symptomatic asthma: the VIDA (vitamin D add-on therapy enhances corticosteroid responsiveness in asthma) study (65). Nevertheless, in studies from the Hawrylowicz group it has been showed that a short course of vitamin D can increase responsiveness to corticosteroids (66) and that IL-17A production in severe asthma is decreased by vitamin D (67). It is now recognised that suboptimal levels of vitamin D are present in many people in the UK during the winter, and dietary supplementation is recommended for pregnant women, children and high risk groups (68). Taking serum levels of 25-hydroxy-vitamin D it has been found that 87% of UK adults have suboptimal vitamin D levels at <75nmoles/l in winter (69)
   In nasal micro-curettage samples it is possible to measure gene expression, which assesses levels of thousands of mRNA molecules. The Hansel group has recently found that there are profound changes in circadian-associated genes in the nasal mucosa after NAC, and that these genes are inhibited by corticosteroids. Circadian genes regulate innate aspects of the immune system by actions involving the BMAL1-CLOCK complex of nuclear transcription factors, receptors and enzymes (70) (71;72). The circadian clock was first identified with regard to lipopolysaccharide(LPS) responses varying with the time of the day, and these circadian responses have been shown to be present in murine macrophage responses to endotoxin (73;74) and more recently in murine inflammatory monocytes (75). In addition, a bronchiolar epithelial clock controls pulmonary inflammation and glucocorticoid action in mice, as shown by genetic ablation of the mouse clock gene Bmal1 in bronchiolar epithelial cells (76).
   Our demonstration in the human nose of allergen-induced changes in circadian-associated genes in the presence of innate inflammatory pathways, suggests that the circadian-associate genes could be controlling gene expression and protein synthesis involved in these innate inflammatory pathways. Furthermore, these allergen–induced circadian changes are reversed by a single preceding oral dose of prednisone, consistent with

known actions of endogenous cortisol (77) (76) implying that the corticosteroid may be acting on circadian-associated genes in order to resolve inflammation.
Since the NAC model with grass pollen is exquisitely sensitive to studying the effects of therapy, we have designed a clinical study to assess whether vitamin D supplementation can alter NAC responses through effects on circadian–associated genes and pathways of inflammation in asthma (78). After a review of the literature we propose a dose of 4000 IU per day for 4 weeks, since this level of dietary supplementation has been safely used in pregnant women and in a large clinical study in asthma (79;80) (65). We shall assess whether there are effects of vitamin D on inflammation produced after NAC: looking at levels of cytokines, chemokines, cells and gene expression, with a focus on circadian–associated genes (78).

B. Allergen (Timothy Grass Pollen): An additional aim is to identify the cells that are responsible for production of IL-5 and IL-13 in MLF, suspecting that these may be Th2 cells or natural helper cells (nuocytes). We wish to perform NAC with appropriate saline control challenge in allergic and non-allergic subjects. Serial samples will be taken to assess chemokines and cytokines in nasal MLF, with measurement of transcriptomics and leukocyte flow cytometry on nasal epithelial curette samples.

Research Question and Hypotheses

Following nasal spray challenge with various agents can we develop a safe system that mimics the response of the airways to microbes (pathogen-associated molecular patterns) and allergens?

Will different challenge agents cause characteristic and differing nasal mucosal responses? Following nasal challenge we shall take serial non-invasive samples and be able to document the inflammatory response to challenges in terms of cytokines and chemokines in mucosal lining fluid, and in terms of cells and gene expression in nasal epithelial curettage samples.

Will supplementary Vitamin D alter levels of inflammatory mediators, gene expression and modulate circadian-associated gene expression following nasal allergen challenge (NAC) with Timothy grass pollen extract?

By understanding the nasal response to challenge agents, we will have a system for identifying biomarkers of disease, targets for new drugs, and be able to provide a clinical challenge test for testing the efficacy of new drugs.

2. STUDY OBJECTIVES

2.1 PRIMARY AND SECONDARY OBJECTIVES

- To develop a set of well-tolerated, robust and reproducible (validated) nasal challenge systems with microbial constituents and allergen. To identify biomarker signatures following different challenges.

- To document a dose response and time course to nasal challenge in terms of cytokine and chemokine release, and nasal epithelial curettage (Rhinoprobe) mRNA expression, cell influx by flow cytometry.

- To provide a set of nasal challenge methods to enable a "proof of target pharmacology" for a novel therapy or vaccine. This may also permit dosage optimisation in relation to clinical efficacy studies.

- To develop real time sampling utilising a special probe that touches the surface of the nasal lining and is linked to a mass spectrometer.

  To assess nasal cytokines/chemokines in the nasal mucosa and assess levels of circadian–associated gene expression (BMAL-CLOCK genes) in curettage samples after NAC before and after Vitamin D supplementation.

3. STUDY DESIGN (See Figures above)

Group 1: Nasal challenge with TLR Agonists: This part of the study has a total of 96 subjects divided into two parts; Part A and B.

Part A: We shall have 3 cohorts of 8 healthy volunteers (4 of the 8 having atopy). There will be a separate cohort of 8 subjects for each challenge agent (MPLA, poly-ICLC, and resiquimod).
A careful incremental ascending dose tolerability study will be performed: starting with saline and then giving single doses of 10, 100,500µg and up to 1000µg of a given challenge agent in volumes ranging from 100µl to 500µl per nostril.

Part A, N= 24

a. MPLA n = 8
b. Poly-ICLC n = 8
c. Resiquimod n = 8

For each challenge agent we shall initially take serial SAM samples over 8 hours, and assess the cytokine and chemokine response.

Part B: When we have defined the top safe dose, we shall study up to a further 16 subjects for each challenge agent (up to 46 for resiquimod): and include extra sampling for nasal curettage and blood flow cytometry. For those volunteers in the resiquimod arm of the study, we will invite some of the participants (up to 6 in each group) for a repeat challenge at least 6 weeks after the prior challenge. In January 2016 we updated the protocol to move to a weight based dosing regimen for administering resiquimod. We intend to challenge 6 volunteers (mix of healthy volunteers an those with allergic rhinitis) at a dose of 0.02µg/kg per nostril, which equates to 1µg/100µl per nostril in a 50kg volunteer. For the asthma group, we intend to challenge 4 people at half the dose given to the healthy group. The maximum tolerated dose will then be used for the remaining subjects in the study, We will not use resiquimod at a dose higher than 10µg/100µl per nostril for the remainder of the study.

Part B, N = 90

a. MPLA n = 16
b. Poly-IC n = 16
c. Poly-ICLC n = 16
d. Resiquimod n = 46 (12 healthy, 12 atopic, 16 allergic asthmatic, 6 non-allergic asthma)

Group 2: Nasal challenge with tuberculin: This part of the study has a total of 32 subjects divided into two parts; Part A and B.

Part A: This involves 4 cohorts of subjects with defined latent TB.
A careful incremental ascending dose tolerability study will be performed: starting with single doses of:

Part A, N=16
A. Subjects with Latent TB
   a. 0.1 TU/100µl/nostril, n = 4
   b. 1.0 TU /100µl/nostril, n = 4
   c. 2.0 TU /100µl/nostril, n = 4
   d. 5.0 TU /100µl/nostril, n = 4

For each agent we shall look at tolerability and initially take serial SAM samples over the next 7 days to assess the cytokine and chemokine response.

Part B: When we have defined the top safe dose, we shall study up to a further 16 subjects with the top tuberculin dose: and include extra sampling for nasal real time mass spectrometry, nasal curettage and blood flow cytometry.

Part B, N=16
A. Subjects with Latent TB,
   a. 10.0 TU /100µl/nostril, n = 8
B. Healthy volunteers,
   b. 10.0 TU /100µl/nostril, n = 8

Group 3: Nasal challenge with allergen (Timothy grass pollen): This part of the study has a total of 70 subjects divided into two parts; Part A and B.

Part A Nasal challenge with allergen and vitamin D3 supplementation or placebo**:**
This involves a total of 24 subjects who are allergic to grass pollen and are vitamin D insufficient. Participants are divided into 2 groups (vitamin D supplementation and placebo arms).This is a randomized, double-blind, placebo-controlled study. They will have two NAC; one before and one after supplementation. At each NAC visit, subjects will be given a single dose of Timothy grass pollen (*Phleum P5*) allergen:
The *Phleum P5* is given as 5000 SQ-U = 1µg/100µl per nostril.

After being randomized into one of the two groups they will receive their first NAC and subsequently take vitamin D3, 4000IU (n=18) or placebo (n=6) orally once daily at approximately the same time each morning until their second NAC. This second NAC will take place between 28-35 days. During both days of NAC, serial SAM samples will be taken and nasal curettage performed. Blood samples will also be taken for routine safety tests, peripheral blood mononuclear cells (PBMC) *in vitro* assays, vitamin D, calcium, cortisol, melatonin and whole blood transcriptomics (circadian-associated genes).

Part A, N=24
A. Subjects with grass pollen allergy and insufficient Vitamin D3
   a. Subjects taking Vitamin D3 supplementation n = 18
   b. Subjects on placebo n = 6

Part B: Nasal challenge with allergen involves a total of 46 subjects:
This is divided into 4 groups of subjects given a single dose of Timothy grass pollen (*Phleum P5*) allergen:
The *Phleum P5* is given as 5000 SQ-U = 1µg/100µl/nostril

Subjects with hay fever out of season (n=12)
Subjects with allergic asthma out of season (n=12): half taking no anti-inflammatory drugs, half receiving combined inhaled corticosteroids and long-acting beta-agonists (ICS/LABA)
Non-atopic healthy controls (n=6)
Subjects with hay fever out of season who will trial a novel nasal special sampling probe (SSP) for real time mass spectrometry (n=16)

N= 46

a. People with hay fever, n = 12
b. People with allergic asthma, n = 12
c. Healthy, non-atopic people, n = 6
d. People with hay fever, n =16

3.1 STUDY OUTCOME MEASURES

- Clinical symptoms and tolerability
- Inspection of nasal mucosa by a clinician: erythema, discharge, ulceration
- Mucosal lining fluid cytokines and chemokines
- Blood and nasal flow cytometry and transcriptomics
- Nasal lining fluid metabolites

4. PARTICIPANT ENTRY

4.1 PRE-REGISTRATION EVALUATIONS: SCREENING HISTORY AND TESTS

Before patients are considered for inclusion in the study we shall consider whether they have asthma or hay fever, or a history of latent tuberculosis (TB), or whether they are healthy volunteers (HVs) with none of these conditions.

Screening tests may include intra-epidermal skin allergen prick tests and an intradermal tuberculin skin test.

4.2 MIXED INCLUSION & EXCLUSION CRITERIA (FOR ALLERGEN CHALLENGE ARMS ONLY)

General: for all subjects

- Males and females aged 18 to 60 years
- Current non-smokers for last year, maximum of 10 cigs per month, with a smoking history of <5 pack years

- Body mass index in the range18-39
- No systemic illnesses that might affect nasal immune responses
- No recent viral infections in past month: including colds and flu.
- No nasal anatomical abnormalities, sinus infections, polyps, nasal mucosal abnormalities
- No prescribed anti-inflammatory drug therapy

A. Healthy non-atopic volunteers

- No clinical history of allergic rhinitis, allergic asthma or eczema.
- Negative skin prick tests to a range of 6 common aeroallergens: cat, dog, grass pollen, tree pollen, house dust mite, fungal spores
- Normal blood eosinophil count

B. Atopic subjects with Timothy Grass Pollen sensitivity & Vitamin D Insufficiency

- A clinical history of seasonal grass pollen allergic rhinitis: sneezing, running and itching nose, nasal drip in the UK grass pollen summer season (May-July).
- Specific allergy confirmed by positive intra-epidermal skin prick test to Timothy grass pollen extract (Soluprick, P*hleum pratense*; ALK, Horsholm, Denmark), a positive reaction being a raised wheal of diameter ≥3mm larger than a negative saline control.
- Vitamin D insufficiency: serum levels of 25-hydroxy vitaminD3 (25-OHD3) <30ng/ml or <30µg/L or <75nmoles/L
  - No use of sun lamps, vitamin D supplements (including fish oils), no holidays abroad in sunny locations (including beach holidays and skiing) for the duration of participation
  - No clinical history of tree pollen allergy from February to April

C. Asthmatic subjects with Timothy Grass Pollen sensitivity

- Seasonal grass pollen allergic rhinitis: sneezing, running and itching nose, nasal drip in the UK grass pollen summer season (May-July).
- Specific allergy confirmed by positive intra-epidermal skin prick test to Timothy grass pollen extract (Soluprick, P*hleum pratense*; ALK, Horsholm, Denmark), a positive reaction being a raised wheal of diameter ≥3mm larger than a negative saline control.
- Half the asthmatics have clinical history and diagnosis of asthma, requiring therapy with occasional inhaled beta-agonists, but no inhaled corticosteroids for the past 28 days. Half the asthmatics receive regular combined inhaled corticosteroids and long-acting beta-agonists (ICS/LABA)

4.3 MIXED INCLUSION & EXCLUSION CRITERIA (FOR TLR AGONIST ARM ONLY)

General: for all subjects

- Males and females aged 18 to 60 years
- Current non-smokers for last year, maximum of 10 cigs per month, with a smoking history of <5 pack years
- Body mass index in the range18-39
- No systemic illnesses that might affect nasal immune responses
- No recent viral infections in past month: including colds and flu.

- No nasal anatomical abnormalities, sinus infections, polyps, nasal mucosal abnormalities
- No prescribed anti-inflammatory drug therapy

A. Healthy non-atopic volunteers

- No clinical history of allergic rhinitis, allergic asthma or eczema.
- Negative skin prick tests to a range of 6 common aeroallergens: cat, dog, grass pollen, tree pollen, house dust mite, fungal spores
- Normal blood eosinophil count
- Normal baseline forced expiratory volume (FEV1) i.e. ≥80%
- Negative methacholine challenge (PC_{20}> 8mg/ml)

B. Atopic subjects with Timothy Grass Pollen sensitivity

- A clinical history of seasonal grass pollen allergic rhinitis: sneezing, running and itching nose, nasal drip in the UK grass pollen summer season (May-July).
- Specific allergy confirmed by positive intra-epidermal skin prick test to Timothy grass pollen extract (Soluprick, P*hleum pratense*; ALK, Horsholm, Denmark), a positive reaction being a raised wheal of diameter ≥3mm larger than a negative saline control.
- Normal baseline forced expiratory volume (FEV1) i.e. ≥80%

C. Asthmatic subjects with Timothy Grass Pollen sensitivity

- Physician diagnosed asthma up to stage 2 BTS/GINA guidelines
- Seasonal grass pollen allergic rhinitis: sneezing, running and itching nose, nasal drip in the UK grass pollen summer season (May-July).
- Specific allergy confirmed by positive intra-epidermal skin prick test to Timothy grass pollen extract (Soluprick, P*hleum pratense*; ALK, Horsholm, Denmark), a positive reaction being a raised wheal of diameter ≥3mm larger than a negative saline control.
- Baseline forced expiratory volume (FEV1) ≥75%
- Positive methacholine challenge (PC_{20}< 8mg/ml)

D. Asthmatic subjects with no allergies

- Physician diagnosed asthma up to stage 2 BTS/GINA guidelines
- No clinical history of allergic rhinitis or eczema
- Negative skin prick tests to a range of 6 common aeroallergens: cat, dog, grass pollen, tree pollen, house dust mite, fungal spores
- Normal blood eosinophil count
- Baseline forced expiratory volume (FEV1) ≥75%
- Positive methacholine challenge (PC_{20}< 8mg/ml)

EXCLUSION CRITERIA

Nasal and Respiratory

- Recent infections in past 14 days before screening: especially upper respiratory tract illnesses (including colds and influenza), sore throats, sinusitis, infective conjunctivitis.
- Lower respiratory tract infection in past 28 days
- Signs or symptoms of significant nasal anatomical defects, hypertrophy of turbinates, major septum deviation, nasal polyposis or recurrent sinusitis
- Nasal mucosal defects, injury, ulceration
- Previous nasal or sinus surgery

Therapy

- Medical therapy other than that permitted for contraception.
- Treatment with local or systemic corticosteroids during the previous 2 months (other than low dose inhaled corticosteroids for asthmatic subjects)
- Anti-inflammatory therapy: including non-steroidal anti-inflammatory drugs (NSAIDs)

Concomitant diseases

Clinically significant diseases are not permitted

- tuberculosis at any stage in life
- active infectious disease
- cardiovascular diseases
- respiratory (other than hay fever or asthma where specified)
- hepatic, gastrointestinal, renal, endocrine, infective, haematological, autoimmune, rheumatological, neurological, dermatological,
- neoplastic conditions
- metabolic diseases and extreme obesity
- depression and psychiatric disorders

Miscellaneous

- Non-smokers: up to 10 cigarettes a year is permitted
- Participation in a therapeutic drug trial in the prior 30 days.
- Inability or unwillingness to use contraception if the patient is a female of child-bearing age.
- Pregnant or breast feeding women
- Inability to provide informed consent

4.4 MIXED INCLUSION & EXCLUSION CRITERIA FOR TUBERCULIN CHALLENGE ARM ONLY

VOLUNTEERS WITH LATENT TB INFECTION

INCLUSION CRITERIA

- Males and females aged 18 to 60 years
- Body mass index in the range 18-39
- Positive blood Interferon-γ release assay (IGRA): Quantiferon TB Gold-in-Tube (QFT-it), **>0.35 IU/ml** IFN-γ versus control

- Tuberculin skin test (TST), using RT23 tuberculin purified protein derivative (PPD), from Statens Serum Institut (SSI) of Copenhagen.
- 2 tuberculin units (TU) in 0.1ml injected intradermally (id) : **≥6mm to ≤25mm** of induration at 48-120h OR **≥15mm to ≤25mm** in subjects with BCG vaccination
- Normal chest X-ray (CXR) or CT scan if performed routinely for clinical reasons
- Previous chemoprophylaxis for TB is permitted.

HEALTHY INTERFERON-Γ RELEASE ASSAY (IGRA) NEGATIVE VOLUNTEERS

INCLUSION CRITERIA

- Age and sex matched to latent TB subjects
- Body mass index in the range 18-39
- No clinical history of TB infection
- Healthy with no lung nor systemic symptoms
- No active nasal allergy
- No BCG vaccination
- Negative blood Interferon-γ release assay (IGRA): Quantiferon TB Gold-in-Tube (QFT-it), **<0.35 IU/ml** IFN-γ versus control
- Tuberculin skin test (TST), using RT23 tuberculin purified protein derivative (PPD), from Statens Serum Institut (SSI) of Copenhagen.
- 2 tuberculin units (TU) in 1ml injected intradermally (id): **≤6mm** of induration at 48-120h.
- Chest X-ray is not required

EXCLUSION CRITERIA (FOR ALL VOLUNTEERS)

Nasal and Respiratory

- Recent infections in past 14 days before screening: especially upper respiratory tract illnesses (including colds and influenza), sore throats, sinusitis, infective conjunctivitis.
- Lower respiratory tract infection in past 28 days
- Signs or symptoms of significant nasal anatomical defects, hypertrophy of turbinates, major septum deviation, nasal polyposis or recurrent sinusitis
- Nasal mucosal defects, injury, ulceration
- Previous nasal or sinus surgery
- No clinical history of allergic rhinitis, allergic asthma or eczema.

Concomitant diseases

- Active tuberculosis at any stage in life
- ENT disease
- Active infectious disease including hepatitis
- Respiratory disease
- Renal disease
- Autoimmune disease
- Rheumatological disease & vasculitis
- Dermatological disease
- Neoplastic conditions

- Metabolic diseases and extreme obesity
- Severe depression and psychiatric disorders

Therapy

- Medical therapy other than that permitted for contraception or for chronic conditions unlikely to affect the results of the study as determined by the study physician.
- Treatment with local or systemic corticosteroids during the previous 2 months
- Anti-inflammatory therapy: including non-steroidal anti-inflammatory drugs (NSAIDs)

Miscellaneous

- Participation in a therapeutic drug trial in the prior 30 days.
- Inability or unwillingness to use contraception if the patient is female of child-bearing age.
- Pregnant or breast feeding women
- Inability to provide informed consent

NB: Smokers permitted – but must be willing to stop smoking for duration of the study

4.5 WITHDRAWAL CRITERIA

In the case of the ascending dose tolerability studies, all clinical activities will discontinue if there is a significant level of adverse events that is judged to be related to the nasal challenge as judged by the patient, and in the opinion of the Study Physician and/or the Principal Investigator.

5. ADVERSE EVENTS

5.1 DEFINITIONS

Adverse Event (AE): any untoward medical occurrence in a patient or clinical study subject.

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:

- **Results in death**
- **Is life-threatening** – *refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe*
- **Requires hospitalisation, or prolongation of existing inpatients' hospitalisation**
- **Results in persistent or significant disability or incapacity**
- **Is a congenital anomaly or birth defect**

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

5.2 REPORTING PROCEDURES

All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator in the first instance.

5.2.1 Non serious AEs

All such events, whether expected or not, should be recorded.

5.2.2 Serious AEs

An SAE form should be completed and faxed to the Chief Investigator and the Sponsor within 24 hours. However, relapse and death due to an independent condition, and hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.

All SAEs should be reported to the NRES Committee London-Harrow where the Chief Investigator considers the event was:

- 'related', i.e. resulted from the administration of any of the research procedures; and
- 'unexpected', i.e. an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies. The Chief Investigator must also notify the Sponsor of all SAEs.
Local investigators should report any SAEs as required by their Local Research Ethics Committee, Sponsor and/or Research & Development Office.

Contact details for reporting SAEs

Fax: 020 331 25750, attention Dr Trevor Hansel and/or Dr Akhilesh Jha
Please send SAE forms to: Imperial Clinical Respiratory Research Unit (ICRRU)
St, Mary's Hospital
Mint Wing, First Floor, Entrance C
Paddington
London
W2 1NY
Tel: 020 331 25744 (Mon to Fri 09.00 – 17.00)

6. ASSESSMENT AND FOLLOW-UP:

DOSAGE JUSTIFICATION

SAFETY MEASURES

SAMPLING SCHEDULE

MPLA

We are proposing nasal administration of MPLA at incremental ascending single doses from 10 to 100 to 500µg/100µl/nostril.

- We have recently completed a study in 15 subjects with nasal highly purified LPS at doses of up to 100µg (in 100 µl), in which there were no symptoms or adverse events caused by the nasal LPS.
- MPLA is at least 100 fold less toxic than LPS, and has been administered to more than 300,000 human subjects.

- MPLA has been used as an allergen immunotherapy adjuvant at doses of 50µg sub-lingually and by subcutaneous injection (36;46;47).
- MPLA has been given to rodents at doses of 2500µg/kg by injection (81).

Poly-IC

Poly-IC is a synthetic viral poly-inosine/cytosine molecule that stimulates TLR-3. Unmodified poly-IC given systemically for cancer caused toxic side effects and this was probably caused by IL-12 production (82). High molecular weight poly-IC is employed for *in vitro* stimulation of human cells at doses of 5-100 µg/ml (83) (84) (85) (86) (87).

- A pilot study of Poly-IC nasal challenge was published recently by a group from Lund University, Sweden (53). Seven healthy atopic volunteers were challenged with a total of 800µg Poly-IC, administered as 8 separate doses of 100µl into one nostril. Four doses were given over an hour on day 1 and four doses 24h later on day 2. Each volunteer was challenged twice - once outside the allergen season and once during the Swedish birch pollen allergy season. The challenge was tolerated well, causing no changes in total nasal symptom scores at this dose. Nasal lining cells (epithelial cells) were taken using an interdental brush, and the RNA levels analysed.

- Our study similarly will recruit healthy volunteers, both atopic and non-atopic. We will not recruit atopic volunteers during allergy season, to avoid any confounding effects of allergy on results. Our study will have additional scientific merit to that published as we repeatedly sample the mucosal lining fluid and look directly at the proteins secreted, as well as the RNA. Alongside total nasal symptom scores, we also assess volunteer responses using peak nasal inspiratory flow measurements. This will allow both subjective and objective clinical measurements that we can correlate with the immune data generated.

- Based on the successfully tolerated dosage with measurable mucosal immune responses reported by Brandelius et al. (53), we therefore propose omitting Part A (Poly-IC incremental dose escalation from 10µg to 500µg) from the study, and move directly onto Part B with Poly-IC top dose 500µg. We intend to administer a total of 500µg Poly-IC into each nostril over 1 hour, in 5 equal doses.

Poly-ICLC

Poly-ICLC is poly-IC stabilised with poly-L-lysine and carboxymethylcellulose, and this molecule has more RNAse resistance, and is better able to stimulate intracellular endosomal TLR3. Poly-ICLC has been developed by a Pharmaceutical Company (Oncovir of Washington DC, USA) and has been administered to man in a range of clinical studies, including as a vaccine adjuvant (48;54).

We propose to administer poly-ICLC at incremental ascending single nasal spray doses from 10 to 100 to 500µg to 1000µg/in up to 500µl, topically onto the nasal mucosa. We have obtained poly-ICLC from Oncovir and will use doses previously well tolerated in man.

- Nasal poly-ICLC. John Beigel of Baltimore, MD, USA has performed a human phase I dose escalation clinical study with nasally administered poly-ICLC. The study has not been published, but data has been sent to the FDA. Poly-ICLC was well tolerated at all doses between 0.25mg and 2mg (2000µg) per nostril. This was a simple nasal instillation, not a spray.

- Intramuscular poly-ICLC at 20µg/kg has been safely given to man, being a 2mg dose in a 100kg man (54)

  Subcutaneous administration of 1.6mg (1600µg) of poly-ICLC in man caused some redness and induration at the site of injection, and some mild-to-moderate transient flu-like symptoms (48). In this study there was upregulation of blood innate transcriptional signals (48).

Resiquimod (R848)

We will employ resiquimod as a solution, proceeding in careful incremental steps up to a maximum dose of 500µg in 100µl saline as a nasal spray, to each nostril.

- Resiquimod was well tolerated in mice and rats in extensive tolerability studies (57;89).
- Topical dermal resiquimod is safe and effective as a skin cream for the treatment of anogenital warts, actinic keratosis and skin cancer (55). Human studies have been carried out giving topical resiquimod to inflamed skin with actinic keratosis at doses up to 0.25%.

  0.25g in 100ml= 250,000µg/100ml = 2500µg/ml = 250µg/100µl
- Oral resiquimod has been given to patients with chronic hepatitis C virus (HCV) infection (90). Resiquimod was well tolerated orally at doses of 0.01mg/kg, equating to 1mg (1000µg) for a 100kg man.

Tuberculin

Table. TUBERCULIN DOSE JUSTIFICATION: Relative Skin, Inhaled and Nasal Doses

	Skin	Nasal	Inhaled
Tuberculin PPD supplied as 20 TU/ml 100 TU/ml	Intradermal injection of 100µl 2, 5, 10 TU	Proposal for 0.1, 1, 2, 5, 10 TU per 100µl nasal spray in separate cohorts of subjects with latent TB	Bronchoscopic administration of 0.5 TU in 10 ml to one broncho-pulmonary segment in patients with latent TB

We propose nasal tuberculin challenge in a cautious incremental ascending dose regimen. We shall begin at 0.1TU since this is below the Minimum Anticipated Biological Effect Level (MABEL). In the next cohort we shall give 1.0TU, followed by a cohort at 2.0TU, 5.0TU and finally 10.0TU.

The nasal dose is delivered by a nasal spray and will be administered as a fine mist diffusely to the upper respiratory tract nasal passages.

- For tuberculin injected intradermally at up to 10TU, there is a high local concentration within a bleb.
- On the basis of skin and nasal tuberculin doses being in the same range, we shall give tuberculin at a top dose of 10TU/100µl spray.
- Bronchial installation: Tuberculin has been administered safely to subjects with latent TB by bronchosegmental challenge at 0.5TU per 10ml.

In the studies of Richard Silver and colleagues in Ohio, 0.5TU tuberculin PPD was administered into a subsegment of the lingula in 10ml, covering an area of alveoli and small airways estimated at 100cm^2 (58) (59) (60).

We estimate our nasal spray is delivered over each nostril to an equivalent area of approximately 100cm^2, that corresponds to the area of the nasal mucosa in a nostril. Hence we begin at a lower dose of 0.1TU per nostril spray.

Allergen
The dose of Timothy grass pollen allergen has previously been safely employed in a range of clinical studies in patients with allergic rhinitis and asthma (91).

Vitamin D3
This is a widely used nutritional supplement with an excellent safety profile with no adverse events reported at doses of 2000-4000 IU/day given orally. To study the actions of vitamin D3 we propose looking at the effects of NAC with Timothy grass pollen in people with grass pollen allergy outside the summer grass pollen season (May-July).

We will use Vitamin D3 at a dose of 4000IU/tablet taken orally. This dose has been widely used in research studies and was used on a daily basis for 28 days in the VIDA Study: Vitamin D Supplementation in Asthma (65). There is no additional effect of increasing the dose further and very few studies go above 4000IU/day.

The supplement used will be sourced from Lamberts Healthcare Ltd and is certified to be

- Manufactured according to good medical practice guidelines (GMP)
- Verified for quality (vitamin D levels) to British Pharmacopoiea (BP) standards
- Licensed as a food supplement

Serum calcium levels will be monitored as rarely hypercalcaemia can be an adverse event.

Placebo
This will be prepared by an NHS pharmacy in a similar format to vitamin D3 and will be taken by subjects in an identical manner to the vitamin D3 supplementation.

DOSING SCHEDULE: SAFETY PRECAUTIONS

A. Nasal Challenge with TLR-Agonists

For each dosage cohort, a single subject will first be assessed for safety over 24h. We shall monitor all subjects for local and systemic symptoms, and record vital signs, for at least 6 hours following each nasal challenge. For asthmatic subjects we will additionally monitor their forced expiratory volume (FEV_1) and should respiratory symptoms or spirometry deteriorate significantly, this will be promptly treated by clinical staff, e.g. by administration of inhaled B_2 agonists via inhaler or nebulizer. For the dose escalation phase of the study (Part A), we will additionally make a telephone call at 24 hours to ensure there are no persistent symptoms. We shall then proceed to further subjects if there are no adverse events.

Then the second and third subjects will be observed over 24h, again only proceeding if there are no adverse events.

Then the 4 to 8th subjects can be given nasal challenge simultaneously.

There will be at least 4 to 7 days interval between each challenge agent in any one volunteer subject.

Dosage Regimen for poly-ICLC

- First dose 10µg per 100µl per nostril
- Second dose 100µg per 100µl per nostril
- Third dose 500µg per 300µl per nostril
- Fourth dose of 1000µg in 500µl per nostril administered over 1 hour

Dosage Regimen for poly-IC

- Single dose of 500µg per 500µl per nostril administered over 1 hour

Dosage Regimen for MPLA and Resiquimod

- First dose 10µg per 100µl per nostril
- Second dose 100µg per 100µl per nostril
- Third dose 500µg per 500µl per nostril administered over 1 hour
- Jan 2016 update: due to the development of pyrexia in a volunteer given the 10µg/nostril dose – we are moving to a weight based dosing regimen. This will involve using a dose between 0.02µg/kg to 0.05µg/kg per nostril dose in remaining subjects.

B. Nasal Challenge with Tuberculin

Visits to ICRRU

Each subject shall be observed for up to 7 days following nasal administration of tuberculin

Visits to ICRRU will be made at:

- Screening
- challenge day (with observation up to 8h after administration)
- 24h, 48h (2d), 3d, 4d, 7d

Intervals between Patients

In the ascending dose part of the study we study groups of 4 people given a single dose, before moving incrementally to the next group of 4 given a higher dose.

For each dosage cohort, a single subject will first be observed for **≥96h**. We shall then proceed to further subjects at the same dose, if there are no adverse events

Then the second subject will be observed over **≥48h**, again only proceeding if there are no adverse events in the first 2 subjects

Then the 3rd and 4th subjects can be given nasal challenge simultaneously.

There will be follow-up for all subjects given nasal challenge at 7d after challenge.

Ascending Dose Protocol in Subjects with Latent TB (LTB)

Single dose of tuberculin PPD per subject (spray each nostril),
4 subjects per dose without placebo initially, 4 groups of 4:

- 0.1TU/nostril, from tuberculin 20U/ml, n=4
  clinical observations: nasal examination and systemic features (BP, pulse, temperature) every 30min for 2h

- 1.0TU/nostril, from tuberculin 20U/ml, n=4
  clinical observations, nasal examination and systemic features (BP, pulse, temperature)

- 2.0TU/nostril, from tuberculin 20U/ml, n=4
  clinical observations, nasal examination and systemic features (BP, pulse, temperature)

- 5.0TU/nostril, from tuberculin 100U/ml, n=4
  clinical observations nasal examination and systemic features (BP, pulse, temperature)

After 5.0TU has been administered clinical data and MSD analyses of nasosorption eluates from all doses will be made.
Then the decision will be made on whether to continue 5.0TUnostril or move up to 10.0TU/nostril, from tuberculin 100U/ml

Highest dose nasal tuberculin cohort

1. Decision on top dose.
   N=8 on top dose with latent TB, 6 versus 2 placebo
   N=8 healthy IGRA test negative, 6 versus 2 placebo

2. Nasosorption:

   - cytokines and chempokines: - elute with buffer
   - lipidomics, prostanoids, resolvins, metabolomics – spin off neat

3. Nasal scrapes for:
   - flow cytometry on T cells and DCs
   - transcriptomics

7. STATISTICS AND DATA ANALYSIS

Data and all appropriate documentation will be stored for a minimum of 10 years after the completion of the study, including the follow-up period.

The major data will be levels of cytokines and chemokines in nasal SAM eluates.

We shall perform a test of distribution of data (Shapiro-Wilks test), although nasal levels of chemokines and cytokines are generally non parametric in distribution.

Raw Data:
We shall make plots for individual cytokines with raw data for individuals connected by lines. These “spaghetti plots” generally have different colours for different individuals.

Summary Statistics
Normal data with graphs with means and standard error of means (SEM)
Non-normal data with graphs with medians, quartiles and ranges (box-whisker)

Non-parametric tests are used to compare challenge effects with placebo at different times:
Willcoxon signed-rank test for paired and Mann-Whitney for unpaired data.

The differences from baseline and difference compared with that from placebo (active-placebo) at each time point and the differences from placebo (active-placebo) will be calculated, and the significance determined.

We shall also analyse area under the curve (AUC), by selecting specified times in the early or late phase or for the entire response.
e.g. PGD2 from 10min to 2h
e.g. for IL-4 at 4 to 8h.

A *P* value of <0.05 is considered significant.
Randomization

For the ViDAR study randomization will be supervised by the pharmacy at St Mary’s Hospital. The pharmacy will organise the packaging and have sole access to the randomization codes until the completion of all the clinical aspects of the study.

8. REGULATORY ISSUES

Topical Nasal Challenge Agents

All our challenge agents are used to elicit an inflammatory response, and not employed for therapeutic benefit.
Hence these agents are regarded by the UK regulatory authority (the Medicines and Healthcare Regulatory Authority, MHRA) as Non-Investigative Medicinal Products (non-IMP) (92).
For this reason, the challenge agents do not have to be manufactured according to Good Manufacturing Practice (GMP).
A proposal to employ these nasal challenges does not require supervision by the MHRA, where we have contacted the Clinical Trials Dept and Dr Elaine Godfrey.

Vitamin D3

We have obtained confirmation from the MHRA and the manufacturers of Vitamin D3 (Lamberts Healthcare Ltd) that the vitamin D3 is classified as a food supplement and not an Investigative Medicinal Product (IMP).

8.1 ETHICS APPROVAL

The Chief Investigator has obtained approval from the Research Ethics Committee. The study must be submitted for Site Specific Assessment (SSA) at each participating NHS Trust. The Chief Investigator will require a copy of the Trust R&D approval letter before

accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

Only for subjects receiving tuberculin we shall propose a limited range of genetic tests. This is because the immune response to TB is strongly influenced by a limited number of genes (61;93). These samples will be handled strictly under the Human Tissue Act, and data handled in a confidential manner, with no reporting back to the subject.

8.2 CONSENT

- Anonymised samples will be aliquoted and stored prior to analysis in all cases.
- An option for the participant to provide blood samples for DNA analysis has been included for Part B
- An option for the participant to take part in a reproducibility element has been included for Part B
- All DNA blood samples will be stored as per Human Tissue Act regulations.
- All publications will be anonymised.

Consent to enter the study must be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent should be obtained. If potential volunteers are identified from sites other than Imperial College Healthcare NHS trust, they will be asked to sign a pre-screening consent form that seeks permission for their identifiable demographic and medical data to be transferred from their base hospital to Imperial College Healthcare NHS trust. This data will be transferred on a password protected spread sheet via secure NHS mail and will subsequently be stored on a secure trust network that is restricted to researchers from the Imperial Clinical Respiratory Research Unit (ICRRU) based at St Mary's Hospital, Paddington, London. The right of the participant to refuse to participate without giving reasons must be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

8.3 CONFIDENTIALITY

The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

8.4 INDEMNITY

Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study.
The College holds two insurance policies which apply to healthcare research.

1. Public Liability (negligent harm)

The College holds Zurich Municipal Public Liability insurance which provides cover in the event that Imperial College is held legally liable for negligent harm caused to a research subject.

2. Clinical Trial (non-negligent harm)

Cover for non-negligent harm arising from an Imperial project is provided by the College's Zurich Municipal Clinical Trials insurance policy. The policy document defines a "clinical trial" very broadly as: "Any investigation or series of investigations conducted on any person for a medicinal purpose".

Consequently, this policy will be relevant if your study involves direct interventions with a research subject.

The non-negligent harm policy specifically excludes cover for claims arising from children aged under five and pregnant women. It has a number of other important exclusions, including, but not limited to, claims arising directly from HIV/AIDS, Hepatitis, Creutzfeldt-Jakob disease, or the use of products or devices designed by Imperial College.

It is usually possible to have the pregnant women and children aged under five exclusions waived on a study-by-study basis following liaison with the Insurer.

http://www3.imperial.ac.uk/clinicalresearchgovernanceoffice/projectplanning/clinicalresearchofficeapproval/insurance

8.5 SPONSOR

Imperial College London will act as the main Sponsor for this study. Delegated responsibilities will be assigned to the NHS Trust taking part in this study.

8.6 FUNDING

Imperial NIHR BRC are funding this study

8.7 AUDITS

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

9. STUDY MANAGEMENT

The day-to-day management of the study will be co-ordinated through Tanushree Tunstall (unit coordinator), Dr Akhilesh Jha (Clinical Research Fellow) and Jennifer Brimley (Research Nurse) with close support from Dr Trevor T. Hansel of ICRRU at St Mary's Hospital.

10. PUBLICATION POLICY

Our aim is to ensure the full dissemination of all study results in medical publications of the highest impact and citation factor. Individual subjects will be strictly anonymised.

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APPENDICES

Appendix 1: Schedule of Events: Summary of Screening Procedures

Depending on the part of the study, a range of these procedures will be performed.

	Pre-challenge
Telephone screening	X
Clinical history	X
Physical exam	X
Skin allergen test	X
Tuberculin skin test	X
Interferon-gamma release assay (IGRA)	X
FBC, U&E, LFT, IgE	X
Chest X-ray and ECG	X
Informed Consent	X
Nasal and Pharyngeal Swab Sample	
Calcium, 25-OH Vitamin D levels, melatonin	X
Pregnancy test (if applicable)	X
Methacholine bronchoprovocation	X

Appendix 2a: Summary of Procedures for Nasal Toll-Like Receptor (TLR) Agonist Challenge

PART A: Incremental Ascending Dose Study based on Tolerability and SAM

Time 2a	**Total duration = 30 min** Nasal sampling before NC			Nasal Challenge (NC)	**Total duration=9h** Hourly nasal sampling post NC				
	-30m	-20m	-10m		15m	30m	45m	1h	2h – 8h
Nasal challenge Saline/TLR agonist (variable doses)				X					
Nasal lavage (Washing only)		L/R							
Nasal mucosal examination	X				X	X	X	X	X
Total nasal symptom scores (TNSS) & PNIF	X				X	X	X	X	X
Nasosorption (L and R)	L/R		L/R		L/R	L/R	L/R	L/R	L/R

- LEFT nostril: Nasosorption neat: to include from lipidomics, mass spectrometry, prostanoids, PGD2, tryptase
- RIGHT nostril: Nasosorption in assay buffer: for chemokines and cytokines, complement C3a & 5a

Appendix 2b: Summary of Procedures for Nasal Toll-Like Receptor (TLR) Agonist Challenge

PART B: Highest Dose (1000µg over 1h, or highest single dose)

Time 2b	**Screening**	**Total duration = 30 min** Nasal sampling before NC			**Total duration = 1h** Nasal Challenge (NC) 200µg/nostril on 5 occasions over 1h					**Total duration=8h** Hourly nasal sampling post NC					**Total duration = 2h**
	D -7	-30m	-20m	-10m	0m	15m	30m	45 m	60m	75m	90m	105m	120m	3h – 9h	24h
Nasal challenge Saline / TLR agonist					X	X	X	X	X						
Nasal lavage (Washing only)	X		L /R												
Nasal mucosal examination		X				X	X	X	X	X	X	X	X	X	X
Total nasal symptom scores (TNSS) & PNIF		X				X	X	X	X	X	X	X	X	X	X
Nasosorption (NS)		L /R		L /R						L /R	L /R	L /R	L /R	L /R	X
Nasal curettage	X Up to 4 samples													(9h) 4L	X 4R
Blood (20ml)			X												X
OPTIONAL: Nasal SSP (Real time MS)		X	X	X	X	X	X	X	X	X	X	X	X	X	X

- LEFT nostril: Nasosorption neat: to include from lipidomics, mass spectrometry, prostanoids, PGD2, tryptase
- RIGHT nostril: Nasosorption in assay buffer: for chemokines and cytokines, complement C3a & 5a

Appendix 2c: Summary of Procedures for Nasal Resiquimod Challenge

PART B: Highest Dose (10µg/100µl)[Jan 2016: move to a weight based dosing regimen between 0.02µg/kg to 0.05µg/kg]

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Time 2c	**Screening**	**Nasal sampling before NC** Total duration = 30 min			**Nasal Challenge** (NC)	**Hourly nasal sampling post NC** Total duration=8h					**Final Sample**
	D -7	-30m	-20m	-10m	0m	15m	30m	45m	60 m	1-10h	24h
Nasal challenge Saline / Resiquimod					X	X	X	X	X		
Nasal lavage (Washing only)	X		L /R								
Nasal mucosal examination		X				X	X	X	X	X	X
Total nasal symptom scores (TNSS) & PNIF		X				X	X	X	X	X	X
Nasosorption (NS)		L /R		L /R		L /R	L /R	L /R	L /R	L /R	X
Nasal curettage	X Up to 4 samples									(6-8h) 4L	X 4R
Blood (up to 20ml)	X		X							X (4h) X (8h)	X
Spirometry (for asthmatics)	X	X								1,2,4,8h	x

Appendix 3a: Part A Summary of Procedures for Tuberculin Nasal Challenge

PART A: Nasal ascending dose (0.1TU, 1.0TU, 2.0TU, 5.0TU) tolerability and SAM

Time 3a	S D -7	-30m	-20m	-10m	Nasal Challenge (NC)	1h – 8h	d1	d2	d3	d4	d7
Nasal challenge Saline/Tuberculin (variable doses)					X	X					
Bilateral nasal lavage for washing purposes	X		X			X					
Nasal mucosal examination		X				X	X	X	X	X	X
Total nasal symptom scores (TNSS) & PNIF		X				X	X	X	X	X	X
Nasosorption		X 2L 2R		X 2L 2R		X 2L 2R	X 2L 2R	X 2L 2R	X 4L	X 4R	X 4R
OPTIONAL Nasal curettage	X							X 4R		X 4L	X 4R

- LEFT nostril: Nasosorption neat: to include from lipidomics, mass spectrometry, prostanoids, PGD2, tryptase
- RIGHT nostril: Nasosorption in assay buffer: for chemokines and cytokines

Appendix 3b: Part B Summary of Procedures for Tuberculin Nasal Challenge

PART B: Top dose (10.0TU) with Additional Sampling

Time 3b	S D -7	-30m	-20m	-10m	Nasal Challenge	1h – 8h	d1	d2	d3	d4	d7
Nasal challenge Placebo/Tuberculin (Top dose)					X						
Bilateral nasal lavage for washing purposes	X		X								
Nasal mucosal examination		X				X	X	X	X	X	X
Total nasal symptom scores (TNSS) & PNIF		X				X	X	X	X	X	X
Nasosorption		X 2L 2R		X 2L 2R		X 2L 2R	X 2L 2R	X 2L 2R	X 4L	X 4L	X 4R
Nasal curettage	X							X 4R		X 4L	X 4R
Blood (20ml)		X						X		X	X
OPTIONAL Nasal SSP							X				

- LEFT nostril: Nasosorption neat: to include from lipidomics, mass spectrometry, prostanoids, PGD2, tryptase
- RIGHT nostril: Nasosorption in assay buffer: for chemokines and cytokines

Appendix 4A: **Vitamin D3 in Nasal Allergic Rhinitis (ViDAR)**
Summary of Procedures for Nasal Allergen Challenge (NAC) Days

Procedure	-30m	-20m	-10m	Nasal Challenge	5m	15m	30m	45m	1h	Hourly 2-8h
Nasal allergen challenge Timothy grass pollen *Phleum P5*				X ~ 9AM						
Bilateral nasal lavage for washing purposes (discard)		X								
Oral capsule: Day 28: vitamin D or placebo	X									
Total nasal symptom scores (TNSS) and peak nasal inspiratory flow (PNIF)			X		X	X	X	X	X	X
Nasosorption (SAM)	L/R		L/R		L/R	L/R	L/R	L/R	L/R	L/R
Blood (75ml)*	X									
Nasal curettage (rhinoprobe)										2L/2R At 8 hours (~5PM)

*Blood for full blood count (5ml); peripheral blood mononuclear cells (PBMC) (50ml); serum vit D, calcium, cortisol, melatonin (10ml); whole blood transcriptomics (10ml)

Appendix 4B: Summary of Procedures for Nasal Allergen Challenge (NAC)

Time 4	-30m	-20m	-10m	NC	5m	10m	15m	30m	45m	1h	2h	3h	4h	5h	6h
Nasal challenge Saline/Allergen				X											
Bilateral nasal lavage for washing purposes		X													
Nasal mucosal examination	X		X		X	X	X	X	X	X	X	X	X	X	X
Total nasal symptom scores (TNSS) & PNIF	X				X		X	X	X	X	X	X	X	X	X
Nasosorption	X 2L 2R		X 2L 2R		X 2L 2R	X 2L 2R	X 2L 2R	X 2L 2R	X 2L 2R	X 2L 2R	X 2L 2R	X 2L 2R	X 2L 2R	X 2L 2R	X 2L 2R
Blood	X														X
Nasal curettage	X 4L														X 4R
OPTIONAL Nasal SSP	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

- LEFT nostril: Nasosorption neat: to include from lipidomics, mass spectrometry, prostanoids, PGD2, tryptase
- RIGHT nostril: Nasosorption in assay buffer: for chemokines and cytokines

Appendix 5: Pharmaceutical Quality of Agents delivered by Nasal Spray as Nasal Challenges or taken orally

Challenge/Oral Agent	Manufacturer Contact person	Catalogue Dose	Production Purity
Timothy grass pollen allergen licensed for intraepidermal and subcutaneous skin testing Aquagen SQ Timothy grass Pollen *Phleum pratense* number 4	Manufacturer: ALK-Abello: TEL 01488 686 016 Andrew Young TEL 01488 688 337 MOB 07917 191 468 andrew.young@alk-abello.com Supplier: Healthcare Logistics TEL 01234 248 736, Mandy Meinertzhagen.	Aquagen SQ Timothy grass Pollen, internal code 1001862 product code 225 156 *(ALK 225)*	ALK Abello freeze dried extract.
Tuberculin purified protein derivative (PPD) licensed internationally for intradermal skin testing In the Mantoux test		• 20 units/ml, 1.5ml vial • 100 units/ml, 1.5ml vial	Heat-treated products of growth and lysis of appropriate *Mycobacterium spp*.
Poly-ICLC (poly inosine-cytosine stabilised with poly-L-lysine) Hiltonol Vaccine component (HTN code 3002200**)** Registered with FDA under IND 43984, held by Oncovir Inc. Lot PJ215-10-01	Dalton Chemical Laboratories for Oncovir Andres M. Salazar, MD CEO & Scientific Director Oncovir, Inc., 3203 Cleveland Ave, NW Washington, DC 200008-3450, USA asalazar@oncovir.com TEL 001 202 342 1726	2mg/ml 1ml/vial	Chemical synthesis. Non-biological origin: does not contain any animal nor human components
Poly-IC (inosine-cytosine), high molecular weight, ultrapure Synthetic analogue of double stranded RNA	Invivogen, California 3950 Sorrento Valley Boulevard, Suite 100, San Diego, CA 92121, USA www.invivogen.com	Tlrl-pic 10mg and 50mg Lyophilised powder Reconstitute in endotoxin-free physiological water	Chemical synthesis. Non-biological origin: does not contain any animal nor human componentsEndotoxin level <1.25 EU/mg
MPLA (monophosphoryl lipid A) Synthetic vaccine grade	Invivogen, California 3950 Sorrento Valley Boulevard, Suite 100, San Diego, CA 92121, USA www.invivogen.com	I mg MPLA VacciGrade supplied as a clear lipidic film. CAS 1246298-63-4 Reconstitute in endotoxin-free physiological water	Chemical synthesis. Non-biological origin: Comprises lipid A from *E.coli* serotype R515, with 6 fatty acyl groups
Resiquimod R848 VacciGrade TLR 7/8 agonist Synthetic vaccine grade	Invivogen, California 3950 Sorrento Valley Boulevard, Suite 100, San Diego, CA 92121, USA www.invivogen.com	5 mg lyophilized R848 Reconstitute in endotoxin-free physiological water CAS 144875-48-9	Non-biological origin: does not contain any animal nor human components. Endotoxin <1.25 EU/mg

Vitamin D3	Vitamin D3 from Lamberts Healthcare Ltd http://www.lambertshealthcare.co.uk	4000IU	Manufactured according to GMP & meets British Pharmacopoiea (BP) standards for Vitamin D levels