Identification of autoantigens and their potential post-translational modification in EGPA and Severe Eosinophilic Asthma.

JRMO Research Protocol for Research Studies

Full Title	Identification of autoantigens and their potential post-translational modification in EGPA and Severe Eosinophilic Asthma.
Short Title	IDentification of EGPA Autoantigens (IDEA)
Sponsor	Queen Mary University of London Contact person: Tumi Kaminskas Research Governance and Performance Manager Joint Research Management Office Research Services Dept W 69-89 Mile End Road, London E1 4UJ Phone: 020 7882 7275 Email: research.governance@qmul.ac.uk
IRAS Number	274097
REC Reference	20/PR/0004
Chief Investigator	Dr Myles Lewis, Senior Lecturer and Consultant Rheumatologist, Queen Mary University of London and Barts Health NHS Trust, UK William Harvey Research Institute, Charterhouse Square, London EC1M 6BQ, UK myles.lewis@qmul.ac.uk +44(0) 20 7882 3305

List of sites	Patient and Healthy Control Recruitment: St Bartholomew's Hospital, Mile End Hospital and Royal London Hospital, Barts Health NHS Trust Royal Free London NHS Trust Research Laboratories: William Harvey Research Institute, Queen Mary University of London

1. Contents

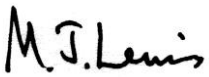
2. Glossary

ANCA	Anti-Neutrophil Cytoplasmic Antibodies
AUC	Area Under the Curve
CI	Chief Investigator
COPD	Chronic Obstructive Pulmonary Disease
CRF	Case Report Form
EGPA	Eosinophilic Granulomatous PolyAngiitis
ELISA	Enzyme-Linked ImmunoSorbent Assay
FeNO	Fractional exhaled Nitric Oxide
ICF	Informed Consent Form
IL	Interleukin
ILC	Innate Lymphoid Cell
JRMO	Joint Research Management Office
NHS REC	National Health Service Research Ethics Committee
NHS R&D	National Health Service Research & Development
Participant	An individual who takes part in a clinical trial
PBMC	Peripheral blood mononuclear cells
PI	Principal Investigator
PIS	Participant Information Sheet
QMUL	Queen Mary University of London
REC	Research Ethics Committee
ROC	Receiver Operating Characteristic
SAE	Serious Adverse Event

3. Signature page

Chief Investigator Agreement

The study, as detailed within this Research Protocol, will be conducted in accordance with the principles of Good Clinical Practice, the UK Policy Framework for Health and Social Care Research, and the Declaration of Helsinki and any other applicable regulations. I agree to take responsibility for the statistical analysis and oversight of this study.

Chief Investigator Name:	**Dr Myles Lewis**
Signature:	
Date:	**20th June 2022**

4. Summary and synopsis

Short title	IDentification of EGPA Autoantigens (IDEA)
Methodology	Laboratory-based research study using blood and sputum samples from healthy controls and patients with EGPA, Severe Eosinophilic Asthma and other eosinophilic diseases.
Objectives / aims	To determine the presence, or not, of auto-antibodies to self-peptides in severe eosinophilic asthma and EGPA.
Number of participants	120 participants (90 patients and 30 healthy controls)
Inclusion and exclusion criteria	Major Inclusion Criteria: Patients: Patients with Severe Eosinophilic Asthma and/or EGPA and/or eosinophilic COPD and/or eosinophilic oesophagitis, age 18 yo and over, and able to give consent. Healthy Controls: age 18 yo and over, and able to give consent. Exclusion criteria: Pregnancy; anaemia; rituximab, plasmapheresis or IVIG infusion ever
Study duration	30 months (18 months recruitment)

5. Introduction

5.1. Background

The pivotal importance of eosinophils in the pathology of severe asthma and eosinophilic granulomatous polyangiitis (EGPA) is increasingly evident given the success of anti-eosinophil biologic therapies. The source of eosinophil stimulating IL-5 in severe asthma is now thought to be ILC2 cells. However the persistent chronicity of the airways inflammation in the absence of an exogenous stimulus in most patients is unexplained and of major research importance. This suggests the presence of an endogenous stimulus of persistent auto-reactive airways inflammation - an auto-immune hypothesis for severe asthma pathology - which with molecular spread could then potentially lead to the systemic disease of EGPA (Mukherjee and Nair 2018).

Auto-antibody responses have been reported in patients with asthma (Mukherjee and Nair 2018). For example Lott and colleagues have detected auto-antibodies to type V collagen in asthmatic individuals (Lott, Sehra et al. 2013). Similarly Liu and colleagues have reported auto-antibodies to type V collagen in asthmatic patients, as well auto-antibodies to other proteins such as Activin A receptor, with clinical correlates to markers of asthma severity (Liu, Subramanian et al. 2012). However auto-antibodies within the pulmonary compartment, as detected in sputum, may be more important than auto-antibodies in serum (Qin, Long et al. 2019). In particular auto-antibodies to eosinophil peroxidase in sputum is a feature of severe eosinophilic asthma (Mukherjee, Bulir et al. 2018).

Anti-neutrophil cytoplasmic antibodies (ANCA), especially anti-myeloperoxidase antibodies, are present in a proportion of patients with EGPA but absent in a similar proportion. Similar perinuclear ANCA is also present in inflammatory bowel disease. These issues raise the question of whether ANCA in EGPA is an epiphenomenon or pathological.

Previous research at our centre and with collaborators has shown that post-translational modification of potential auto-antigens is a key step in breaking self-tolerance and development of autoimmune disease (Ryan, Nissim et al. 2014) (Eggleton, Nissim et al. 2013). For example we have shown auto-antibodies to oxidised type II collagen are much more frequent than those to native type II collagen in rheumatoid arthritis (Nissim, Winyard et al. 2005). Similarly in type 1 diabetes mellitus auto-antibodies to insulin that has undergone oxidative post-translational modification are significantly more common than those to native insulin (Strollo, Vinci et al. 2015).

Oxidative stress, implicated in the formation of oxidised auto-antigens, is a major feature of uncontrolled asthma inflammation (Mak, Ho et al. 2013). However, to date the potential role of modified auto-antigens in the airways has not been studied in severe eosinophilic asthma, EGPA and other related diseases.

In this project we will look for auto-antibodies to relevant proteins both in native form and importantly in post-translationally modified forms. Potential modified auto-antigens are eosinophil proteins (analogous to the cytoplasmic neutrophil proteins identified in vasculitides such as Granulomatosis with Polyangiitis (formerly known as Wegener's) and alternatively structural proteins such as collagen V. As well as advancing our understanding of asthma pathology, identifying a serum auto-antibody that could then be used as a clinical blood test, analogous to anti-CCP antibodies in rheumatoid arthritis, may revolutionise diagnosis of severe eosinophilic asthma and EGPA. There is a considerable burden of undiagnosed severe eosinophilic asthma in part due to difficulties in definitive diagnosis and a diagnostic blood test would help diagnose these patients, allowing them to receive necessary treatment.

We have previous experience of identifying novel autoantigens in Systemic Lupus Erythematosus (SLE) (Lewis et al. 2018), using an advanced protein microarray developed by Oxford Gene Technology, now known as Sengenics. This protein microarray technology benefits from using correctly folded proteins with preserved native epitopes, and the technology permits on chip modification of proteins such as citrullination or oxidation.

5.2. Rationale

We hypothesise the presence of auto-antibodies to self-peptides in severe eosinophilic asthma and EGPA, underpinning persistent airways inflammation in patients with severe eosinophilic asthma refractory to standard therapies. In particular we hypothesise the auto-antigens will be to eosinophil proteins and/or basement membrane proteins that are post-translationally modified.

In this study we will collect blood and sputum samples from well-phenotyped patients with EGPA, severe eosinophilic asthma and other eosinophilic diseases. The presence or not of auto-antibodies to relevant self-peptides, in native and post-translationally modified form, will be determined with appropriate assays.

5.3. Risks / benefits

Previous research has shown the profound benefit of translational research and potential for such studies to advance our understanding of disease leading to new treatments. The only participant involvement in this study is the consent process, the blood donation (venipuncture and collection of up to 50 mls blood in to sterile containers using standard phlebotomy practice at the recruiting NHS Trust), and sputum donation in patients producing sputum. As such risks are low and potential benefits high.

6. Study objectives

We will approach the research question with parallel agnostic and targeted approaches.

In the agnostic approach the presence of auto-antibodies in patient serum and sputum to inactive and activated eosinophils, with and without post-translational modification, will be examined by indirect immunofluorescence.

In the targeted approach we will examine by ELISA the presence/absence of antibodies to pre-selected candidate eosinophil and base membrane proteins both in native form and post-translationally modified. Proteins to examine will be chosen based on literature review (e.g. eosinophil peroxidase and collagen V) and eosinophil-specific proteins identified by FANTOM5 geneset analysis (FANTOM Consortium et al. 2014).

Both blood and sputum samples from highly-characterised patients with severe eosinophilic asthma and/or EGPA will be examined given the possibility of compartment-specific immune responses.

Once candidate auto-antigens have been identified in the selected group of patients with severe eosinophilic asthma and EGPA we will then examine their prevalence in serum samples from a wider selection of patients with eosinophilic airways diseases including mild-to-moderate asthma, severe eosinophilic asthma, EGPA, nasal polyposis and eosinophilic COPD as well as healthy controls. Length of disease, atopy, presence/absence nasal polyps, gender, age will be examined as co-variates. Correlations with highest blood eosinophil counts, requirement for oral corticosteroids and presence of other auto-antibodies, e.g. anti-MPO ANCA, will be examined. In particular we will look for the presence of novel autoantibodies in specific patient subsets: i) ANCA negative, ii) ANCA positive by immunofluorescence but negative for anti-MPO and anti-PR3 antibodies, iii) ANA positive but ANCA and ENA negative; since patients in all three groups may have novel, as yet undetermined autoantibodies. ROC AUC analyses will be conducted to ascertain the predictive value of blood auto-antibodies for diagnosis of eosinophilic airways disease.

6.1. Study Outcomes / Endpoints

Primary objectives

- The presence of serum and/or sputum auto-antibodies to eosinophil and/or basement membrane proteins will be established in severe eosinophilic asthma and EGPA cohorts, and patients with other asthma phenotypes and related eosinophilic diseases.

- Whether auto-antibodies to post-translationally modified self-antigens are more prevalent than to native self-antigens will be examined, and which post-translational

modifications are important to be studied (including particulate matter air pollution as a potential combined stimulus of eosinophil activation and protein modification).

Primary outcomes

- Correlations of auto-antibodies to clinical variables such as spirometry (FEV1 % predicted), pattern of organ involvement, age of onset, requirement for maintenance of oral corticosteroids, eosinophil count, highest recorded eosinophil count and FeNO will be studied.

- Predictive ability to diagnose severe eosinophilic asthma / EGPA of relevant auto-antibodies will be examined by ROC AUC analysis.

Secondary outcomes

- Exploratory Outcomes: Further experimental assays may be conducted to examine immunological mechanisms underpinning associations of auto-antibodies to disease.

7. Study population

90 patients under the care of the Barts Health NHS Trust and Royal Free London NHS Trust with severe eosinophilic asthma, EGPA, eosinophilic COPD and other related pathologies will be recruited.

At least 60 patients will have severe eosinophilic asthma and/or EGPA, and will be recruited from the regional North Central and East London Severe Asthma Service, the Royal Free London NHS Trust and from the Barts Arthritis Centre, with multi-disciplinary consensus diagnoses of severe eosinophilic asthma and/or EGPA respectively. Patients will be phenotyped with blood leucocyte differential, lung function tests and FeNO as appropriate in conjunction with detailed medical history.

Up to a further 30 patients with milder asthma, other vasculitides, eosinophilic COPD, and/or eosinophilic oesophagitis will be recruited from other clinics at Barts Health NHS Trust and the Royal Free London NHS Trust.

In a future extension of this research our intention is to verify positive findings with samples from a replication sample of patients with severe eosinophilic asthma from the wider network of the North Central and East London Severe Asthma Service (principally through our satellite clinic at Royal Free London NHS Trust). These samples will be collected under a separate ethics application.

30 healthy controls will be recruited from volunteers at Queen Mary University of London via poster advertising. Healthy controls will also be recruited from Bart's Health NHS Trust and the Royal Free London NHS Trust.

7.1. Inclusion criteria

- *Informed consent*
- *Age 18 years and over*

- *Patients:*
  - *Severe Eosinophilic Asthma*
    (with multi-disciplinary diagnosis as per ERS/ATS Criteria, blood eosinophils ≥ 0.3 x10^9/L on inhaled corticosteroids); or
  - *EGPA*
    (as per American College of Rheumatology (ACR) Criteria); or
  - *Eosinophilic COPD*
    (post-bronchodilator FEV1/FVC < 70% predicted, absence of bronchodilator reversibility, > 20 pack year smoking history, no medical history of current asthma, blood eosinophils ≥ 0.3 x10^9/L); or
  - *Eosinophilic oesophagitis*
    (with diagnostic histology); or
  - *Granulomatosis with Polyangiitis (GPA, formerly called Wegener's)*
    (as per American College of Rheumatology (ACR) Criteria)

- *Healthy Controls:*
  - *No acute medical illness.*
  - *No diagnosed chronic respiratory disease, allergy or infective condition, including no history of asthma.*
  - *No history of EGPA or other vasculitis,*
  - *No systemic immunosuppressive medication.*

7.2. Exclusion criteria

- *Any inclusion criteria not met*
- *Known Pregnancy*
- *Anaemia*
- *Donation of more than 240mls blood in the last sixteen weeks (four months) to any other research study or as a donation to the National Blood Transfusion Service*
- *Rituximab, plasmapharesis or polyclonal immunoglobulin infusion (ever)*

8. Study design

Study Visit (Patients)

This is a single study visit encompassing patient recruitment, consent, data collection and provision of a peripheral blood sample with/without concurrent sputum sample, with no follow-up visits.

Patients will be recruited via Outpatient Clinics conducted at St Bartholomew's (Bart's) Hospital, The Royal London Hospital, and Mile End Hospital (all Bart's Health NHS Trust), Royal Free London NHS Trust, as well as from inpatients at these hospitals under the care of the Respiratory Medicine and Rheumatology teams (including those under other services needing inpatient respiratory medicine or rheumatology review).

Potential patients will be identified by their clinical team and invited to participate. If interested they will be given a Participant Information Sheet (PIS). Informed Consent and Screening will be undertaken by a GCP-trained member of the clinical team or GCP-trained researcher. An anonymised data collection case report form (CRF) will be completed by a member of the clinical team and a linked (anonymised) blood sample collected by a staff member trained in phlebotomy. If the patient is able to spontaneously produce sputum, and there is no clinical concern about TB infection, then they will be asked to cough a sample in to a sterile sample pot.

The linked anonymised CRF and samples will then be taken to the research laboratories at QMUL for immune cell analysis. Laboratory immunology studies will be conducted by as per protocol (Section 14) by a dedicated Post-Doctoral Research Assistant.

Study Visit (Healthy Controls)

There is a single study visit encompassing healthy control recruitment, consent, data collection and taking of blood sample, with no follow-up visits.

Healthy controls will be recruited at QMUL, Bart's Health and Royal Free sites via poster advertisement.

Healthy controls will be identified by the research team and invited to participate. If interested they will be given a Participant Information Sheet (PIS). Informed Consent and Screening will be undertaken by a GCP-trained researcher. A limited anonymised data collection case report form (CRF) will be completed by a member of the research team and a linked (anonymised) blood sample collected by a staff member trained in phlebotomy. The linked anonymised CRF and blood sample will then be transferred to the research laboratories at QMUL for immune cell analysis.

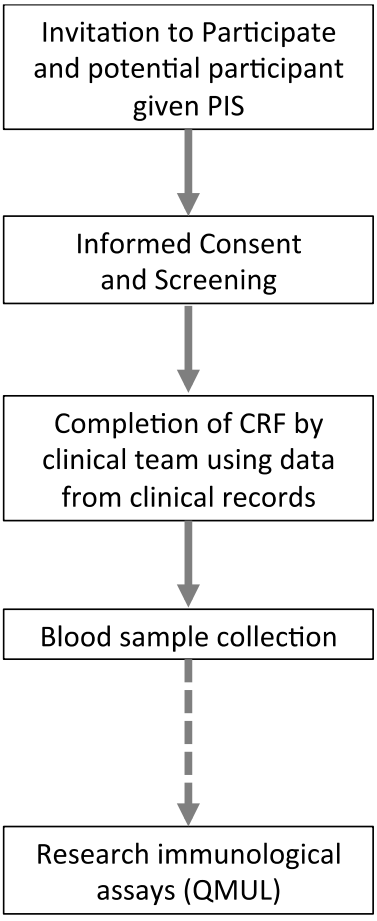


Figure 1: Study design graphic

9. Study procedures

Invitation to Participate:

Invitation to Participate will be by members of their clinical team for Patients and by members of the research team for Healthy Controls. Before participants are invited to participate, capacity to process samples from that type of participant will be discussed with the research team. Invited participants will be given a copy of the Participant Information Sheet (PIS) at invitation to participate.

Informed Consent Procedures and Screening:

Potential participants interested in the study will be given time to read the PIS and ask questions from a GCP-trained member of the research team who may or may not be a member of the clinical team treating the participant. After questions have been answered, potential participants will be asked to complete the written informed Consent Form. Consenting participants will then be briefly screened to check they fulfil inclusion/exclusion criteria.

Data Collection (CRF):

A member of the clinical team (for patient participants) and the research team (for healthy control participants) will complete the case report form (CRF) which contains only pseudoanonymised information but with a study code to link the patient to the samples (and to Consent Form for governance purposes).

Sample Collection:

A peripheral blood sample (up to 50ml total volume) will be collected using aseptic technique into vacutainers or anti-coagulated syringe by staff trained in phlebotomy techniques and securely transported to the research laboratories for analysis.

If the patient is able to spontaneously produce sputum, and there is no clinical concern about TB infection, then they will be asked to cough a sample in to a sterile sample pot.

Samples will be analysed as per Section 14.

Subject Withdrawal:

In the event that a participant asks to withdraw from the study before the end of the Study Visit then the CRF and samples will be securely destroyed and no recording / analysis of data from that patient undertaken. In the event a participant asks to withdraw from the study after the first Study Visit has been completed the research team will be asked to withdraw any unreported linked data from that participant from the study.

Recruitment Time-Frame

Recruitment will be completed within 21 months. Laboratory analysis of collected samples will continue up to 30 months. Residual samples may be stored for future ethically approved studies.

End of Study Definition

The end of the study is at completion of laboratory sample analysis for the last participant recruited. Statistical analysis of data may continue after the End of Study.

10. Statistical considerations

10.1. Sample size

As a pilot laboratory research study with aim of revealing novel associations, a formal power calculation is not possible, since no previous data is available. The sample size is based on previous experience with similar projects and feasibility. The data from this study will be used to calculate sample sizes for future larger scale studies.

10.2. Method of analysis

For the major body of translational laboratory experiments appropriate parametric and non-parametric tests will be conducted using GraphPad Prism. For experiments assessing associations of detected auto-antibodies to clinical variables, linear regression analyses will be undertaken in R, and ROC AUC analysis of capacity of serum auto-antibodies to detect severe eosinophilic diseases undertaken.

11. Ethics

The Chief Investigator will ensure that the study is carried out in accordance with the ethical principles of the UK policy framework for health and social care research (2017) and its subsequent amendments as applicable, as well as applicable legal and regulatory requirements.

The research team has no financial or other competing interests for this study.

Human genome (gDNA) analysis will not be conducted as part of this research project.

Clinically defined immunodeficiencies will not be tested for as part of the immunological analyses.

The CRF and samples for immunological analysis will be anonymised so donors are not known to the research team or anyone reviewing the analyses and results.

Invitation to participate and consent will be undertaken on the same day. Potential participants will be given as long as they require to review the PIS and ask questions before deciding whether to participate. The study clinical procedure (taking of a blood sample of 50ml maximum volume) carries minimal risk. Donor decisions to participate or not in the study will not affect their health or clinical care. We therefore feel this is an appropriate period for informed consent for this low-risk study. Requiring potential volunteers to come back on a second occasion to give informed consent and samples would not facilitate ethical recruitment of patients, many of whom need to travel significant distances to Barts Health/Royal Free Hospitals for appointments and many of whom have significant chronic ill health.

11.1. Annual Safety Reporting

The CI will send an Annual Progress Report to the REC and the sponsor using the HRA template on the anniversary of the REC "favourable opinion".

12. Public involvement

There has been no specific public involvement in the development of this protocol for exploratory translational laboratory research. However this research is commensurate with the research strategies suggested by patient advocacy groups such as Asthma UK and the European Asthma Research and Innovation Partnership (EARIP) asthma research roadmap which themselves had extensive public and patient involvement.

13. Data handling and record keeping

13.1. Data management

The personal data of each patient will be stored in hard copies using paper-based consent forms, paper CRFs and associated data in a secure locked filling cabinet in a locked room. Only members of the research team, monitors and/or regulatory bodies will have access to the physical hard copy of this data and are authorised to do so.

13.2. Source data

Case Report Form (Patients) data variable list:

1. Age
2. Gender
3. Ethnicity (optional)
4. Occupational history
5. Confirmation participant meets Inclusion and Exclusion criteria (Yes/No)
6. Smoking history
7. Diagnosed medical condition(s) and basis for diagnoses with focus on diagnostic criteria for Severe Eosinophilic Asthma, EGPA
8. Suspected medical conditions(s)
9. Immunosuppressive medication(s) dose regimes and history
10. Previous treatment history, especially corticosteroid history
11. Full blood count (haematology)
12. Most recent and previous FeNO breath tests, if done
13. Auto-immune serology results
14. Spirometry and gas transfer tests, if done
15. Highest previous blood eosinophil count, ANCA serology (immunofluorescence, anti-PR3 and anti-MPO antibody titre) and trend
16. Investigations for target organ involvement in patients with suspected vasculitis including histology
17. Clinical disease activity measures (for example Birmingham Vasculitis Activity Score (BVAS), Asthma Control Questionnaire (ACQ)-6)

Case Report Form (Healthy Control) data variable list:

1. Age
2. Gender
3. Ethnicity (optional)
4. Occupational history
5. Confirmation participant meets Inclusion and Exclusion criteria (Yes/No)
6. Smoking history

13.3. Confidentiality

Information related to participants will be kept confidential and managed in accordance with the Data Protection Act, the General Data Protection Regulation (GDPR), NHS Caldecott Principles, UK policy framework for health and social care research (2017), and the conditions of Research Ethics Committee Approval.

Each patient will be assigned a unique study number. Patients' initials and date of birth in the format dd/mm/yy will be used as a means of confirming patient identity for audit checks. This information will be kept in an encrypted screening log, which will be updated throughout the study and stored on a secure server. Only patient study IDs will be used on paper CRFs and samples (pseudoanonymisation). The subject will be identified by a study specific subject number. The patient's name will NOT be included in any CRF or sample.

13.4. Record Retention and Archiving

When the research trial is complete, records will be kept for a further 5 years.

14. Laboratories

Samples will be analysed at the EMR laboratory, Queen Mary University of London:
EMR Laboratory (QMUL)
Dr. Rebecca Hands (Laboratory Manager)
Centre for Experimental Medicine and Rheumatology
2nd floor, John Vane Science Centre, William Harvey Research Institute Barts and the London School of Medicine and Dentistry
Queen Mary University of London, Charterhouse Square
London, EC1M 6BQ
Samples will be processed on day of receipt of the samples (within 24 hours of sampling). Following processing, all samples will be stored immediately in -80C freezers or subject to analysis as described below within 48 hours.

- *Samples will be analysed in the QMUL laboratories at William Harvey Research Institute as follows to investigate the major Study Outcomes:*

INDIRECT IMMUNOFLUORESCENCE ASSAYS

Eosinophils will be cultured in preparatory experiments from the EoL-1 eosinophil cell line and in definitive experiments isolated from patients with eosinophilia in the context of severe asthma. Slides of cell smears will then be prepared of inactive eosinophils and those activated to undergo piecemeal degranulation and cytolysis with eosinophil extracellular trap formation. Cell smears will be examined unmodified and after post-translational modification of proteins. Fixed slides will then be incubated with dilutions of serum and sol phase sputum supernatant from patients and healthy controls, and incubated with fluorescein-conjugated anti-human immunoglobulin antibody before being examined by fluorescent microscopy and fluorescence intensity measured. Confocal microscopy and complementary fluorescein-conjugated antibody markers will be used to examine characteristics of target antigens for patient serum auto-antibodies.

TARGET PROTEIN ASSAYS

Eosinophil proteins such as those previously identified as of importance (e.g. eosinophil-peroxidase, *EPX*) and also novel candidate proteins specific to eosinophils (including *ARFIP1*, *BCL2A1*, *PPCDC*, *SLC12A6* etc) as identified by FANTOM5 geneset analysis will be purchased or synthesised in Expi-293 cells, by obtaining tagged clones from Origene and developing stable expressing cell lines. Protein synthesis be confirmed by western blot. Proteins will be used to coat ELISA plates with/without prior post-translational modification. Presence of donor serum antibodies in serum and in sputum will then be examined by ELISA using appropriate enzyme-

linked secondary antibodies. Similar extracellular matrix proteins such as collagen V will be investigated.

POST-TRANSLATIONAL MODIFICATION OF PEPTIDES

Eosinophil suspensions / slides and commercially sourced / synthesised target proteins (e.g. eosinophil peroxidase and collagen V) will be modified as follows:

Oxidative modification – by hydroxyl radical exposure with hypochlorous acid using our established methodology (Strollo, Vinci et al. 2015). Citrullination – by recombinant peptidylarginine deiminase (PAD).

Ambient pollutants and nanopollutants are known to be able to induce both oxidative stress and protein citrullination as well as potentially direct eosinophil activation. Given the known association between asthma and ambient pollution we will additionally examine potential serum antibodies to ambient pollution particulate matter stimulated/modified eosinophil and collagen proteins.

- *Samples may also be analysed to address associated Exploratory Outcomes, to examine immunological mechanisms underpinning auto-antibody disease associations. Applicable experimental techniques to include:*

Blood and sputum leukocyte phenotyping *ex vivo* by flow-cytometry (FACS) analysis of circulating immune cell subsets (including myeloid-lineage cells, conventional/unconventional lymphocyte populations, and granulocyte populations).

In vitro culture of blood and sputum leukocytes (whole sample cultures, fractionated peripheral blood mononuclear cells, and magnetically separated / FACS-sorted leukocyte subsets) to assess responses to a variety of subset-specific agonists and stimuli. Positive and negative controls to be included as appropriate. Key mediators of these responses may be identified using small molecule inhibitors, blocking antibodies, recombinant cytokines/proteins, essential supplements/chelators, and other equivalent culture additives according to cell type and function of interest. Responses to be assessed using multicolour flow-cytometry methods, advanced imaging techniques (INCell analysis, immunofluorescence and confocal microscopy, ImageStream), quantification of cytokine/chemokine/antibody release by ELISA / multiplex bead array, and measurement of gene expression by PCR / RNA-seq methodology (mRNA but not gDNA). To include B-ELISpot (B lymphocyte enzyme-linked immune absorbent spot) assays to further examine auto-antigen specific immune responses.

Isolated proteins may be examined by immunoblotting, proteomics, mass spectrometry, surface plasmon resonance / microscale thermophoresis, and other equivalent binding studies.

14.1. Study Workflow

Blood samples will be collected in up to 4 Serum Separator Tube (SST) vacutainer tubes and up to 4 Acid Citrate Dextose (ACD) vacutainer tubes.

Serum samples from SST tubes will be aliquoted and stored at -80°C pending analyses as above.

Peripheral blood samples from ACD tubes will be used to store peripheral blood mononuclear cells (PBMC) (with/without sorting of eosinophils and/or other cell subsets such as neutrophils prior to freezing) as viably-frozen cells in freezing medium or in RNA preservation buffer.

Sputum will be processed to isolate sputum plugs from saliva contamination, diluted with buffered saline before centrifugation to separate supernatant and cellular components. Aliquots of supernatant will be stored at -80°C pending analyses as above. The cellular fraction will be treated with dithiothreitol, filtered and final cell suspension stored at -80°C for further analysis or in RNA preservation buffer.

15. Safety reporting

The only patient contact in this study is the consent process and the sample donation (venipuncture and collection of blood into sterile containers using standard phlebotomy practice at the recruiting NHS Trust, with/without collection of spontaneously produced sputum). As such Adverse Events (AE) and Serious Adverse Events (SAE) are extremely unlikely. In the event of an AE or SAE then this will reported to the CI by any research team members, and escalated as appropriate by the CI.

Notification and Reporting of Serious Adverse Events

Serious Adverse Event (SAEs) that are considered to be 'related' and 'unexpected' will be reported to the sponsor within 24 hours of learning of the event and to the Main REC within 15 days in line with the required timeframe.

16. Monitoring and auditing

The Sponsor or delegate retains the right to audit any study, study site or central facility. In addition, any part of the study may be audited by the funders where applicable.

As this is exploratory laboratory research, no formal monitoring plan is planned.

17. Study committees

There is no trial committee for this study. Members of the research team are as follows:

Chief Investigator (CI)	*Dr Myles Lewis* *Senior Clinical Lecturer and* *Consultant Rheumatologist,* *William Harvey Research Institute,* *Queen Mary University of London, UK*
Co-Investigator	*Dr Paul E Pfeffer* *Consultant Respiratory Physician and* *Hon Senior Lecturer,* *Barts Health NHS Trust and Queen Mary University of London, UK*
Other Collaborating Investigators	*Prof Ahuva Nissim,* *Professor in Antibody and Therapeutic Engineering,* *William Harvey Research Institute,* *Queen Mary University of London, UK*

18. Finance and funding

This research study has been designed by Dr Paul Pfeffer (p.pfeffer@qmul.ac.uk), Dr Myles Lewis (myles.lewis@qmul.ac.uk) & Prof Ahuva Nissim (a.nissim@qmul.ac.uk) at Barts Health NHS Trust and Queen Mary University of London. It is being funded by GlaxoSmithKine (GSK) through its Supported Studies Programme. None of the doctors or investigators involved in the study will receive any direct payment dependent on recruiting participants for this study.

19. Insurance and indemnity

The insurance that Queen Mary University of London has in place provides cover for the design and management of the study as well as "No Fault Compensation" for participants, which provides an indemnity to participants for negligent and non-negligent harm.

20. Dissemination of research findings

Results will be presented at national and international meetings, and published in international peer-reviewed scientific journals.

21. References

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