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

Ilaria Esposito,[1] Ioanna Kontra,[1] Chiara Giacomassi,[1,2] Sotiria Manou-Stathopoulou,[1] James Brown,[2] Richard Stratton,[2,3] Galateia Verykokou,[4] Roberto Buccafusca,[5] Mike Stevens,[4,6] Ahuva Nissim,[1,* †] Myles J. Lewis,[1,* †] Paul E. Pfeffer,[1,4,*†]

[1] William Harvey Research Institute, Queen Mary University of London, London, UK
[2] Royal Free NHS Foundation Trust, London, UK
[3] University College London, London, UK
[4] Barts Health NHS Trust, London, UK
[5] School of Physical and Chemical Sciences, Queen Mary University of London, London, UK
[6] University Hospitals Sussex NHS Foundation Trust, Brighton, UK
[†] These authors contributed equally to this work and share last authorship

[*] **Corresponding Authors:**
Myles J. Lewis myles.lewis@qmul.ac.uk
Paul E. Pfeffer p.pfeffer@qmul.ac.uk

Word Count: 4307

Keywords: eosinophil, autoantibodies, autoantigens, oxidative modification, diagnostic test, TREM1, eosinophil peroxidase, NETosis

Abstract

Background: The chronic airways inflammation in severe eosinophilic asthma (SEA) suggests potential autoimmune aetiology with unidentified autoantibodies analogous to myeloperoxidase (MPO) in ANCA-positive EGPA (eosinophilic granulomatosis with polyangiitis). Previous research has shown that oxidative post-translational modification (oxPTM) of proteins is an important mechanism by which autoantibody responses may escape immune tolerance. Autoantibodies to oxPTM autoantigens in SEA have not previously been studied.

Methods: Patients with EGPA and SEA were recruited as well as healthy control participants. Autoantigen agnostic approach: Participant serum was incubated with slides of unstimulated and PMA-stimulated neutrophils and eosinophils, and autoantibodies to granulocytes identified by immunofluorescence with anti-human IgG FITC antibody. Target autoantigen approach: Candidate proteins were identified from previous literature and FANTOM-5 geneset analysis for eosinophil expressed proteins. Serum IgG autoantibodies to these proteins, in native and oxPTM form, were detected by indirect ELISA.

Results: Immunofluorescence studies showed serum from patients with known ANCA stained for IgG against neutrophils as expected. In addition, serum from 9 of 17 tested SEA patients stained for IgG to PMA-stimulated neutrophils undergoing NETosis. Immunofluorescent staining of eosinophil slides was evident with serum from all participants (healthy and with eosinophilic disease) with diffuse cytoplasmic staining except for one SEA individual in which subtle nuclear staining was evident. FANTOM-5 geneset analysis identified TREM1 (triggering receptor expressed on myeloid cells 1) and IL-1 Receptor 2 (IL1R2) as eosinophil specific targets to test for autoantibody responses in addition to MPO, eosinophil peroxidase (EPX) and Collagen-V identified from previous literature. Indirect ELISAs found high concentrations of serum autoantibodies to Collagen-V, MPO and TREM1 in a higher proportion of SEA patients than healthy controls. High concentrations of serum autoantibodies to EPX were evident in serum from both healthy and SEA participants. The proportion of patients with positive autoantibody ELISAs was not increased when examining oxPTM compared to native proteins.

Discussion: Although none of the target proteins studied showed high sensitivity for SEA, the high proportion of patients positive for at least one serum autoantibody shows the potential with more research for autoantibody serology to improve diagnostic testing for severe asthma.

Introduction

The pivotal importance of eosinophils in the pathology of severe asthma and eosinophilic granulomatosis with polyangiitis (EGPA) is increasingly evident given the success of anti-eosinophil biologic therapies (1, 2). However the persistent chronicity of the eosinophilic airways inflammation in the absence of a known chronic, exogenous stimulus in many patients is unexplained and 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 (3).

Autoantibody responses have been reported in patients with asthma (3). Lott and colleagues detected autoantibodies to collagen in asthmatic individuals (4). Similarly, Liu and colleagues have reported autoantibodies to collagen in asthmatic patients, as well autoantibodies to other proteins such as Activin A receptor, with clinical correlates to markers of asthma severity (5). Anti-neutrophil cytoplasmic antibodies (ANCA), especially anti-myeloperoxidase (MPO) 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, raising the question of whether ANCA in EGPA is an epiphenomenon or pathological. Interestingly, recent research has reported in severe asthma sputum autoantibodies to eosinophil peroxidase (EPX), an enzyme closely related to myeloperoxidase (6-8). However, none of these autoantibodies yet reported are present in the serum at high prevalence in severe asthma.

Previous research at our centre and with collaborators has shown that post-translational modification of potential autoantigens is a key step in breaking self-tolerance and development of autoimmune disease (9, 10). We have shown serum autoantibodies to oxidative post-translationally modified type II collagen are more frequent than those to native type II collagen in rheumatoid arthritis (11). Similarly in type 1 diabetes mellitus autoantibodies to insulin that has undergone oxidative post-translational modification are significantly more common than those to native insulin (12). Oxidative stress, implicated in the formation of post-translationally modified autoantigens, is a major feature of uncontrolled asthma inflammation (13). However, to date the potential role of modified autoantigens in the airways has not been studied in severe eosinophilic asthma, EGPA and other related diseases.

Identification of prevalent autoantibodies in severe asthma, EGPA and related diseases is not only important for understanding disease pathogenesis but also in terms of a potential diagnostic test. Diagnosis of asthma and severe asthma, including of patients needing biologic therapy, is often difficult with delays in many cases leading to a significant unmet need (14-

16). In rheumatoid arthritis and other types of inflammatory arthritis the discovery of serum autoantibodies to cyclic citrullinated protein (CCP) and other autoantigens has revolutionised disease diagnosis facilitating early management (17, 18). The identification of similarly diagnostic, serum autoantibodies in severe asthma would greatly advance end-to-end pathway management of these patients.

In this research we have looked for potential serum autoantibodies to relevant proteins both in native form and in oxidative post-translationally modified (oxPTM) form, using antigen-agnostic and targeted approaches. In the agnostic approach we aimed to detect autoantibodies to stimulated granulocytes, whereby cellular induction of reactive oxygen species (ROS) in neutrophil/eosinophil extracellular trap (NET/EET) formation can cause oxidation of granulocyte self-antigens (19, 20). In the targeted approach, we have investigated autoantibodies against specific proteins of interest from prior literature (collagen V, MPO, EPX) and other proteins highly-expressed in eosinophils as identified by FANTOM5 geneset analysis (FANTOM Consortium 2014). The success of eosinophil suppressing biologic therapies suggests eosinophils are a prime candidate as a possible source of autoantigens in severe asthma in some individuals.

Materials and methods

Participant Recruitment and Sampling

Patients with severe eosinophilic asthma (severe asthma with/without nasal polyps), patients with EGPA, healthy control participants, and a group of patients with other similar diseases for comparison (patients with mild-to-moderate asthma, eosinophilic chronic obstructive pulmonary disease (COPD), and those with other vasculitides) were recruited with informed consent (NHS REC ethics approval 20/PR/0004). Severe eosinophilic asthma (SEA) patients had a diagnosis as per ERS/ATS criteria confirmed by specialist clinic multi-disciplinary consensus as per standard UK practice (21, 22), with a recorded blood eosinophil count of ≥0.3 $x10^9$/L on inhaled corticosteroids. For ELISA analyses these patients were subdivided by presence or not of nasal polyps; as severe eosinophilic asthma with nasal polyps (SEA+NP) or without nasal polyps (SEA-NP). Patients with EGPA had a clinical diagnosis of such and were confirmed to meet the research criteria suggested by Wechsler and colleagues (23). Patients with chronic obstructive pulmonary disease (COPD) and those with granulomatosis with polyangiitis or ANCA-associated vasculitis other than EGPA (GPA/AAV) were diagnosed as

127 such by their specialist clinical teams. Healthy controls were required to have no acute medical
128 illness; no diagnosed chronic respiratory disease, allergy or infective condition, including no
129 history of asthma; no history of EGPA or other vasculitis; and to not be taking any systemic
130 immunosuppressive medication. Rituximab, plasmapheresis and/or polyclonal
131 immunoglobulin infusion (ever) were ineligibility criteria for study participation. The recruited
132 patient populations included both those with a new diagnosis prior to definitive treatment and
133 those already established on definitive treatment such as a biologic or steroid-sparing
134 immunosuppressant medication.
135 Clinical data for participants was extracted from electronic medical records by their clinical
136 teams and used to confirm patient protocol eligibility and for phenotyping patients. Blood was
137 collected in appropriate serum separator tubes, and serum aliquoted and stored at -80°C
138 pending use in assays. Due to the COVID pandemic there were delays in completing
139 recruitment and some experimental assays had to be run before all participants were recruited
140 (in particular anti-MPO and anti-Collagen V ELISAs; Supplemental Table S1).
141
142 **Neutrophil Immunofluorescence**
143 Venous blood from healthy human volunteers (separate to the study serum donors) was
144 collected in EDTA vacutainer tubes (BD Biosciences) and neutrophils isolated using the
145 density gradient medium Polymorphprep (Proteogenix) as per manufacturer's instructions.
146 Isolation of neutrophils was confirmed by flow-cytometry using a PerCP/Cyanine5.5 anti-
147 human CD11b antibody (BioLegend) and a PE/Cyanine7 anti-human CD16 antibody
148 (BioLegend). Neutrophils were plated on microscope slides (Hendley-Essex) at concentration
149 of 2 x 10^6 cells/ml (100 µl/well) and incubated for 30 minutes at 37°C with 5% CO_2 to allow
150 adherence. For NETosis slides, cells were stimulated with 100µl PMA (final concentration
151 400nM) in HBSS buffer (Gibco) containing 2mM $CaCl_2$ (Sigma) for 4 hours at 37°C with 5%
152 CO_2. Unstimulated neutrophils were incubated for 4 hours in HBSS buffer, in parallel.
153 Neutrophils were then fixed and permeabilized with 95% ethanol for 15 minutes at -20°C,
154 washed with PBS and incubated with a 1:20 dilution of serum from study participants in PBS
155 for 20 minutes at room temperature. After washing, a 1:320 dilution of anti-human IgG FITC
156 conjugate antibody (Dako) in PBS was applied to each well for 20 minutes at room temperature
157 in the dark. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) for 20 minutes at
158 room temperature in the dark. The stained cells were imaged using LSM800 Zeiss fluorescence
159 microscope using the program ZenBlue. At x20 optical magnification, three random fields

160 were captured for each sample and the images were acquired. These images were then analysed
161 using ImageJ software by a clinical immunologist experienced in reading clinical ANCA slides,
162 and blinded to the disease status of study participants. Exposure times of each channel (blue
163 or green) were kept constant throughout the analysis.
164
165 **Eosinophil Immunofluorescence**
166 Whole blood from healthy human volunteers was collected in ACD vacutainer tubes (BD
167 Biosciences) and layered onto Lymphoprep (STEMCELL Technologies) as per manufacturer's
168 instructions and centrifuged at 2000 rpm for 30 min at room temperature with break off. The
169 plasma layer, mononuclear cell band and the density gradient medium were discarded to leave
170 the red blood cell / polymorphonuclear pellet. Lysis of red blood cells in the pellet was
171 performed using ACK lysis solution (Sigma), 10mM potassium bicarbonate and 97.3µM
172 EDTA. Eosinophils were isolated from the total polymorphonuclear cells by magnetic
173 selection using the EasySep Human Eosinophil Isolation Kit (STEMCELL Technologies) as
174 per manufacturer's instructions. Isolation of eosinophils was confirmed by flow-cytometry
175 using a PerCP/Cyanine5.5 anti-human CD11b antibody (BioLegend) and a Pacific Blue anti-
176 human CRTH2 antibody (BioLegend). Eosinophils were plated on microscope slides
177 (Hendley-Essex) at concentration of 2×10^6 cells/ml (100 µl/well) and incubated for 4 hours at
178 37°C with 5% CO_2. Eosinophils were fixed and permeabilized with 95% ethanol for 15
179 minutes at -20°C, washed with PBS and incubated with 0.1M glycine for 10 minutes at room
180 temperature. The cells were blocked using a serum free Protein Block (Agilent Dako) for 30
181 minutes at room temperature before incubating with serum diluted 1:20 in Antibody Diluent
182 (Agilent Dako) for 20 minutes at room temperature. After washing, a 1:320 dilution of anti-
183 human IgG FITC conjugate antibody (Dako) was applied to each well for 20 minutes at room
184 temperature in the dark, followed by DAPI staining. Eosinophil slides were then read as per
185 neutrophil slides.
186
187 **Analysis of FANTOM5 Dataset for Eosinophil Specific Genes**
188 RLE normalized FANTOM5 data (24) were downloaded from
189 http://fantom.gsc.riken.jp/5/data/ and analysed as previously described (25). In brief, data were
190 subsetted to include only unmanipulated and uncultured primary tissues (derived cells,
191 stimulated cells, and cell lines were excluded) and restricted to NCBI gene transcripts. For each
192 gene the CAGE peak with the highest mean expression was used. Data were Z-score
193 normalized across all primary tissues and expression of each gene ranked across all tissues. A

194 specificity score was determined for all genes by counting the number of tissues showing
195 increased gene expression Z score >3 (i.e. more than 3 SD above the mean expression across
196 all tissues), so that the most tissue specific genes would have the lowest specificity scores.
197 After different cut-offs were tested for robustness, genes were considered specific to a tissue
198 type using the following criteria: i) the level of gene expression in that tissue was in the top
199 three tissues (i.e., rank 1-3); ii) Z score >5 (i.e. >5 SD above the mean expression across all
200 tissues); iii) specificity score <10 tissues. Gene modules for different cell types were consistent
201 with lists of genes previously published by the FANTOM5 consortium for several cell types
202 (26) (27).
203
204 **Target Proteins and Oxidation**
205 Human recombinant myeloperoxidase (MPO; cat# BA1078, Origene), Interleukin-1 Receptor
206 2 (IL1R2; cat# 10111-HCCH, Sino Biological), Triggering Receptor Expressed On Myeloid
207 Cells 1 protein (TREM1; cat# 10511-H08H, Sino Biological), Collagen V (ColV; cat# C3657,
208 Sigma), full-length Eosinophil Peroxidase (EPX; cat# abx620092, Abbexa) and light-chain
209 EPX (cat# abx653287, Abbexa) were sourced as 'native' target antigens for indirect ELISAs.
210 Oxidative post-translational modification of target proteins was also undertaken to produce
211 oxPTM target antigens by incubation overnight at 37°C with sodium hypochlorite at
212 respectively these concentrations: 0.1mM, 1mM, 0.5mM, 0.5mM and 0.4mM (oxPTM light-
213 chain EPX not undertaken). Protein modifications were monitored by gradient 4-20% reducing
214 sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by
215 staining with Coomassie blue (Abcam). Protein modification with EPX was monitored by
216 mass spectroscopy (Supplemental Methods and Figures S1-S4).
217
218 **Autoantibody Indirect ELISAs**
219 Polystyrene 96-well plates (Nunc MaxiSorp, Thermo Fisher) were coated with 500ng/well of
220 native / oxPTM proteins in 50mM pH 9.6 sodium bicarbonate and incubated overnight at 4°C.
221 Plates were washed and blocked with 2% (wt./vol.) dry milk powder (ChemCruz) in 1x 0.05%
222 PBS-Tween-20 for 2 hours at room temperature under agitation at 200 rpm. Human sera
223 samples diluted 1:50 in 2% (wt./vol.) dry milk powder in PBS-Tween were added in duplicate
224 (100µl in each well), followed by 2 hours incubation at room temperature.
225 Secondary goat anti-human IgG alkaline phosphatase (ALP) conjugate antibody (Jackson
226 Immunoresearch) diluted 1:5000 or secondary goat anti-human IgG- horseradish peroxidase
227 (HRP)-conjugate antibody (Jackson Immunoresearch) diluted 1:1000 in 2% (wt./vol.) dry milk

228 powder in 1x PBS /Tween-20 0.05% was added to detect IL1R2, MPO and EPX, or Collagen
229 V and TREM1 proteins respectively and incubated for 1 hour at room temperature avoiding
230 light exposure.
231 HRP ELISAs were subsequently incubated with TMB reagent before being stopped with 1M
232 sulphuric acid and optical density (OD) for each sample read at 450 nm using a Fluostar Omega
233 Plate reader (BMG LABTECH). ALP ELISAs were incubated with p-nitrophenyl phosphate
234 reagent and optical density (OD) read at 405nm.
235
236 **Statistics**
237 The upper limit of normal (ULN) for ELISA ODs was set as 1.96 x SD above the mean for
238 healthy participants. Statistics were analysed in R (version 4.1.2; www.r-project.org) as
239 described in the text using the following additional packages: ggplot2, dplyr, cowplot, tabyl,
240 janitor, vcd. Statistical comparisons between two groups were performed using two-tailed
241 unpaired Wilcoxon test and between three or more groups using Kruskal-Wallis test. P-values
242 < 0.05 were considered significant.
243
244
245 **Results**
246
247 **Participants**
248 Patients with severe eosinophilic asthma (SEA) and those with EGPA were recruited from
249 specialist clinics in London, UK, as was a comparison group of patients with other similar
250 diseases, for example patients with COPD, with granulomatosis with polyangiitis or ANCA-
251 associated vasculitis other than EGPA (GPA/AAV). Healthy control (HC) participants were
252 recruited from the same geographical area.
253 63 patients with SEA were recruited of whom 30 had nasal polyps. 17 patients with EGPA
254 were recruited of whom 6 were ANCA-positive when clinically tested at disease diagnosis and
255 7 had nasal polyps. 10 patients were recruited to the comparison group with other similar
256 diseases (1 with moderate asthma (MA), 4 with GPA/AAV and 5 with COPD). 30 HC
257 participants were recruited. Participant characteristics are shown in Table 1.
258
259 **Granulocyte Autoantibody Immunofluorescence**
260 To assess for serum autoantibodies against granulocyte, and in particular eosinophil, antigens
261 in patients with SEA / EGPA and healthy controls in an unbiased, autoantigen-agnostic manner

262 we conducted immunofluorescence studies in the manner of clinical ANCA
263 immunofluorescence tests.
264 In initial experiments, slides of unstimulated neutrophils, and PMA-stimulated neutrophils with
265 induced NETosis, from healthy donors separate to the HC participant group, were prepared.
266 Slides were incubated with participant serum and then presence of IgG in patient / HC serum
267 to antigens on neutrophil slides examined by immunofluorescence. Serum was tested from 29
268 participants (7 healthy controls, 17 patients with severe eosinophilic asthma (SEA)
269 with/without nasal polyps, and 5 with EGPA; Supplemental Table S2). For unstimulated
270 neutrophil slides, strongly positive nuclear staining was evident with serum from 2 patients, 1
271 of whom had ANCA-positive (clinical lab test) EGPA (Figure 1A) and the other severe
272 eosinophilic asthma with positive ANCA (clinical lab test) but not meeting clinical criteria for
273 EGPA. Weakly positive staining of borderline significance was evident with serum from 3
274 further patients (2 with SEA, 1 with EGPA), with the remaining samples negative (example
275 Figure 1B). For PMA-stimulated slides with evident NETosis, strongly positive IgG staining
276 was evident with serum from 7 donors (5 severe eosinophilic asthma, 2 EGPA; Figure 1B),
277 weakly positive staining with serum from 5 participants (4 severe eosinophilic asthma, 1
278 healthy control), with the remaining 17 negative (example Figure 1A). The
279 immunofluorescence from positive PMA-stimulated neutrophil slides was predominantly of
280 clusters of speckles, not associated with cell nuclei. Of the two patients with nuclear staining
281 of unstimulated neutrophils, one had weakly positive staining of the PMA-stimulated
282 neutrophil slide and the other no significant staining. Considering the 7 healthy controls as a
283 group, none had a positive neutrophil immunofluorescence slide except one with a weakly
284 positive staining for a PMA-stimulated slide. Considering the 17 SEA patients as a group (not
285 including those with EGPA), 9 had a positive or weakly positive neutrophil
286 immunofluorescence slide.
287 In subsequent experiments we aimed to examine eosinophil slides in a similar manner.
288 However, use of an equivalent protocol for slide staining led to non-specific staining of
289 eosinophils (positive immunofluorescence with anti-human IgG FITC conjugate antibody in
290 the absence of addition of participant serum). Addition of a protein block to the protocol
291 abrogated most of the non-specific staining (Figure 2A), and serum was tested from 11
292 participants (5 healthy controls, 2 patients with ANCA-positive EGPA, 1 with SEA, 1 with
293 moderate asthma (MA), 1 with COPD and 1 with GPA/AAV; Supplemental Table S2) using
294 the adapted protocol. Diffuse cytoplasmic staining was evident with all eosinophil
295 immunofluorescence slides, including those with serum from healthy controls (Figure 2B) and

296 patients (Figure 2C), with subtle nuclear staining evident with serum from 1 patient with SEA
297 known to be ANCA positive (Figure 2D). Given the non-specific immunofluorescence evident
298 with healthy control eosinophil slides, and subtle difference with slide exhibiting nuclear
299 staining, diagnostic use of staining for anti-eosinophil cytoplasmic antibodies (AECA) was not
300 felt pragmatic and therefore attention shifted to a targeted protein approach.
301
302 **Indirect ELISA for Serum Autoantibodies to Target Proteins**
303 Candidate target proteins for the indirect ELISA included those previously identified of interest
304 in asthma and other eosinophil highly-expressed targets were identified by reanalysis of the
305 FANTOM5 tissue repository geneset (Figure 3A) (24). Triggering Receptor Expressed On
306 Myeloid Cells 1 protein (TREM1) and Interleukin-1 Receptor 2 (IL1R2) were identified as
307 novel highly eosinophil specific genes compared to other peripheral blood subset cell types
308 (Figure 3B). We also compared these results with proteins considered to be granulocyte
309 expressed including MPO and EPX. It was noteworthy that *MPO* transcript showed moderately
310 specific expression in eosinophils with higher transcript levels compared to neutrophils.
311 However, EPX which is considered an eosinophil specific protein had negligible transcript
312 levels in eosinophils and showed generally low expression in all cell types analysed. This is
313 plausible because secreted proteins stored in granules, including numerous cytokines, may
314 commonly demonstrate very low mRNA transcript levels once sufficient protein has been
315 synthesised and stored in relevant cells within secretory granules.
316 ELISA optical densities (ODs), as a measure of autoantibody serum concentrations, to
317 Collagen V (ColV) above the Upper Limit of Normal (ULN, defined using ODs from healthy
318 controls) – here after termed positive tests - were evident with serum from 4 SEA individuals
319 (out of 50 tested; 8.0%) but none of the 23 healthy controls tested (Figure 4A, Table 2, and
320 Supplemental Figure S5). Serum samples positive for antibodies to oxPTM-ColV were evident
321 in 3 individuals – 2 with SEA and 1 with EGPA, and no healthy controls (Figure 4B).
322 9 of 52 participants (17.3%) with SEA had positive serum autoantibody status to unmodified
323 MPO, and 3 of 13 participants (23.1%) with EGPA, compared to 2 of 24 (8.3%) healthy
324 controls (Figure 4C). With oxPTM-MPO proportions were similar; 5 of 52 with SEA (9.6%),
325 4 of 13 with EGPA (30.1%), and 1 of 24 healthy controls (4.2%) (Figure 4D).
326 8 of 63 SEA patients tested (12.7%) had positive serum autoantibodies to TREM1 in contrast
327 to 0 of 16 patients with EGPA (0.0%) and 1 of 30 healthy controls (3.3%) (Figure 4E). With
328 oxPTM-TREM1 proportions were lower with 2 of 63 patients positive (3.2%) and 1 of 30
329 healthy controls (3.3%) (Figure 4F). 3 of 63 SEA patients (4.8%) and 2 of 30 healthy controls

330 (6.7%) had positive serum autoantibodies to IL1R2 (Figure 4G) with similar proportions
331 positive for oxPTM-IL1R2 (Figure 4H).
332 In ELISAs for autoantibodies to EPX and oxPTM-EPX, ODs for both healthy controls and
333 patients were notably higher than with other target protein ELISAs despite other aspects of the
334 ELISA protocols being unchanged (Figure 4I). No patients had higher concentrations of serum
335 autoantibodies to EPX than the ULN from the healthy controls. Results with oxPTM-EPX
336 were similar (Figure 4J).
337 To examine whether the higher ODs with the EPX ELISAs might be due to non-specific
338 reactions to the EPX protein used in the ELISA, in a small subset of patients we conducted
339 ELISAs using a different protein preparation of EPX (light chain only) and compared results.
340 There was a strong positive correlation between ELISA ODs using the different EPX protein
341 preparations (Figure 5A) consistent with the ELISAs measuring high concentrations of IgG to
342 EPX in the serum of both healthy controls and patients, rather than non-specific signal.
343 Comparing ELISA ODs to EPX and MPO it was apparent that many patients with positive IgG
344 to MPO also had high serum concentrations of IgG to EPX (Figure 5B).
345 In further analyses we sought to understand within the group of patients with SEA and EGPA
346 whether there were particular characteristics associated with higher concentrations (as
347 measured by ELISA OD) for serum autoantibodies to TREM1 and EPX. There were no
348 differences in concentrations by patient gender (Figure 5C) or patient age (Figure 5D). With
349 regard to smoking status, the median anti-EPX OD was higher in never smokers than those
350 with a smoking history but distribution was not significantly different (Figure 5E). However,
351 non-atopic patients had significantly higher serum concentrations for anti-EPX IgG than atopic
352 patients (Wilcoxon test, $p=0.034$; Figure 5F). Whether or not the patient was on definitive
353 treatment (with a biologic or steroid-sparing immunosuppressant) did not appear to have major
354 impact on anti-TREM1 and anti-EPX serology, and neither did presence/absence of current
355 blood eosinophilia (Supplemental Figure S6).
356 Of the 9 severe asthma patients with positive immunofluorescence for PMA-stimulated
357 neutrophil slides, 6 had a positive autoantibody ELISA though there was no clear association
358 with a specific autoantigen.
359 Finally, we examined whether the proportion of participants positive to one or more of ColV,
360 MPO, TREM1 and IL1R2 was higher in patients with SEA compared to healthy controls
361 (Figure 6). Patients with EGPA were excluded from this analysis due to potential selection
362 bias in that group in favour of ANCA positive individuals. Of 46 SEA patients who had been

tested against all four potential autoantigens, 19 were positive to at least one compared to 4 of 25 healthy controls (Chi-Squared test p=0.030).

Discussion

In this research we set out to identify potential serum autoantibody responses in patients with severe eosinophilic asthma and EGPA using an approach agnostic to candidate autoantigens (granulocyte immunofluorescence) and a targeted approach (indirect ELISAs), and in particular to oxidative post-translationally modified autoantigens. In the agnostic approach serum from a high proportion of severe eosinophilic asthma patients yielded IgG staining of PMA-stimulated neutrophils that had undergone NETosis in a manner indicative of serum antibodies against potentially oxidised products of NETosis. However severe eosinophilic asthma is characterised by eosinophilic rather than neutrophilic inflammation and we therefore proceeded to look for serum antibodies to eosinophils using similar methodology. Nuclear eosinophil immunofluorescence was evident with serum from an ANCA-positive severe asthma patient but more notable was the diffuse cytoplasmic staining evident with all healthy controls and other patients. Given the aim of developing a clinical blood test to aid diagnosis of severe eosinophilic asthma and EGPA, and the subtlety of the difference in eosinophil staining pattern, we proceeded to the target protein approach. Indirect ELISAs found serum autoantibodies to ColV, MPO and TREM1 in a higher proportion of SEA patients than healthy controls, and to IL1R2 in similar proportions. High titres of IgG to EPX were present in high number of both patients and healthy controls. The proportion of SEA patients with autoantibodies positive to at least one of ColV, MPO, TREM1 and/or IL1R2 was significantly greater than the proportion of healthy controls, though no single autoantibody ELISA showed high sensitivity.

Whether elevated titres of autoantibodies to TREM1 in severe eosinophilic asthma, as identified in this research, is an epiphenomenon or whether these autoantibodies have a functional role in asthma pathology will require more research. TREM1 is a transmembrane protein expressed by immune cells with functional role in amplifying certain immune responses. Interestingly associations between asthma and TREM1 pathway signalling activity

396 have been reported by several groups (28, 29), and relative suppression of the TREM1
397 signalling pathway has been reported in eosinophilic nasal polyposis (30, 31).
398
399 We hypothesised that serum autoantibodies in severe asthma and EGPA may be to antigens
400 post-translationally modified by oxidation in the airways. Oxidative burst is a feature of
401 granulocyte degranulation and NETosis/EETosis, and the presence of antibodies in the serum
402 of many patients with severe eosinophilic asthma to products of NETosis may be to oxidised
403 neutrophil-derived proteins. However, none of the candidate protein autoantibodies were more
404 prevalent to the oxidised than the native unmodified form. Although post-translational
405 modification of proteins may not be a major mechanism in the development of auto-immune
406 responses in the airway, protein oxidation is only one of many different types of post-
407 translational modification that proteins may undergo and other types of PTM may be more
408 important. For example, the action of eosinophil peroxidase has been linked with
409 carbamylation of proteins (32). Histone citrullination is a feature of granulocyte extracellular
410 trap formation and potentially other bystander proteins may also be citrullinated (33).
411 In this study we have used stimulation of NET/EETosis as a physiological mechanism for
412 generation of post-translationally modified granulocyte proteins. NETs and EETs have active
413 functions *in vivo*. Of particular relevance to this research is their capacity to be immunogenic
414 – NETs have been shown to facilitate uptake by dendritic cells of neutrophil antigens and
415 thereby induction of ANCA autoantibodies (34). NETs may also be able to facilitate release
416 of potential autoantigens by epithelial cells (35), an action that might be shared with EETs,
417 which have been shown to similarly have effects on epithelial cells as well as activating other
418 eosinophils (36). Products of NETosis can promote type 2 inflammation in murine models
419 (37), and have recently been suggested as a potential biomarker in asthma (38).
420 However, the pathological role for autoantibodies may differ between anti-MPO positive and
421 ANCA negative EGPA. Genomic research shows anti-MPO positive EGPA to have a strong
422 association at *HLA-DQ* consistent with autoantibody pathology whilst ANCA negative EGPA
423 has a separate but weaker association in the *HLA* region (39). Anti-PR3 ANCA associated
424 vasculitis has different genomic associations, in particular in the *HLA-DP* region (40, 41).
425
426 In our targeted autoantigen approach, we selected for assay proteins encoded by genes
427 identified as being highly expressed with high specificity in eosinophils in the FANTOM5
428 Dataset. The advantage of this approach was the ability to identify potential novel eosinophil-
429 associated auto-antigen proteins, in a dataset generated from primary cells rather than cell lines.

430 We only investigated a proportion of the identified targets in this proof-of-principle study. A
431 limitation of the FANTOM5 Dataset is that it identifies gene transcripts rather than expressed
432 proteins – not all transcripts are translated into proteins and the genes for some proteins are not
433 continually expressed. An alternative would have been to use a protein dataset and this should
434 be considered in future research.
435 Our research indicates large-scale screening of different proteins and post-translational
436 modifications may yet identify serum autoantibodies, alone or in combination as a panel, with
437 good sensitivity and specificity for diagnosis of severe asthma and (ANCA negative) EGPA.
438 It is important for any clinical test to have both good sensitivity for the disease but also high
439 specificity. In this case it would need good specificity for severe asthma, to differentiate from
440 other causes of breathlessness and other eosinophilic conditions. Severe asthma is a
441 heterogenous condition and individual autoantibodies may show specificity for particular
442 endotypes of severe asthma. To address this, and reflect real-world clinical conditions, we
443 recruited a broad SEA patient group, without restricting patient characteristics further than
444 being of an eosinophilic endotype. Clinical data was used to further characterise patients by
445 presence/absence of nasal polyps, atopy and smoking history. COVID pandemic spirometry
446 restrictions unfortunately prevented characterisation by presence/absence of persistent airflow
447 limitation. Our patient population was also heterogenous in terms of disease stage, including
448 both new patients who had recently completed initial assessment and those on definitive
449 treatment with a biologic / steroid-sparing immunosuppressant – this was in keeping with our
450 interest in a possible autoantibody diagnostic test (as such not masked by treatment status)
451 rather than a disease activity biomarker. However, the clinical data to address the question of
452 whether prevalence of seropositivity increases in severe asthma as a function of duration of
453 disease was not collected.
454

455 The diffuse cytoplasmic staining of eosinophils with healthy control / patient serum and FITC-
456 labelled anti-IgG, and the high prevalence of serum IgG to eosinophil proteins such as EPX in
457 both serum from healthy controls and patients, suggests serum anti-eosinophil autoantibodies
458 may potentially be common in both health and disease. A limitation of our experiments is that
459 we only looked for IgG autoantibodies, and not IgA/IgM/IgE, and could not determine the
460 protein epitope to which the autoantibodies may bind nor the IgG subclass. Elevated IgG4
461 subclass immunoglobulin can in particular be a feature of EGPA (42). Potentially differences
462 in these characteristics of any anti-eosinophil protein antibodies may determine whether they
463 have pathological action. A number of our asthma patients had positive anti-MPO antibodies

464 in our experimental assays, and clinical ANCA results, but did not have other aspects of EGPA
465 to support a diagnosis of the systemic, vasculitic autoimmune disease. Potentially the epitope
466 for anti-MPO antibodies is different in these patients to those with EGPA (43).
467 However, autoantibodies within the pulmonary compartment, as detected in sputum, may be
468 more important than autoantibodies in serum (44, 45). In particular autoantibodies to
469 eosinophil peroxidase in sputum is a feature of severe eosinophilic asthma and not apparent in
470 sputum from healthy controls (6). Possibly it is not the development of autoantibodies to
471 eosinophil proteins that is abnormal in severe asthma, but local production of the autoantibody
472 in the lungs is the issue. This is consistent with the published negative association between
473 peripheral blood lymphocyte counts and sputum autoantibodies, suggestive that migration of
474 B lymphocytes to inflamed lungs is a determinant of airway autoimmune responses (7).
475
476 In this research we focussed on potential autoantibodies to eosinophils with the hypothesis that
477 the clinical effectiveness of eosinophil-suppressing anti-IL-5 therapies may in part be the
478 reduction of a circulating eosinophil autoantigen. Since this research was initiated, sputum
479 autoantibody responses to macrophage receptor with collagenous structure (MARCO) have
480 recently been reported in severe asthma (7, 46), challenging the criticality of eosinophils in
481 asthma immunology. Importantly IL-5 has actions on cell types other than eosinophils,
482 including B cells (47, 48), and the effects of anti-IL-5 biologics to block IL-5 dependent actions
483 on these cell types may be of underappreciated importance.
484
485 In conclusion, although we did not find serum autoantibody responses to oxidative post-
486 translationally modified proteins to be frequent in severe eosinophilic asthma and EGPA, we
487 did find high proportions of patients to have autoantibodies to TREM1 and to PMA-stimulated
488 neutrophils undergoing NETosis. It is increasingly apparent that severe asthma is a
489 heterogeneous condition and different endotypes may be associated with different auto-
490 antibodies. As such a serological diagnostic test may require a panel of autoantigens rather
491 than a single antigen, as has been reported in other diseases (49, 50), and consistent with that
492 we found the proportion of participants positive to one or more of ColV, MPO, TREM1 and
493 IL1R2 was higher in patients with severe eosinophilic asthma than in healthy controls.
494
495
496
497 **Contribution to the field statement**

Severe eosinophilic asthma and eosinophilic granulomatosis with polyangiitis (EGPA) share features, such as persistent steroid-refractory inflammation, suggestive of potential autoimmune aetiology. Autoantibody blood tests have revolutionised our diagnosis and understanding of many autoimmune diseases, however, serum autoantibodies have not been described to-date in the vast majority of cases of severe asthma and autoantibodies such as ANCA are only reported in around 30% of EGPA patients across studies.

In this research we have taken two approaches to identifying new autoantibodies in these diseases to eosinophils, a cell type known to be critical for these pathologies, and closely related neutrophils. Additionally, we examined whether potential autoantibody responses might be to antigens modified by oxidation.

Autoantibody responses were identified in asthma patients to a novel autoantigen, TREM1 protein, in addition to other self-antigens, although antibodies were not more prevalent to oxidatively modified antigens. Serum autoantibody responses were also evident in severe asthma patients to stimulated neutrophils undergoing NETosis.

Although positivity rates to each of the autoantibodies tested individually were relatively low, a significantly greater proportion of severe asthma patients than healthy controls showed autoantibody seropositivity to a panel of four potential autoantigens including TREM1. This suggests that even if a single autoantigen used solely is insufficient for diagnosis, there is definite potential for a composite diagnostic autoantibody panel for severe asthma and EGPA, with further autoantigens to be identified in future to improve sensitivity and specificity.

Funding: This research was funded by GSK as an Investigator Supported Study (reference 10969).

Study Registration: ClinicalTrials.gov Identifier: NCT0467144

Contributions: The research was conceived by PEP, ML, AN. IE and IK conducted the majority of experiments. MS and RB supported specific experimental methodologies. PEP, ML, CG, SMS, JB, RS, GV contributed to patient recruitment and data collection as well as contributing to translation of immunology findings to clinical pathology. All authors contributed to and reviewed this manuscript.

Declarations of Interest: PEP has attended advisory board for AstraZeneca, GlaxoSmithKline and Sanofi; has given lectures at meetings with/without lecture honoraria supported by AstraZeneca and GlaxoSmithKline; has attended international conferences with AstraZeneca; has taken part in clinical trials sponsored by AstraZeneca, GlaxoSmithKline, Novartis and Sanofi.

Acknowledgements: We would like to acknowledge help from the CMR Advanced Bio-Imaging Facility of QMUL microscope facility at Queen Mary University of London for the use, help and advice with immunofluorescent microscopy.

540 **Tables**

541

	Healthy Control	Severe Eosinophilic Asthma	EGPA	Other
Number of Participants	30	63	17	10 (MA=1, GPA/AAV=4, COPD=5)
Gender				
Female	16	29	7	4
Male	14	34	10	6
Age (mean)	42.0	54.4	56.6	63.1
Smoking status				
Never Smoker	25	36	11	4
Ex-smoker	2	26	4	3
Current smoker	0	1	0	2
Treatment status				
On long-term oral corticosteroid	N/A	19	14	3
On biologic	N/A	41	5	0
On steroid-sparing immunosuppressant	N/A	0	15	1

542

543 **Table 1: Participant Characteristics**

544 Numbers of participants except Age (mean years). EGPA; eosinophilic granulomatosis with
545 polyangiitis. MA; moderate asthma. GPA/AAV; granulomatosis with polyangiitis or ANCA-
546 associated vasculitis other than EGPA. COPD; chronic obstructive pulmonary disease. N/A;
547 not applicable.

548

Protein	HC	SEA (SEA-NP, SEA+NP)	EGPA
ColV	0.0%	8.0% (10.3%, 4.8%)	0.0%
oxPTM-ColV	0.0%	4.0% (6.9%, 0%)	8.3%
MPO	8.3%	17.3% (13.3%, 22.7%)	23.1%
oxPTM-MPO	4.2%	9.6% (10.0%, 9.1%)	30.8%
TREM1	3.3%	12.7% (9.1%, 16.7%)	0.0%
oxPTM-TREM1	3.3%	3.2% (6.1%, 0.0%)	0.0%
IL1R2	6.7%	4.8% (9.1%, 0.0%)	0.0%
oxPTM-IL1R2	6.7%	6.3% (12.1%, 0.0%)	0.0%
EPX	6.7%	0.0% (0.0%, 0.0%)	0.0%
oxPTM-EPX	6.7%	3.3% (3.0%, 3.6%)	0.0%

549

550 **Table 2: Serum Autoantibody Positivity Across Participant Groups**

551 Percentage of participants by group with positive serum autoantibodies to each target protein
552 as identified by indirect ELISA. Positive result; ELISA OD over the ULN as determined from
553 the healthy control participant group. HC; healthy control. SEA; severe eosinophilic asthma.
554 SEA-NP; severe eosinophilic asthma without nasal polyps. SEA+NP; severe eosinophilic
555 asthma with nasal polyps. EGPA; eosinophilic granulomatosis with polyangiitis.

556 **Figure Legends**

557

558 **Figure 1: Immunofluorescent staining of serum IgG to unstimulated neutrophils and to**
559 **PMA-stimulated neutrophils undergoing NETosis.**
560 Immunofluorescent staining with participant serum from (A) a patient with ANCA-positive
561 EGPA, exhibiting IgG to unstimulated neutrophils but not to PMA-stimulated neutrophils, and
562 from (B) a patient with severe eosinophilic asthma, exhibiting IgG to PMA-stimulated
563 neutrophils but not unstimulated neutrophils. DAPI; 4′,6-diamidino-2-phenylindole staining.
564 FITC; anti-human IgG FITC conjugate antibody staining. Photomicrographs of representative
565 field of views with x20 optical microscopy. Insets shows x4 digital zoom images of
566 characteristic features.

567

568 **Figure 2: Immunofluorescent staining of serum IgG to eosinophils from healthy control**
569 **participants and patients with severe eosinophilic asthma and EGPA.**
570 Eosinophil slide immunofluorescence with anti-human IgG FITC conjugate antibody staining;
571 (A) in the absence of participant serum, (B) with serum from healthy control participants, (C)
572 with serum from patients with ANCA-positive EGPA, and (D) with serum from ANCA-
573 positive patient with severe eosinophilic asthma. Photomicrographs of representative field of
574 views with x20 optical microscopy. Insets shows x4 digital zoom images of characteristic
575 features.

576

577 **Figure 3: Analysis of FANTOM5 CAGE-sequencing tissue repository dataset**
578 (A) Heatmap showing CAGE-Seq expression of cell-specific genesets in peripheral blood
579 subsets from FANTOM5. (B) Bar charts comparing transcript expression levels of eosinophil-
580 specific genes *MPO*, *EPX*, *TREM1* and *IL1R2* in human peripheral blood subsets.

581

582 **Figure 4: Serum autoantibodies to target native and oxidative post-translationally**
583 **modified (oxPTM) proteins as measured by indirect ELISA.**
584 ELISA optical density (OD) on a logarithmic scale (*y*-axis) as measure of serum IgG
585 autoantibodies to target proteins in native and oxidative post-translationally modified form
586 (oxPTM) in different participant groups (*x*-axis). HC; healthy control participants. SEA-NP;
587 severe eosinophilic asthma without nasal polyps; SEA+NP; severe eosinophilic asthma with
588 nasal polyps. EGPA; eosinophilic granulomatosis with polyangiitis. *y*-axis intercept at the
589 upper limit of normal (ULN) for healthy control participant ELISA OD.

590

591 **Figure 5: Analyses of serum autoantibody ELISA ODs relative to patient characteristics.**
592 (A) ELISA OD for serum IgG to full length EPX protein compared to for serum IgG to EPX
593 light chain protein. (B) ELISA OD for serum IgG to full length EPX protein compared to for
594 serum IgG to MPO protein. Scatter plot points for individual participants formatted as per
595 Figure 4 with axis-intercepts at upper limits of normal (ULN) for healthy control participants.
596 (C-F) Effects of (C) Gender, (D) Age, (E) Smoking Status and (F) Atopic Status on measures
597 of serum IgG to TREM1 and EPX in patients with Severe Eosinophilic Asthma and EGPA.
598

599 **Figure 6: Mosaic plot of proportions of participants with positive autoantibody status**
600 Mosaic plot of proportions of healthy control participants (HC) and severe eosinophilic asthma
601 (SEA) patients negative or positive for serum autoantibodies to at least one of ColV, MPO,
602 TREM1 or IL1R2. Positive defined as an ELISA OD above ULN. Boxes sized relative to
603 patient number. P-value for Chi-Squared test.

References

1. Pavord ID, Bel EH, Bourdin A, Chan R, Han JK, Keene ON, et al. From DREAM to REALITI-A and beyond: Mepolizumab for the treatment of eosinophil-driven diseases. *Allergy* (2022) 77(3):778-97.

2. Redmond C, Heaney LG, Chaudhuri R, Jackson DJ, Menzies-Gow A, Pfeffer P, et al. Benefits of specialist severe asthma management: demographic and geographic disparities. *Eur Respir J* (2022) 60(6):2200660.

3. Mukherjee M, Nair P. Autoimmune Responses in Severe Asthma. *Allergy Asthma Immunol Res* (2018) 10(5):428-47.

4. Lott JM, Sehra S, Mehrotra P, Mickler EA, Fisher AJ, Zhang W, et al. Type V Collagen–induced Tolerance Prevents Airway Hyperresponsiveness. *Am J Respir Crit Care Med* (2013) 187(4):454-7.

5. Liu M, Subramanian V, Christie C, Castro M, Mohanakumar T. Immune responses to self-antigens in asthma patients: clinical and immunopathological implications. *Hum Immunol* (2012) 73(5):511-6.

6. Mukherjee M, Bulir DC, Radford K, Kjarsgaard M, Huang CM, Jacobsen EA, et al. Sputum autoantibodies in patients with severe eosinophilic asthma. *J Allergy Clin Immunol* (2018) 141(4):1269-79.

7. Salter B, Zhao N, Son K, Suray Tan N, Dvorkin-Gheva A, Radford K, et al. Airway autoantibodies are determinants of asthma severity. *Eur Respir J* (2022). 60(6):2200442.

8. Klein DK, Silberbrandt A, Frøssing L, Hvidtfeldt M, von Bülow A, Nair P, et al. Impact of former smoking exposure on airway eosinophilic activation and autoimmunity in patients with severe asthma. *Eur Respir J* (2022) 60(4):2102446.

9. Ryan BJ, Nissim A, Winyard PG. Oxidative post-translational modifications and their involvement in the pathogenesis of autoimmune diseases. *Redox Biol* (2014) 2:715-24.

10. Eggleton P, Nissim A, Ryan BJ, Whiteman M, Winyard PG. Detection and isolation of human serum autoantibodies that recognize oxidatively modified autoantigens. *Free Radic Biol Med* (2013) 57:79-91.

11. Nissim A, Winyard PG, Corrigall V, Fatah R, Perrett D, Panayi G, et al. Generation of neoantigenic epitopes after posttranslational modification of type II collagen by factors present within the inflamed joint. *Arthritis Rheum* (2005) 52(12):3829-38.

12. Strollo R, Vinci C, Arshad MH, Perrett D, Tiberti C, Chiarelli F, et al. Antibodies to post-translationally modified insulin in type 1 diabetes. *Diabetologia* (2015) 58:2851-60.

13. Mak JC, Ho SP, Ho AS, Law BK, Cheung AH, Ho JC, et al. Sustained elevation of systemic oxidative stress and inflammation in exacerbation and remission of asthma. *ISRN Allergy* (2013) 2013:561831.

640 14. Miravitlles M, Andreu I, Romero Y, Sitjar S, Altés A, Anton E. Difficulties in
641 differential diagnosis of COPD and asthma in primary care. *Br J Gen Pract* (2012)
642 62(595):e68-75.

643 15. Lynch BA, Van Norman CA, Jacobson RM, Weaver AL, Juhn YJ. Impact of delay in
644 asthma diagnosis on health care service use. *Allergy Asthma Proc* (2010) 31(4):e48-e52.

645 16. Ryan D, Heatley H, Heaney LG, Jackson DJ, Pfeffer PE, Busby J, et al. Potential Severe
646 Asthma Hidden in UK Primary Care. *J Allergy Clin Immunol Pract* (2021) 9(4):1612-
647 23.e9.

648 17. van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP Antibody, a Marker for the Early
649 Detection of Rheumatoid Arthritis. *Ann N Y Acad Sci* (2008) 1143:268-85.

650 18. Aggarwal R, Liao K, Nair R, Ringold S, Costenbader KH. Anti-citrullinated peptide
651 antibody assays and their role in the diagnosis of rheumatoid arthritis. *Arthritis Rheum*
652 (2009) 61(11):1472-83.

653 19. Dahlgren C, Karlsson A, Bylund J. Intracellular Neutrophil Oxidants: From Laboratory
654 Curiosity to Clinical Reality. *J Immunol* (2019) 202(11):3127-34.

655 20. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al.
656 Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are
657 interferogenic and contribute to lupus-like disease. *Nat Med* (2016) 22(2):146-53.

658 21. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International
659 ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur*
660 *Respir J* (2014) 43(2):343-73.

661 22. Jackson DJ, Busby J, Pfeffer PE, Menzies-Gow A, Brown T, Gore R, et al.
662 Characterisation of patients with severe asthma in the UK Severe Asthma Registry in the
663 biologic era. *Thorax* (2021) 76(3):220-7.

664 23. Wechsler ME, Akuthota P, Jayne D, Khoury P, Klion A, Langford CA, et al.
665 Mepolizumab or Placebo for Eosinophilic Granulomatosis with Polyangiitis. *N Engl J*
666 *Med* (2017) 376(20):1921-32.

667 24. Forrest AR, Kawaji H, Rehli M, Baillie JK, de Hoon MJ, Haberle V, et al. A promoter-
668 level mammalian expression atlas. *Nature* (2014) 507(7493):462-70.

669 25. Lewis MJ, Barnes MR, Blighe K, Goldmann K, Rana S, Hackney JA, et al. Molecular
670 Portraits of Early Rheumatoid Arthritis Identify Clinical and Treatment Response
671 Phenotypes. *Cell Rep* (2019) 28(9):2455-70.e5.

672 26. Motakis E, Guhl S, Ishizu Y, Itoh M, Kawaji H, de Hoon M, et al. Redefinition of the
673 human mast cell transcriptome by deep-CAGE sequencing. *Blood* (2014) 123(17):e58-
674 67.

675 27. Schmidl C, Hansmann L, Lassmann T, Balwierz PJ, Kawaji H, Itoh M, et al. The
676 enhancer and promoter landscape of human regulatory and conventional T-cell
677 subpopulations. *Blood* (2014) 123(17):e68-78.

678 28. Sánchez-Ovando S, Simpson JL, Barker D, Baines KJ, Wark PAB. Transcriptomics of
679 biopsies identifies novel genes and pathways linked to neutrophilic inflammation in
680 severe asthma. *Clin Exp Allergy* (2021) 51(10):1279-94.

681 29. Croteau-Chonka DC, Qiu W, Martinez FD, Strunk RC, Lemanske RF, Liu AH, et al.
682 Gene Expression Profiling in Blood Provides Reproducible Molecular Insights into
683 Asthma Control. *Am J Respir Crit Care Med* (2017) 195(2):179-88.

684 30. Wu D, Mueller SK, Nocera AL, Finn K, Libermann TA, Bleier BS. TREM-1 Neutrophil
685 Activation Pathway Is Suppressed in Eosinophilic Nasal Polyps. *Am J Rhinol Allergy*
686 (2018) 32(5):359-68.

687 31. Yao Y, Yang C, Yi X, Xie S, Sun H. Comparative analysis of inflammatory signature
688 profiles in eosinophilic and noneosinophilic chronic rhinosinusitis with nasal polyposis.
689 *Biosci Rep* (2020) 40(2):BSR20193101.

690 32. Wang Z, DiDonato JA, Buffa J, Comhair SA, Aronica MA, Dweik RA, et al. Eosinophil
691 Peroxidase Catalyzed Protein Carbamylation Participates in Asthma. *J Biol Chem* (2016)
692 291(42):22118-35.

693 33. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev*
694 *Immunol* (2018) 18(2):134-47.

695 34. Sangaletti S, Tripodo C, Chiodoni C, Guarnotta C, Cappetti B, Casalini P, et al.
696 Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to
697 myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood*
698 (2012) 120(15):3007-18.

699 35. Choi Y, Pham LD, Lee DH, Ban GY, Lee JH, Kim SH, et al. Neutrophil Extracellular
700 DNA Traps Induce Autoantigen Production by Airway Epithelial Cells. *Mediators*
701 *Inflamm* (2017) 2017:5675029.

702 36. Choi Y, Le Pham D, Lee DH, Lee SH, Kim SH, Park HS. Biological function of
703 eosinophil extracellular traps in patients with severe eosinophilic asthma. *Exp Mol Med*
704 (2018) 50(8):1-8.

705 37. Toussaint M, Jackson DJ, Swieboda D, Guedán A, Tsourouktsoglou TD, Ching YM, et
706 al. Host DNA released by NETosis promotes rhinovirus-induced type-2 allergic asthma
707 exacerbation. *Nat Med* (2017) 23(6):681-91.

708 38. Varricchi G, Modestino L, Poto R, Cristinziano L, Gentile L, Postiglione L, et al.
709 Neutrophil extracellular traps and neutrophil-derived mediators as possible biomarkers in
710 bronchial asthma. *Clin Exp Med* (2022) 22(2):285-300.

711 39. Lyons PA, Peters JE, Alberici F, Liley J, Coulson RMR, Astle W, et al. Genome-wide
712 association study of eosinophilic granulomatosis with polyangiitis reveals genomic loci
713 stratified by ANCA status. *Nat Commun* (2019) 10(1):5120.

714 40. Xie G, Roshandel D, Sherva R, Monach PA, Lu EY, Kung T, et al. Association of
715 granulomatosis with polyangiitis (Wegener's) with HLA-DPB1*04 and SEMA6A gene
716 variants: evidence from genome-wide analysis. *Arthritis Rheum* (2013) 65(9):2457-68.

717 41. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, et al. Genetically
718 distinct subsets within ANCA-associated vasculitis. *N Engl J Med* (2012) 367(3):214-23.

719 42. Wu Z, Zhang S, Li P, Song N, Zhang F, Li Y. Elevated Serum IgG4 Was Found in
720 Eosinophilic Granulomatosis With Polyangiitis. *J Clin Rheumatol* (2021) 27(8):e501-e4.

721 43. Roth AJ, Ooi JD, Hess JJ, van Timmeren MM, Berg EA, Poulton CE, et al. Epitope
722 specificity determines pathogenicity and detectability in ANCA-associated vasculitis. *J*
723 *Clin Invest* (2013) 123(4):1773-83.

724 44. Mukherjee M, Thomas SR, Radford K, Dvorkin-Gheva A, Davydchenko S, Kjarsgaard
725 M, et al. Sputum Antineutrophil Cytoplasmic Antibodies in Serum Antineutrophil
726 Cytoplasmic Antibody-Negative Eosinophilic Granulomatosis with Polyangiitis. *Am J*
727 *Respir Crit Care Med* (2019) 199(2):158-70.

728 45. Qin R, Long F, Xiao X, Xiao J, Zheng Z, Feng M, et al. Sputum Autoantibodies Are
729 More Relevant in Autoimmune Responses in Asthma than Are Serum Autoantibodies.
730 *Allergy Asthma Immunol Res* (2019) 11(3):406-21.

731 46. Son K, Miyasaki K, Salter B, Loukov D, Chon J, Zhao N, et al. Autoantibody-mediated
732 Macrophage Dysfunction in Severe Asthma Patients with Airway Infections. *Am J*
733 *Respir Crit Care Med* (2023) 207(4):427-437.

734 47. Kariyawasam HH, James LK. Do B cells rather than eosinophils drive chronic
735 rhinosinusitis with nasal polyps? *Lancet Respir Med* (2021) 9(10):e97.

736 48. Sabogal Piñeros YS, Bal SM, van de Pol MA, Dierdorp BS, Dekker T, Dijkhuis A, et al.
737 Anti-IL-5 in Mild Asthma Alters Rhinovirus-induced Macrophage, B-Cell, and
738 Neutrophil Responses (MATERIAL). A Placebo-controlled, Double-Blind Study. *Am J*
739 *Respir Crit Care Med* (2019) 199(4):508-17.

740 49. Tampoia M, Notarnicola A, Abbracciavento L, Fontana A, Giannini M, Louis Humbel
741 R, et al. A New Immunodot Assay for Multiplex Detection of Autoantibodies in a Cohort
742 of Italian Patients With Idiopathic Inflammatory Myopathies. *J Clin Lab Anal* (2016)
743 30(6):859-66.

744 50. Shen L, Suresh L. Autoantibodies, detection methods and panels for diagnosis of
745 Sjögren's syndrome. *Clin Immunol* (2017) 182:24-9.

746