Vulnerability to acid reflux of the airway epithelium in severe asthma

Jeanne-Marie Perotin[1,2,3], Gabrielle Wheway[4,5], Kamran Tariq[1,2], Adnan Azim[1,2], Robert A. Ridley[2], Jonathan A. Ward[6], James P.R. Schofield[7], Clair Barber[1,2], Peter Howarth[1,2], Donna E. Davies[1,2,5,8] and Ratko Djukanovic[1,2,5,8]

[1]NIHR Southampton Biomedical Research Centre, University Hospital Southampton, Southampton, UK. [2]Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK. [3]Dept of Respiratory Diseases, UMRS1250, University Hospital of Reims, Reims, France. [4]Human Development and Health, University of Southampton Faculty of Medicine, Southampton, UK. [5]Institute for Life Sciences, University of Southampton, Southampton, UK. [6]The Histochemistry Research Unit, Faculty of Medicine, University of Southampton, Southampton, UK. [7]Centre for Proteomic Research, Institute for Life Sciences, University of Southampton, Southampton, UK. [8]Joint senior authors.

Corresponding author: Jeanne-Marie Perotin (jmperotin-collard@chu-reims.fr)

Shareable abstract (@ERSpublications)
Using a combination of *in vitro* and *ex vivo* approaches, this study identified reflux causing significant effects on the bronchial epithelial structure and function, which were greater in patients with severe asthma https://bit.ly/31XV9tq

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Abstract

Background Severe asthma is associated with multiple comorbidities, including gastro-oesophageal reflux disease (GORD), which can contribute to exacerbation frequency and poor quality of life. Since epithelial dysfunction is an important feature in asthma, we hypothesised that in severe asthma the bronchial epithelium is more susceptible to the effects of acid reflux.

Methods We developed an *in vitro* model of GORD using differentiated bronchial epithelial cells (BECs) from normal or severe asthmatic donors exposed to a combination of pepsin, acid pH and bile acids using a multiple challenge protocol (MCP-PAB). In addition, we analysed bronchial biopsies and undertook RNA sequencing of bronchial brushings from controls and severe asthmatics without or with GORD.

Results Exposure of BECs to the MCP-PAB caused structural disruption, increased permeability, interleukin (IL)-33 expression, inflammatory mediator release and changes in gene expression for multiple biological processes. Cultures from severe asthmatics were significantly more affected than those from healthy donors. Analysis of bronchial biopsies confirmed increased IL-33 expression in severe asthmatics with GORD. RNA sequencing of bronchial brushings from this group identified 15 of the top 37 dysregulated genes found in MCP-PAB treated BECs, including genes involved in oxidative stress responses.

Conclusions and clinical implication By affecting epithelial permeability, GORD may increase exposure of the airway submucosa to allergens and pathogens, resulting in increased risk of inflammation and exacerbations. These results suggest the need for research into alternative therapeutic management of GORD in severe asthma.

Introduction

Severe asthma is associated with multiple comorbidities, including gastro-oesophageal reflux disease (GORD), which is particularly common and is associated with exacerbation frequency and poor quality of life [1]. Until recently, this association was explained by three mechanisms: vagal reflex [2], neuroinflammation [3] and microaspiration directly triggering airway inflammation [4–7]. While studies of reflux in animal models [4–6, 8] and cultures of bronchial epithelial cells [9, 10] have shown varying impact of gastro-oesophageal refluxate on mediators of inflammation and airway remodelling, direct *in vivo* evidence for these mechanisms in patients with asthma has been limited. We recently undertook an in-depth analysis of sputum proteomics in severe asthmatics and identified 11 proteins differentially

abundant in patients with GORD, including elevated levels of antimicrobial proteins and reduced levels of proteins involved in systemic inflammatory responses and epithelial integrity [11], providing the first direct evidence that reflux is associated with changes in the microenvironment on the epithelial surface of the airways. Recognising that defective epithelial barrier function, dysregulated repair mechanisms and modified epithelial immune responses to pathogens and allergens are important features in asthma [12], we further hypothesised that the presence of GORD in severe asthma would significantly influence global epithelial gene expression. Applying unbiased topological data analysis of microarray data derived from bronchial brushings, we identified a subset of severe asthmatics with a clinical phenotype defined by obesity, presence of GORD and treatment with proton pump inhibitors (PPIs) [13], characterised by upregulated airway remodelling signalling and downregulated mechanisms of immune cell recruitment, possibly linked to both bile acid exposure and PPI treatment [13].

In the current study, we sought to elucidate further the underlying mechanisms of GORD-associated dysregulation of the airway epithelium in severe asthma using a combination of *in vitro* and *ex vivo* approaches. We developed an *in vitro* model of GORD in which fully differentiated air–liquid interface (ALI) cultures of primary bronchial epithelial cells were exposed to a multiple challenge protocol using pepsin, acid pH and bile acids (MCP-PAB). Consistent with our previous *in vivo* observations [13], we observed that *ex vivo* exposure of epithelial cells to refluxate results in significant structural and functional changes. We then extended our studies using bronchial biopsies and bronchial brushings from severe asthmatics with GORD and confirmed the effects on interleukin (IL)-33 and changes in expression of a selection of genes identified from the *in vitro* study.

Methods

Study participants and sample collection

Severe asthmatics (step 4/5 of British Thoracic Society/Scottish Intercollegiate Guidelines Network guidelines) and healthy control participants were recruited prospectively and assessed for GORD using 24-h pH/impedance studies. The severe asthmatics were further stratified into those with documented GORD but no PPI treatment; those with documented GORD and PPI treatment; and those without GORD. Epithelial cells were harvested by bronchoscopic brushings and processed into RNA for subsequent RNA sequencing (RNA-seq) analysis or used in primary bronchial epithelial cell culture [14]. In addition, bronchial biopsies were taken and fixed in paraffin for immunohistochemical analyses.

The study was approved by the South-Central Hampshire A research ethics committee (reference numbers: 13/SC/0182 and 14/WM/1226) and all participants gave their informed consent.

Analysis of the ex vivo effect of MCP-PAB

Initial dose and time-course studies were conducted with 16HBE cells exposed to MCP-PAB (pepsin and chenodeoxycholic acid (CDC) at acidic pH); optimised conditions were subsequently confirmed to be appropriate for fully differentiated ALI cultures (see supplementary material and supplementary figure E1 for full details). MCP-PAB conditions were applied to the apical epithelial surface for 30 min before washing twice. After 4 h recovery, epithelial permeability was measured using transepithelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC) dextran 4 kDa [14]. Apical supernatants were collected for cytokine measurements. Cells were then lysed with TRIzol lysis reagent (Life Technologies, Paisley, UK) and frozen at −80°C until analysis or fixed for immunofluorescence staining or electron microscopy analysis.

Immunostaining and electron microscopy

ALI cultures were fixed with 4% paraformaldehyde and processed for immunofluorescence staining, as described previously [14]. In addition, the cultures were processed for transmission electron microscopy (TEM) and analysed for epithelial integrity.

Bronchial biopsies were processed as described previously [15], embedded into paraffin; sections were stained using goat polyclonal anti-human IL-33 (R&D Systems, Abingdon, UK). Results were expressed as positive nuclei per total number of epithelial cells.

Cytokine analyses

IL-8 concentrations in conditioned media were measured using an IL-8 DuoSet ELISA (R&D, Abingdon, UK) while IL-6, tumour necrosis factor (TNF)-α and IL-1α were measured using VPlex (MSD, MD, USA).

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TABLE 1 Characteristics of participants

	Healthy controls	Severe asthmatics	p-value	Severe asthma, no GORD	Severe asthma and GORD	Severe asthma and GORD + PPI
Participants	15	18		4	6	8
Age, years	41.5±12.9	55.7±8.1	0.0005	52.5±6.2	54.3±9.2	58.25±7.99
Age at asthma onset, years		23.7±21.0		24.0±25.7	27.3±23.6	20.75±19.40
Female/male	11/4	11/7	0.48	3/1	6/0	2/6
Never-smoker	11	8	0.28	3	4	7
BMI, kg·m^{-2}	25.76±3.68	32.55±6.74	0.001	30.28±5.97	37.0±5.5	30.4±6.9
Obese (BMI >30 kg·m^{-2})	1	10	0.004	2	5	3
Diagnosed GORD	0	14	<0.0001	0	6	8
Atopy	4	11	0.08	1	4	6
Sputum						
Eosinophils, % total cells	0.14 (0–0.51)	1.01 (0.27–3.57)	0.10	1.97 (0.26–4.83)	0.51 (0.13–5.23)	1.13 (0.34–2.95)
Neutrophils, % total cells	44.3 (20.1–74.7)	64.1 (37.8–73.1)	0.44	52.9 (31.4–68.3)	65.0(60.3–73.4)	63.4 (11.7–75.3)
FEV_1, % predicted	116.6±23.4	89.7±23.6	0.004	82.5±23.0	105.8±24.4	78.3±15.7
FVC, % predicted	121.7±19.6	110.7±20.0	0.14	99.4±12.1	124.8±25.0	104.1±9.4
FEV_1/FVC	0.80±0.04	0.66±0.12	0.0001	0.69±0.12	0.72±0.08	59.2±12.2
F_{eNO}		24.2±11.1		33.0±17.0	28.8±11.0	19.8±8.8
Exacerbations (in past year)		4.1±3.4		3.3±1.5	3.7±2.0	4.7±4.9
ACQ6		12.8±6.5		6.5±4.8	15.5±6.2	14±5.88
SGRQ						
Activity score	5.16±6.55	58.12±24.52	<0.0001	43.89±12.02	62.10±23.10	72.94±16.66
Impact score	0.32±1.15	36.47±15.82	<0.0001	20.45±10.08	39.10±18.28	41.75±12.94
Symptoms score	8.44±8.93	65.13±22.81	<0.0001	55.51±23.90	65.28±25.56	75.73±13.83
Total score	3.16±2.42	47.79±17.01	<0.0001	30.05±12.85	50.42±17.20	56.84±13.83
Hull Cough Questionnaire	1.0±2.0	36.05±21.97	<0.0001	23.75±13.23	32.50±20.54	34.43±13.10
Treatment						
PPI	0	8	0.0036	0	0	8

Data are presented as n, mean±SD or median (interquartile range), unless otherwise stated. There was no significant difference within the groups of severe asthma patients. GORD: gastro-oesophageal reflux disease; PPI: proton pump inhibitor; BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; F_{eNO}: exhaled nitric oxide fraction; ACQ: Asthma Control Questionnaire; SGRQ: St George's Respiratory Questionnaire.

Analysis of gene expression in epithelial brushings and differentiated cells

Total RNA was extracted from epithelial brushings or cultured cell lysates using miRNeasy Mini Kit and RNase-Free DNase Set (Quiagen, Manchester, UK). cDNA libraries were prepared using NEBNext Ultra (nonstranded) mRNA library prep kit with polyA pulldown for mRNA enrichment. Paired-end 150 bp sequencing to a depth of 20 million reads (epithelial brushings) or 50 million reads (differentiated cells) was performed on an Illumina HiSeq2500 by Novogene (Cambridge, UK). FASTQ files were aligned to human genome build GRCh38 using STARv2.6.0; reads were counted with HTSeq; and differential gene expression analysis conducted with edgeR. Details are given in the supplementary material. Data are deposited in the Gene Expression Omnibus repository.

Statistical analyses

Paired t-tests were applied to transcriptomic data, while clinical and experimental data were analysed using Kruskal–Wallis, Mann–Whitney U-test or t-tests depending on data distribution; $p<0.05$ was considered significant. False discovery rate correction was applied to the transcriptomic data.

Results

MCP-PAB causes epithelial damage and alters barrier and secretory function

To analyse the impact of GORD on the airway epithelium of severe asthmatics, ALI cultures derived from bronchial brushings of participants with severe asthma, GORD and PPI treatment (n=8) and healthy controls (n=5) (table 1) were exposed for 30 min to MCP-PAB conditions consisting of 50 $\mu g \cdot mL^{-1}$ of pepsin and 250 µM CDC at pH 5.

MCP-PAB-induced epithelial damage was characterised by TEM as enlargement of intercellular spaces and beginning of cell detachment (supplementary figure E2). In addition, MCP-PAB-exposed ALI cultures had markedly increased ionic and macromolecular permeability, as shown by decreased TEER (figure 1a) and increased FITC dextran 4 kDa passage, respectively (figure 1b), with a significantly greater impact of MCP-PAB on permeability of cultures from severely asthmatic donors compared with healthy controls. Analysis of epithelial tight junctions and adherens junctions in ALI cultures from severely asthmatic donors showed a marked disruption of the junctions in MCP-PAB-exposed cultures (supplementary figure E3).

In addition to having a marked impact on epithelial structure, MCP-PAB caused an increase in the secretion of CXCL8, IL-1α, and TNF-α (figure 2). These results were supported by analysis of epithelial gene expression (supplementary figure E4).

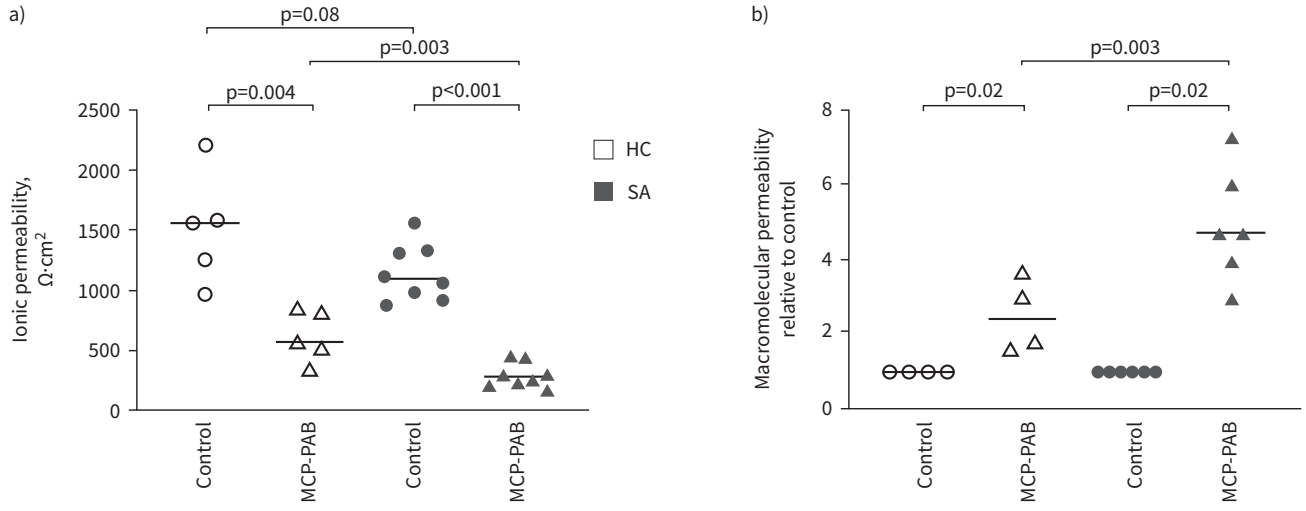

FIGURE 1 Effects of multiple challenge protocol using pepsin, acid pH and bile acid on epithelial permeability. Bronchial epithelial air–liquid interface cultures from healthy controls (HC) (n=5) and severely asthmatic (SA) (n=8) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 h before a) ionic and b) macromolecular permeability were measured.

Artificial refluxate upregulates unfolded protein responses, damage responses and epithelial remodelling mechanisms

To further analyse the mechanisms involved in MCP-PAB-induced epithelial dysregulation in ALI cultures, we analysed mRNA transcriptomes obtained by RNA-seq.

Comparison of gene expression in unstimulated ALI cultures showed 147 genes upregulated and 266 downregulated in cultures from severe asthmatics when compared with healthy controls (figure 3, supplementary table E1). Application of MCP-PAB resulted in a profound effect on gene expression, especially in ALI cultures from severely asthmatic donors, which had a significantly higher number of differentially expressed genes (DEGs) (n=599) compared to cultures from healthy donors (n=87 DEGs). Among the most prominent modulated genes were *IL1RL1* (IL-1 receptor like 1, the receptor for IL-33), *CHAC1* (cation transport regulator-like protein 1), involved in oxidative balance and unfolded protein response (UPR) and *SERPINB9*, a serine protease inhibitor.

Gene ontology analysis (AmiGO) of all MCP-PAB-induced DEGs identified a number of differentially controlled biological processes ($p<0.05$). Taking a cut-off of two-fold increase in gene expression, we found 57 processes upregulated in cultures from severe asthmatic donors and 25 in those from healthy donors. In order to identify the processes with the greatest impact, we undertook a further selection of gene

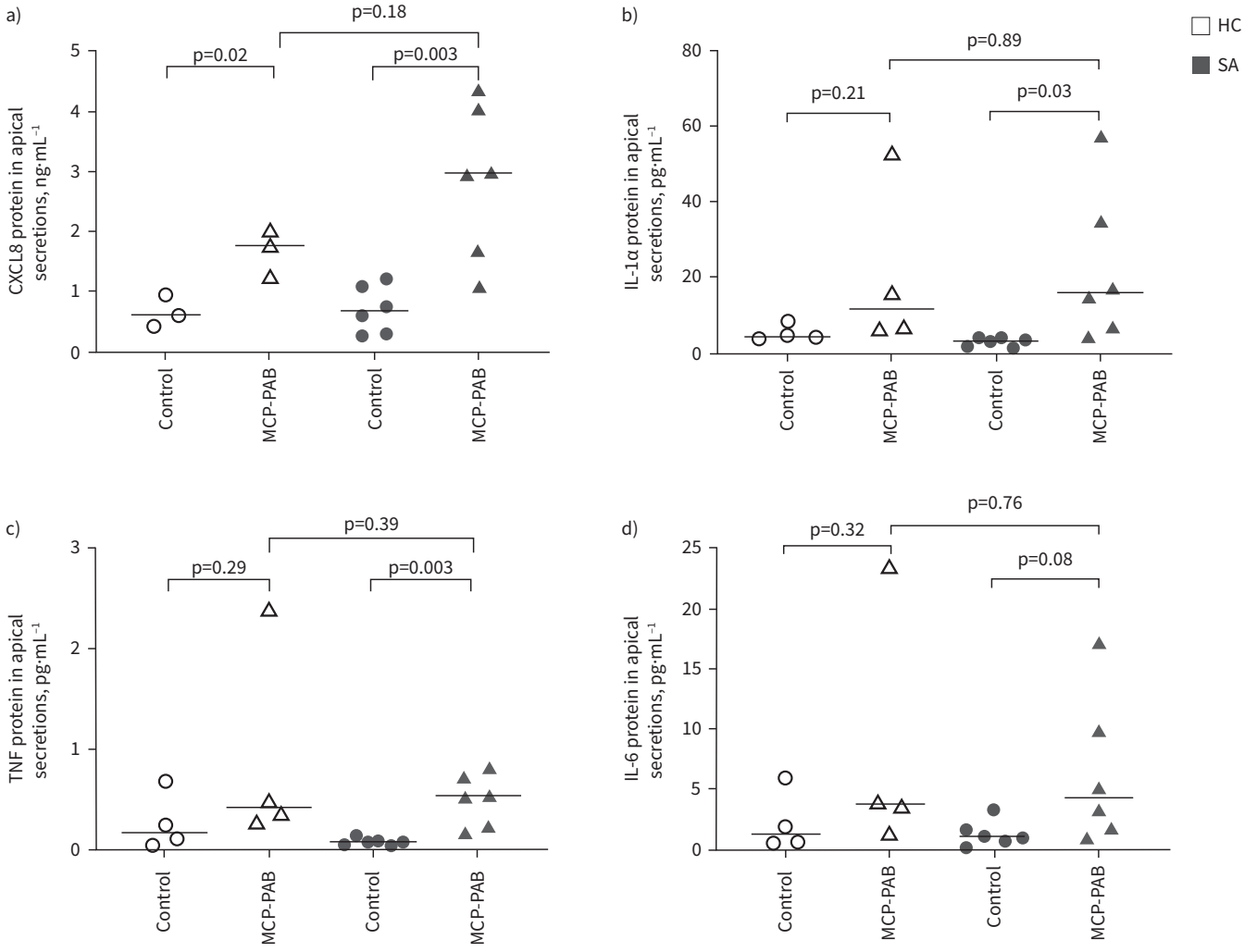

FIGURE 2 Stimulation of epithelial cytokine release by multiple challenge protocol using pepsin, acid pH and bile acid. Bronchial epithelial air–liquid interface cultures from healthy controls (HC) (n=4) and severe asthmatic (SA) (n=6) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 h before a) CXCL8, b) interleukin (IL)-1α, c) tumour necrosis factor (TNF)-α and d) IL-6 protein release was measured in apical secretions.

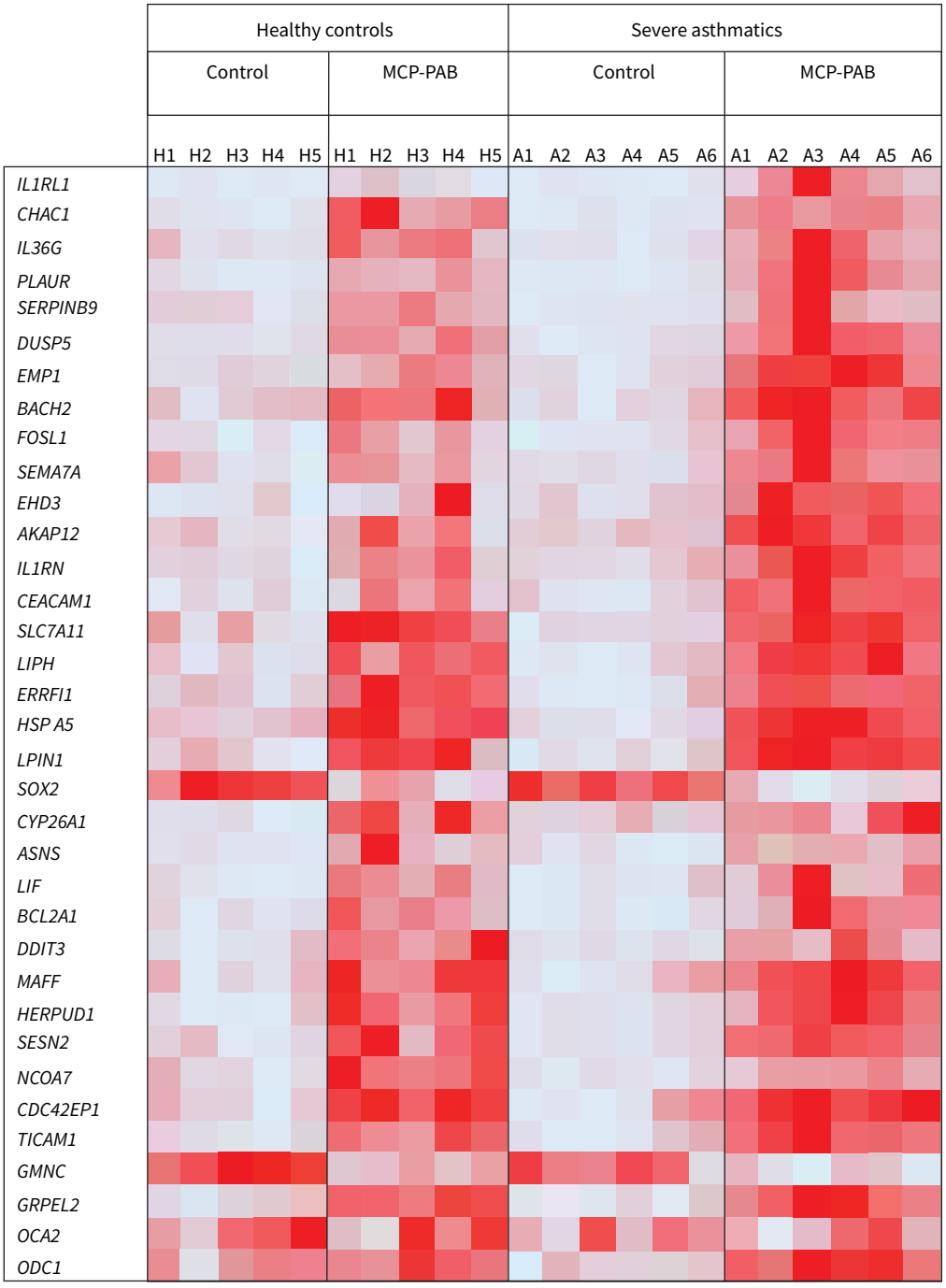

FIGURE 3 Changes in epithelial gene expression caused by multiple challenge protocol using pepsin, acid pH and bile acid *in vitro*. Bronchial epithelial air–liquid interface (ALI) cultures from healthy controls (n=5) and severely asthmatic (n=6) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 h before harvesting for RNA sequencing. Heatmap of the top dysregulated epithelial genes from low (blue) to high (red) levels of expression.

expression with a cut-off of five-fold; this showed 16 processes upregulated in cultures from severe asthmatic donors and 11 in cultures from healthy controls (table 2).

The most significant enrichment due to MCP-PAB exposure was in the protein kinase R-like endoplasmic reticulum kinase (PERK)-mediated UPR; this was significant in cultures from both asthmatics and healthy donors, but was three times greater in healthy participants. Cultures from healthy individuals exposed to

TABLE 2 Top upregulated biological processes in multiple challenge protocol exposed cultures when compared with control cultures (details provided if greater than five-fold enrichment, p<0.05)

	Healthy control		Severe asthma	
	Fold enrichment	p-value	Fold enrichment	p-value
PERK-mediated unfolded protein response (GO:0036499)	>100	0.0000	31.53	0.0020
Cellular response to glucose starvation (GO:0042149)	45.1	0.0243		
Cellular response to biotic stimulus (GO:0071216)	13.21	0.0099		
Negative regulation of intracellular signal transduction (GO:1902532)	7.7	0.0208	3.27	0.0024
Regulation of response to stress (GO:0080134)	6.25	0.0000		
Cellular response to lipopolysaccharide (GO:0071222)			4.55	0.0476
Epidermal growth factor receptor signalling pathway (GO:0007173)			11.58	0.0024
Positive regulation of cytokine biosynthetic process (GO:0042108)			8.22	0.0313
Regulation of epidermal growth factor receptor signalling pathway (GO:0042058)			7.42	0.0210
Regulation of cell junction assembly (GO:1901888)			6.78	0.0439
Positive regulation of epithelial cell migration (GO:0010634)			6.22	0.0015
Positive regulation of vasculature development (GO:1904018)			5.37	0.0013
Regulation of protein kinase B signalling (GO:0051896)			4.48	0.0122
Positive regulation of cellular catabolic process (GO:0031331)			3.39	0.0362

PERK: protein kinase R-like endoplasmic reticulum kinase; GO: Gene Ontology.

MCP-PAB were also enriched in other stress-response processes (table 2). In contrast, MCP-PAB-exposed epithelial cultures derived from severe asthmatic donors were enriched in epidermal growth factor receptor (EGFR) signalling, cell migration and vasculature development, suggesting upregulation of tissue repair and remodelling responses.

Having established MCP-PAB-induced epithelial damage, we next explored the impact of MCP-PAB on damage signalling. We analysed the damage-associated cytokine IL-33 and found that artificial refluxate caused increased nuclear IL-33 staining cultures from severe asthmatic donors (figure 4). We confirmed IL-33 expression in ALI cultures using quantitative PCR (healthy controls n=5; severely asthmatic patients n=6), and showed that MCP-PAB was associated with a 67% increase in IL-33 expression in severe asthma and a −11% change in healthy controls (p=0.01 for between-group comparison).

Comparison of in vitro *findings with* in vivo *epithelial changes in severe asthma with GORD*

To determine the relevance of our *in vitro* findings with epithelial changes in severe asthma with GORD, we first performed immunohistochemistry for IL-33 using bronchial biopsies from severely asthmatic (n=9) or healthy control subjects without GORD (n=4); the severe asthmatics were subgrouped as follows: 1) severe asthma with no GORD (SA-no GORD; n=5), and 2) severe asthma with documented GORD, but who had abstained from their regular PPI treatment for 2 weeks to avoid potential bias of systemic or local (through micro-inhalation) impact of PPI on epithelial gene expression (SA-GORD; n=4). As shown in figure 5, there was a significantly higher number of IL-33-positive nuclei in SA-no GORD compared to healthy controls, with a further significant increase in SA-GORD.

Finally, we analysed mRNA transcriptomes of bronchial brushings from SA-GORD (n=6), SA-no GORD (n=4) and healthy control subjects (n=12). RNA-seq analysis identified that, of the top 37 genes whose expression was modified in ALI cultures in response to MCP-PAB, 15 were similarly modified *ex vivo* in patients with GORD (table 3). Of note, the expression of *CHAC1*, the top upregulated gene involved in the UPR process which was identified as the main mechanism induced by MCP-PAB in ALI cultures (supplementary table E1), was also increased in bronchial brushings obtained from SA-GORD when compared to SA-no GORD, confirming a similar impact of refluxate on epithelial responses to endoplasmic reticulum stress *in vivo*.

Discussion

Using a combination of *in vitro* and *ex vivo* approaches, we have obtained compelling evidence in support of reflux having a significant impact on bronchial epithelial structure and function, with a profound effect on the epithelium of severe asthma patients. Application of MCP-PAB conditions to epithelial ALI cultures caused marked acute structural damage, including disruption of adherens and tight junctions, increased permeability and induction of stress responses, as shown by enrichment of the UPR genes and modulation of the alarmin IL-33 and its receptor *IL1RL1*. These *in vitro* findings were supported by observations in

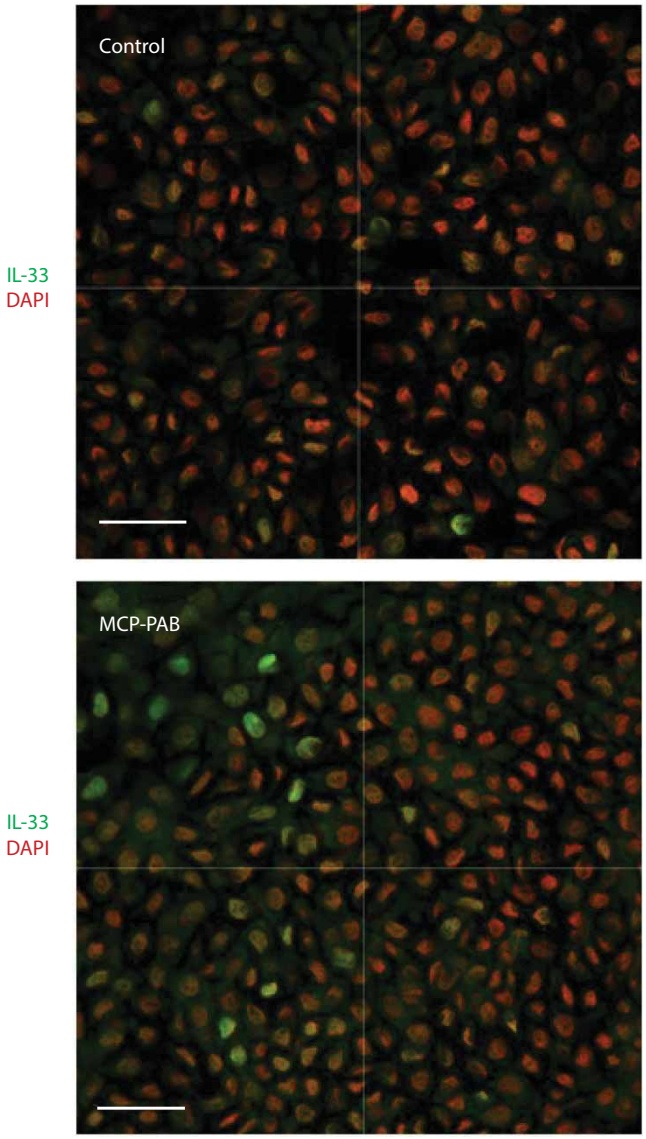

FIGURE 4 Regulation of epithelial expression of interleukin (IL)-33 by multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB). Bronchial epithelial air–liquid interface (ALI) cultures from severely asthmatic donors were untreated (control) or exposed to MCP-PAB for 30 mins, washed and allowed to recover for 4 h before fixing and immunofluorescence staining. Images show IL-33 nuclear staining (green) and 4′,6-diamidino-2-phenylindole (DAPI) (red). Images are representative of experiments using ALI cultures from six donors. Scale bars=25 µm.

bronchial biopsies and by global gene expression analysis of epithelial brushings from severe asthmatics without or with GORD and healthy controls.

GORD is a chronic disorder caused by abnormal reflux of acid, pepsin and bile acids, defined as time of acid exposure >6% during 24-h monitoring of oesophageal pH [16]. Combined with impedance measurement, pH monitoring allows the detection of acidic (pH <4), weakly acidic (pH 4–7) and non-acid reflux (pH >7), the latter occasionally persisting despite treatment with PPIs [17]. Whether and to what extent gastric juice contents penetrate the lungs in subjects with GORD has been uncertain [18], although our own studies have provided evidence that clinical GORD is associated with changes in several biomarkers [11, 13]. When deciding on the composition of the ingredients in the MCP-PAB for *in vitro* testing, we took into account physiological concentrations in gastric secretions of acid [19], pepsin [20] and total bile acids [21] and previous reports of effects of pepsin [9] and bile acids [22] on epithelial cells. Based on dose-ranging experiments, we chose 50 µg·mL^{-1} of pepsin and 250 µM CDC at pH 5 for

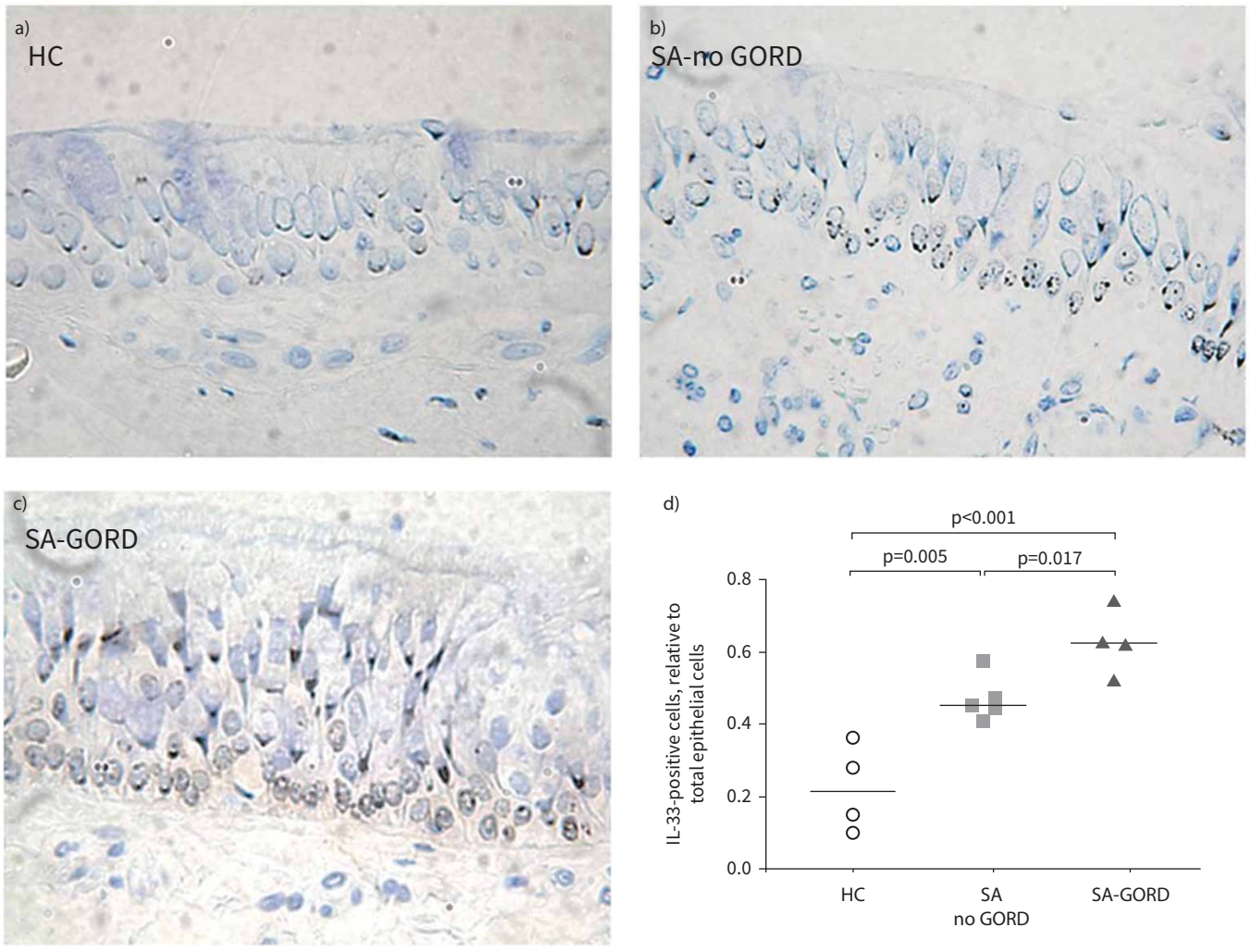

FIGURE 5 Epithelial interleukin (IL)-33 expression is increased severe asthma with gastro-oesophageal reflux disease (GORD). Typical patterns of immunohistochemical staining for IL-33 in bronchial biopsies from a) healthy control participants without GORD (HC); b) severe asthmatics with no documented GORD (SA-no GORD; n=5); and c) severe asthmatics with documented GORD who had abstained from their regular proton pump inhibitor (PPI) treatment for 2 weeks (SA-GORD; n=4). d) Quantitation of positive nuclei expressed as percentage of total epithelial cells.

30 min, because this resulted in measurable damage without causing extreme cytotoxicity. Thus, we observed enlargement of intercellular spaces, disruption of intercellular junctions and increased permeability, effects similar to observations *in vivo* in oesophageal and laryngeal epithelium exposed to chronic refluxate [23, 24]. This, coupled with previous studies showing that ALI cultures derived from severe asthmatic donors exhibit phenotypic features similar to those found *in vivo* [14, 25], led us to conclude that exposure of ALI cultures to MCP-PAB conditions is a reliable model to analyse the effect of reflux on the bronchial epithelium in severe asthma.

Our study revealed a marked reflux-induced increase in the nuclear expression of the alarmin IL-33, as well as upregulation of *IL1RL1*, the gene encoding the IL-33 receptor. IL-33 is a member of the IL-1 cytokine family localised in the nucleus of airway epithelial cells and its release can be triggered by damage caused, for example, by allergens or viruses [26, 27]. It is a known asthma susceptibility gene [28, 29] and plays a crucial role in type-2 innate immunity through activation of group 2 innate lymphoid cells to trigger production of IL-4, IL-5 and IL-13 [30]. Our findings of IL-33 upregulation in bronchial biopsies from severe asthma with GORD are in concordance with the observed upregulation of IL-33 nuclear expression in the oesophageal mucosa in patients with reflux oesophagitis [31] and symptoms of heartburn [32].

TABLE 3 Differential epithelial expression of 37 genes in air–liquid interface (ALI) cultures and expression of these genes in bronchial brushings

	ALI cultures				Bronchial brushings					
	SA MCP *versus* HC CTL		SA MCP *versus* SA CTL		SA-GORD[#] *versus* HC		SA-GORD[#] *versus* SA-no GORD[¶]		SA-no GORD[¶] *versus* HC	
	Fold change	p-value	Fold change	p-value	Fold change	p-value	Fold change	p-value	Fold change	p-value
IL1RL1	14.22	0.0234	11.84	0.014						
CYP26A1	10.31	0.0102	2.26	0.0226	−0.38	0.2119	−0.59	0.015	0.4881	0.2138
CHAC1	6.38	0.0000	8.53	0.0000	0.57	0.0014	0.4	0.078	0.1204	0.3519
PLAUR	5.06	0.0036	5.71	0.0013	0.78	0.1908	0.22	0.7903	0.4588	0.3107
BCL2A1	4.48	0.0174	7.26	0.006	1.19	0.2114	0.42	0.7148	0.5371	0.3406
LIF	4.39	0.0345	3.49	0.0271	0.03	0.8773	0.28	0.4522	−0.1967	0.2768
ASNS	4.37	0.0000	3.77	0.0000						
EHD3	4.16	0.0001	2.5	0.0001	0.43	0.0314	−0.11	0.6064	0.6105	0.0122
DUSP5	4.13	0.001	4.72	0.0003	0.04	0.8965	0.05	0.9213	−0.0097	0.9756
FOSL1	3.89	0.0011	3.6	0.0005						
AKAP12	3.59	0.0000	2.43	0.0000	0.62	0.0429	0.5	0.2501	0.0834	0.7275
EMP1	3.46	0.0001	3.91	0.0000	0.57	0.0126	−0.32	0.272	1.314	0.008
IL36G	3.33	0.0196	5.99	0.0052						
IL1RN	2.83	0.0003	2.15	0.0002	1.54	0.048	0.04	0.9582	1.4514	0.073
BACH2	2.61	0.0001	3.84	0.0000	0.79	0.0242	0.45	0.3752	0.2311	0.3876
CEACAM1	2.55	0.0001	2.14	0.0000	1.05	0.0031	0.77	0.1259	0.1556	0.3661
SEMA7A	2.51	0.0068	3.36	0.0011	0.86	0.0559	0.67	0.2766	0.1151	0.7938
SERPINB9	2.28	0.0586	5.33	0.0151	0.47	0.1701	0.1	0.827	0.3359	0.4284
MAFF	2.08	0.0002	2.18	0.0001	0.1	0.7534	0.38	0.4754	−0.2083	0.4484
LIPH	1.88	0.0001	2.12	0.0000	−0.22	0.046	0.17	0.5231	0.3343	0.0044
TICAM1	1.81	0.0001	1.7	0.0001	0.7	0.0171	0.36	0.3158	0.2518	0.2377
HERPUD1	1.72	0.0009	1.65	0.0003	0.17	0.0537	−0.12	0.3178	0.3201	0.007
DDIT3	1.65	0.0096	1.92	0.0023	0.35	0.037	−0.16	0.335	0.6075	0.0004
CDC42EP1	1.52	0.0000	1.49	0.0001	1.63	0.029	1.91	0.1866	−0.0962	0.7368
SESN2	1.42	0.0000	1	0.0000	0.21	0.1479	−0.11	0.517	0.3598	0.0141
SLC7A11	1.41	0.0002	2.13	0.0000	0.25	0.4279	1.25	0.0364	−0.4458	0.2045
LPIN1	1.38	0.0000	1.61	0.0000	−0.01	0.895	0.19	0.2361	−0.1672	0.0609
ERRFI1	1.32	0.0000	1.9	0.0000	−0.03	0.7968	0.12	0.5169	−0.1333	0.1547
GRPEL2	1.15	0.0009	1.42	0.0002	−0.13	0.3447	0.06	0.7801	−0.185	0.2209
HSPA5	1.12	0.0000	1.62	0.0000	−0.05	0.7581	0.1	0.6946	−0.1369	0.3707
NCOA7	0.59	0.0073	0.85	0.0001	−0.35	0.0361	0.34	0.1864	−0.5129	0.0139
ODC1	0.51	0.0119	1.22	0.0001	−0.25	0.0021	0.2	0.003	−0.0647	0.4584
OCA2	−0.28	0.2787	−0.05	0.8474	0.84	0.0171	−0.37	0.3526	1.9081	0.0177
CYP1B1	−0.32	0.3627	−0.33	0.4407						
CYP1A1	−0.55	0.0343	−0.51	0.1031						
SOX2	−0.7	0.0001	−0.67	0.0000	−0.2	0.038	−0.05	0.7193	−0.1535	0.047
GMNC	−0.81	0.0000	0.72	0.0047	−0.58	0.0002	−0.14	0.6958	−0.5164	0.0075

Fold changes are coloured from blue (downregulation) to red (upregulation): colour intensity reflects magnitude. SA: severe asthma; MCP: multiple challenge protocol; HC: healthy control; CTL: control; GORD: gastro-oesophageal reflux disease. [#]: severe asthmatics with documented GORD who had abstained from their regular proton pump inhibitor treatment for 2 weeks; [¶]: SA-no GORD: severe asthmatics with no documented GORD.

Refluxate-induced damage also included a response to oxidative stress through PERK-mediated UPR [33]. A recent study, using an oesophageal squamous epithelial cell line, identified bile acid-mediated activation of the PERK-mediated UPR [34]. Our study provides the first evidence of refluxate-triggered UPR activation in the airway epithelium. PERK is a type I endoplasmic reticulum (ER) transmembrane protein activated by misfolded proteins inside the ER. Its stimulation induces transcription of UPR-related genes, leading to autophagy, apoptosis and redox homeostasis [35]. UPR is considered a master regulator in inflammatory diseases and its role in asthma development has been suggested [35]. UPR can be activated by various asthma triggers, including allergens, cigarette smoke and viruses, and regulates oxidative stress in asthma [35]. UPR regulates NF-κB activity and NF-κB-mediated inflammation and can induce apoptosis in case of prolonged activation of ER stress. The relatively limited enrichment in the PERK-mediated UPR process that we observed in exposed cells from severe asthmatics may reflect an

ineffective response to multiple types of damage and so explains the vulnerability of the bronchial epithelium in severe asthma to a range of environmental challenges.

We observed MCP-PAB-induced epithelial changes in cultures from both normal and severe asthma donors. However, MCP-PAB had a more pronounced impact on the structural and functional properties of the asthmatic epithelium, including a greater increase in epithelial permeability and a higher number of MCP-PAB-associated DEGs. While our *ex vivo* transcriptome analysis of bronchial brushings did not completely match our *in vitro* results from ALI cultures, this may be because the *in vitro* model represents a single acute stress event caused by exposure to MCP-PAB conditions, which do not fully reflect the complexity of gastric juice, whereas the clinical condition of GORD is characterised by repeated exposures to various components of gastric juice. In addition, severe asthma patients with GORD treated with PPIs may present a dysregulated aerodigestive microbiome, with a potential role in severe asthma [36, 37]. Nonetheless, we were able to identify 15 dysregulated genes in brushings from severe asthmatics with GORD among the 37 top dysregulated genes in MCP-PAB-exposed ALI cultures, with the extent of dysregulation being higher in cultures from severe asthmatics than in those from healthy controls. Among the DEGs were genes involved in oxidative stress responses (*CHAC1*, *BACH2*), cell adhesion (*CEACAM1*), cytoskeleton organisation (*CDC42EP1*) and cilia formation (*GMNC*), pointing to impact on epithelial structure regulation. Exposure of severe asthma cultures to refluxate also caused enrichment in EGFR- and cell migration-related processes that were not changed in cultures from healthy individuals. Our results suggest that refluxate might contribute to defective epithelial barrier function and EGFR-mediated remodelling, key features of asthma [14, 38].

In summary, our study has identified a direct impact of refluxate on the airway epithelial structure, barrier permeability and modulation of gene expression, including UPR, responses to oxidative stress and wound healing processes. This suggests a possible role for GORD in increasing exposure of the subepithelial airway mucosa to allergens and infectious pathogens, resulting in increased risk of inflammation and exacerbations, as well as a possible role in airway remodelling, a key feature of severe asthma. These results suggest the need for research into alternative therapeutic management of GORD in severe asthma.

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Conflict of interest: J-M. Perotin, G. Wheway, K. Tariq, A. Azim, R.A. Ridley, J.A. Ward, C. Barber and D.E. Davies have nothing to disclose. J.P.R. Schofield reports being director and shareholder in TopMD Precision Medicine Ltd. P. Howarth reports personal fees from GSK outside the submitted work. R. Djukanovic reports receiving fees for lectures at symposia organised by Novartis, AstraZeneca and TEVA, consultation for TEVA and Novartis as member of advisory boards, and participation in a scientific discussion about asthma organised by GlaxoSmithKline; and is a co-founder and current consultant, and has shares in Synairgen, a University of Southampton spin-out company.

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