



Early View

Original research article

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Jeanne-Marie Perotin, Gabrielle Wheway, Kamran Tariq, Adnan Azim, Robert A Ridley, Jonathan A Ward, James PR Schofield, Clair Barber, Peter Howarth, Donna E. Davies, Ratko Djukanovic

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Vulnerability to acid reflux of the airway epithelium in severe asthma

Authors: 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 PR Schofield (7), Clair Barber (1, 2), Peter Howarth (1,2), Donna E. Davies* (1,2,5), Ratko Djukanovic* (1,2,5).

* joint senior authors

Affiliations

1. NIHR Southampton Biomedical Research Centre, University Hospital Southampton, UK
2. Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK
3. Department of Respiratory Diseases, UMRS1250, University Hospital of 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

Corresponding author: Prof Jeanne-Marie Perotin, Department of Respiratory Diseases, University Hospital of Reims, 45 rue Cognacq Jay, 51092 REIMS Cedex, France. telephone +33 326787614 ; jmperotin-collard@chu-reims.fr ;

Authors contribution

Substantial contributions to the conception or design of the work: JMP, GW, JPRS, PH, DED, RD

Acquisition, analysis or interpretation of the data: JMP, GW, KT, AA, RAR, JW, CB, DED, RD

Drafting the manuscript or revising it critically for important intellectual content: JMP, DED, RD

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JMP, GW, KT, AA, RAR, JAW, CB, DED have nothing to disclose. JPRS reports being Director and shareholder in TopMD Precision Medicine Ltd. PH reports personal fees from GSK outside the submitted work. RD 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. He is a co-founder and current consultant, and has shares in Synairgen, a University of Southampton spin out company.

Abstract

Background: Severe asthma is associated with multiple co-morbidities, 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). We also 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, 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: By affecting epithelial permeability, GORD may increase exposure of the airway submucosa to allergens and pathogens, resulting in increased risk of inflammation and exacerbations.

Clinical implication: These results suggest the need for research into alternative therapeutic management of GORD in severe asthma.

Take-home message

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 severe asthmatics

Key words

Asthma; Gastroesophageal reflux disease; Airway epithelium; Air liquid interface.

Abbreviations

ALI: air-liquid interface

CDC: chenodeoxycholic acid

DEG: differentially expressed genes

FDR: False discovery rate

GORD: gastro-oesophageal reflux disease

MCP-PAB: multiple challenge protocol with pepsin, acid pH and bile acids

PBEC: primary bronchial epithelial cells

PPI: proton pump inhibitor

TEER: transepithelial electrical resistance

TEM: transmission electron microscopy

UPR: Unfolded protein response

INTRODUCTION

Severe asthma is associated with multiple co-morbidities, 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 anti-microbial 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 (TDA) 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 (PPI) (13), characterized 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 have 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 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 BTS/SIGN Guidelines) and healthy control participants were recruited prospectively and assessed for GORD by 24-hr 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 RNAlater for subsequent RNAseq analysis or used in primary bronchial epithelial cell (PBEC) culture (14). Bronchial biopsies were also taken and fixed in paraffin for immunohistochemical analyses.

The study was approved by the South-Central Hampshire A, Research Ethics Committee, UK (reference numbers: 13/SC/0182 and 14/WM/1226) and all participants gave their informed consent.

Analysis of the *ex vivo* effect of a multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB)

Initial dose and time-course studies were conducted with 16HBE cells exposed to MCP-PAB (pepsin and chenodeoxycholic acid at acidic pHs); optimised conditions were subsequently confirmed to be appropriate for fully differentiated air liquid interface (ALI) cultures (see online supplement and Figure E1 for full details). MCP-PAB conditions were applied to the apical epithelial surface for 30 minutes before washing twice. After 4h recovery, epithelial permeability was measured using transepithelial electrical resistance (TEER) and FITC dextran 4kDa (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 previously described (14). The cultures were also processed for transmission electron microscopy (TEM) and analysed for epithelial integrity.

Bronchial biopsies were processed as previously described (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

Interleukin (IL)-8 concentrations in conditioned media were measured using an IL-8 DuoSet ELISA (R&D, Abingdon, UK) while IL-6, TNF α and IL-1 α were measured using VPLex (MSD, Maryland, USA).

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 (non-stranded) mRNA library prep kit with polyA pulldown for mRNA enrichment. Paired-end 150bp sequencing to a depth of 20M reads (epithelial brushings) or 50M reads (differentiated cells) was performed on an Illumina HiSeq2500 by Novogene Inc (Cambridge, UK). FATSQ 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 in the online supplement. Data are deposited in GEO.

Statistical analyses

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

RESULTS

Multiple challenge protocol using pepsin, acid pH and bile acid (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 50µg/ml of pepsin and 250µM chenodeoxycholic acid (CDC) at pH 5.

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

In addition to having a marked impact on epithelial structure, MCP-PAB caused an increase in the secretion of CXCL8, IL1 α , and TNF α (Figure 2). These results were supported by analysis of epithelial gene expression (Figure E4).

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 RNAseq.

Comparison of gene expression in unstimulated ALI cultures showed 147 genes upregulated and 266 downregulated in cultures from severe asthmatics when compared with healthy control (Figure 3, Table E1). Application of MCP-PAB resulted in a profound effect on gene expression, especially in ALI cultures from severe asthmatic donors which had a significantly higher number of differentially expressed genes (DEGs) (n=599) compared to cultures from healthy donors (n=87 DEGs). Amongst the most prominent modulated genes were *IL1RL1* (Interleukin 1 Receptor Like 1, the receptor for IL33), *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 2-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 expression with a cut-off of 5-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 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 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 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 AR caused increased nuclear IL-33 staining cultures from severe asthmatic donors (Figure 4). We confirmed IL-33 expression in ALI cultures using qPCR (HC, n=5; SA, n=6), and showed that MCP-PAB was associated with a 67% increase in IL-33 expression in SA and a -11% change in HC ($p=0.01$ 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 IHC for IL-33 using bronchial biopsies from severe asthmatic (n=9) or healthy control subjects without GORD (n=4); the severe asthmatics were sub-grouped as follows: (i) SA with no GORD (n=5), and (ii) SA 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 (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 no PPI (n=6), SA-no GORD (n=4) and healthy control subjects (n=12). RNAseq 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 (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 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-hr 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 PPI (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 50µg/ml of pepsin and 250µM chenodeoxycholic acid

(CDC) at pH 5 for 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 localized 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 (ILC-2) to trigger production of IL-4, IL-5, 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).

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 $\text{N}\kappa\text{B}$ activity and $\text{NF}\kappa\text{B}$ -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 characterized by repeated exposures to various components of gastric juice. In addition, severe asthma patients with GORD treated with PPI 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. Amongst the DEGs were genes involved in oxidative stress responses (*CHAC1*, *BACH2*), cell adhesion (*CEACAM1*), cytoskeleton organization (*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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Tables

Table 1. Characteristics of participants

	Healthy controls	Severe asthmatics	p value	SA-no GORD	SA-GORD	SA-GORD+PPI
n	15	18		4	6	8
Age, yrs	41.5 ± 12.9	55.7 ± 8.1	0.0005	52.5 ± 6.2	54.3 ± 9.2	58.25 ± 7.99
Age of asthma onset, yrs		23.7 ± 21.0		24.0 ± 25.7	27.3 ± 23.6	20.75 ± 19.40
Gender (F/M)	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)	1	10	0.004	2	5	3
Diagnosed GORD	0	14	<0.0001	0	6	8
Atopy, n	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]
FEV1, % 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
FEV1/FVC	0.80 ± 0.04	0.66 ± 0.12	0.0001	0.69 ± 0.12	0.72 ± 0.08	59.2 ± 12.2
FeNO		24.2 ± 11.1		33.0 ± 17.0	28.8 ± 11.0	19.8 ± 8.8
Exacerbations (last 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						
Proton pump inhibitor, n	0	8	0.0036	0	0	8

Results are expressed as numbers, mean ± SD or median [IQ25-75]. No significant difference within the groups of severe asthma patients

Table 2. Top upregulated biological processes in multiple challenge protocol exposed cultures when compared with control cultures (details provided if fold enrichment >5, p-value<0.05)

GO biological process	HC		SA	
	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

HC: healthy controls; SA: severe asthmatics

Table 3. Differential epithelial expression of 37 genes in ALI cultures and expression of these genes in bronchial brushings. Foldchanges are coloured from blue (downregulation) to red (upregulation); colour intensity reflects magnitude.

	ALI cultures				Bronchial brushings					
	SA MCP vs HC CTL		SA MCP vs SA CTL		SA-GORD vs Health		SA-GORD vs SA-no GORD		SA-no GORD vs Health	
	fold change	p value	fold change	p value	fold change	p value	fold change	p value	fold change	p value
<i>IL1RL1</i>	14.22	0.0234	11.84	0.014						
<i>CYP26A1</i>	10.31	0.0102	2.26	0.0226	-0.38	0.2119	-0.59	0.015	0.4881	0.2138
<i>CHAC1</i>	6.38	0.0000	8.53	0.0000	0.57	0.0014	0.4	0.078	0.1204	0.3519
<i>PLAUR</i>	5.06	0.0036	5.71	0.0013	0.78	0.1908	0.22	0.7903	0.4588	0.3107
<i>BCL2A1</i>	4.48	0.0174	7.26	0.006	1.19	0.2114	0.42	0.7148	0.5371	0.3406
<i>LIF</i>	4.39	0.0345	3.49	0.0271	0.03	0.8773	0.28	0.4522	-0.1967	0.2768
<i>ASNS</i>	4.37	0.0000	3.77	0.0000						
<i>EHD3</i>	4.16	0.0001	2.5	0.0001	0.43	0.0314	-0.11	0.6064	0.6105	0.0122
<i>DUSP5</i>	4.13	0.001	4.72	0.0003	0.04	0.8965	0.05	0.9213	-0.0097	0.9756
<i>FOSL1</i>	3.89	0.0011	3.6	0.0005						
<i>AKAP12</i>	3.59	0.0000	2.43	0.0000	0.62	0.0429	0.5	0.2501	0.0834	0.7275
<i>EMP1</i>	3.46	0.0001	3.91	0.0000	0.57	0.0126	-0.32	0.272	1.314	0.008
<i>IL36G</i>	3.33	0.0196	5.99	0.0052						
<i>IL1RN</i>	2.83	0.0003	2.15	0.0002	1.54	0.048	0.04	0.9582	1.4514	0.073
<i>BACH2</i>	2.61	0.0001	3.84	0.0000	0.79	0.0242	0.45	0.3752	0.2311	0.3876
<i>CEACAM1</i>	2.55	0.0001	2.14	0.0000	1.05	0.0031	0.77	0.1259	0.1556	0.3661
<i>SEMA7A</i>	2.51	0.0068	3.36	0.0011	0.86	0.0559	0.67	0.2766	0.1151	0.7938
<i>SERPINB9</i>	2.28	0.0586	5.33	0.0151	0.47	0.1701	0.1	0.827	0.3359	0.4284
<i>MAFF</i>	2.08	0.0002	2.18	0.0001	0.1	0.7534	0.38	0.4754	-0.2083	0.4484
<i>LIPH</i>	1.88	0.0001	2.12	0.0000	-0.22	0.046	0.17	0.5231	-0.3343	0.0044
<i>TICAM1</i>	1.81	0.0001	1.7	0.0001	0.7	0.0171	0.36	0.3158	0.2518	0.2377
<i>HERPUD1</i>	1.72	0.0009	1.65	0.0003	0.17	0.0537	-0.12	0.3178	0.3201	0.007
<i>DDIT3</i>	1.65	0.0096	1.92	0.0023	0.35	0.037	-0.16	0.335	0.6075	0.0004
<i>CDC42EP1</i>	1.52	0.0000	1.49	0.0001	1.63	0.029	1.91	0.1866	-0.0962	0.7368
<i>SESN2</i>	1.42	0.0000	1	0.0000	0.21	0.1479	-0.11	0.517	0.3598	0.0141
<i>SLC7A11</i>	1.41	0.0002	2.13	0.0000	0.25	0.4279	1.25	0.0364	-0.4458	0.2045
<i>LPIN1</i>	1.38	0.0000	1.61	0.0000	-0.01	0.895	0.19	0.2361	-0.1672	0.0609
<i>ERRF1</i>	1.32	0.0000	1.9	0.0000	-0.03	0.7968	0.12	0.5169	-0.1333	0.1547
<i>GRPEL2</i>	1.15	0.0009	1.42	0.0002	-0.13	0.3447	0.06	0.7801	-0.185	0.2209
<i>HSPA5</i>	1.12	0.0000	1.62	0.0000	-0.05	0.7581	0.1	0.6946	-0.1369	0.3707
<i>NCOA7</i>	0.59	0.0073	0.85	0.0001	-0.35	0.0361	0.34	0.1864	-0.5129	0.0139
<i>ODC1</i>	0.51	0.0119	1.22	0.0001	-0.25	0.0021	-0.2	0.003	-0.0647	0.4584
<i>OCA2</i>	-0.28	0.2787	-0.05	0.8474	0.84	0.0171	-0.37	0.3526	1.9081	0.0177
<i>CYP1B1</i>	-0.32	0.3627	-0.33	0.4407						
<i>CYP1A1</i>	-0.55	0.0343	-0.51	0.1031						
<i>SOX2</i>	-0.7	0.0001	-0.67	0.0000	-0.2	0.038	-0.05	0.7193	-0.1535	0.047
<i>GMNC</i>	-0.81	0.0000	-0.72	0.0047	-0.58	0.0002	-0.14	0.6958	-0.5164	0.0075

MCP: multiple challenge protocol ; CTL: Control; HC: healthy control; SA-no GORD: severe asthmatics with no documented GORD; SA-GORD: severe asthmatics with documented GORD who had abstained from their regular PPI treatment for 2 weeks.

LEGENDS TO FIGURES

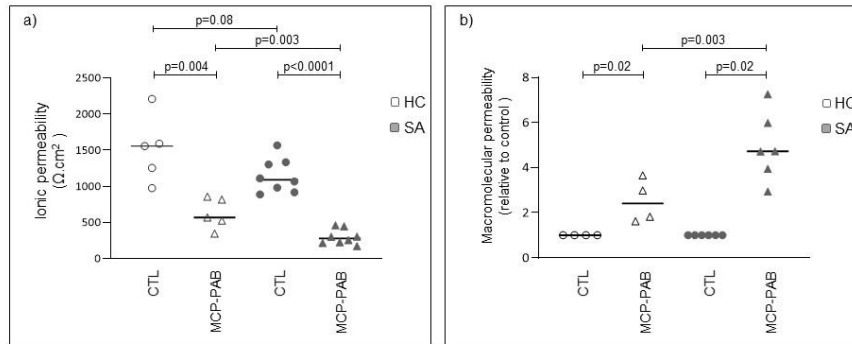


Figure 1. Effects of Multiple Challenge Protocol using pepsin, acid pH and bile acid on Epithelial Permeability. Bronchial epithelial ALI cultures from healthy controls (HC) (N=5) and severe asthmatic (SA) (N=8) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 hours before ionic (a) and macromolecular (b) permeability were measured. * $p<0.05$ vs control; ** $p<0.005$ vs control.

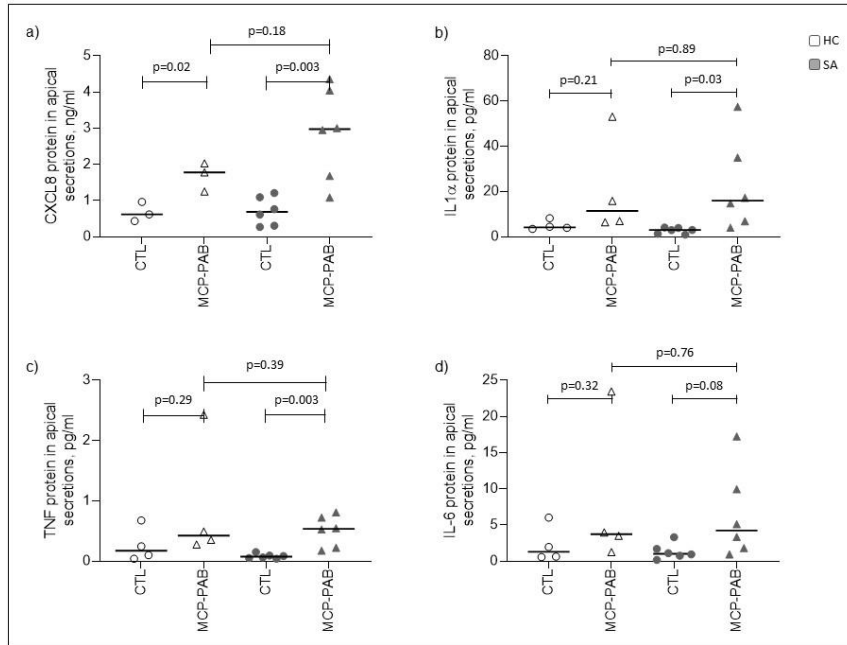


Figure 2. Stimulation of Epithelial Cytokine Release by Multiple Challenge Protocol using pepsin, acid pH and bile acid . Bronchial epithelial ALI cultures from healthy controls (HC) (N=4) and severe asthmatic (SA) (N=6) donors were untreated or exposed to Multiple Challenge Protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 hours before CXCL8 (A), IL-1 α (B), TNF α (C) and IL-6 (D) protein release was measured in apical secretions. * $p < 0.05$ vs control. ** $p < 0.005$ vs control.

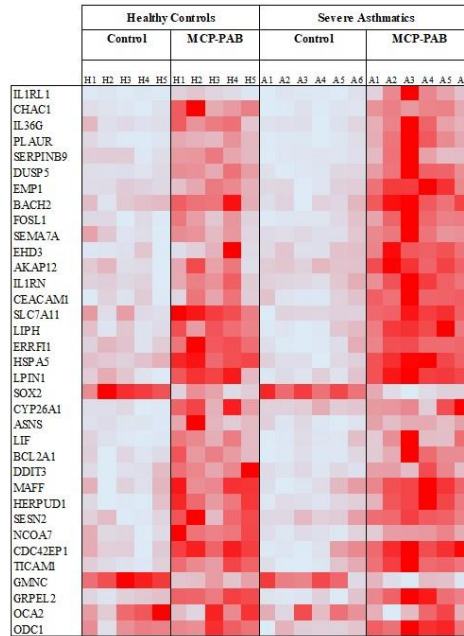


Figure 3. Changes in Epithelial Gene Expression caused by Multiple Challenge Protocol using pepsin, acid pH and bile acid in vitro. Bronchial epithelial ALI cultures from healthy controls (HC) (N=5) and severe asthmatic (SA) (N=6) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid for 30 mins, washed and allowed to recover for 4 hours before harvesting for RNA-seq. Heatmap of the top dysregulated epithelial genes from low (blue) to high (red) levels of expression. For exact fold-change, see Table E2.

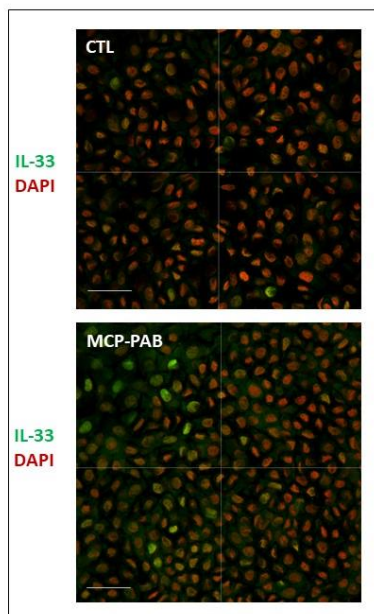


Figure 4. Regulation of Epithelial Expression of IL-33 by Multiple Challenge Protocol using pepsin, acid pH and bile acid . Bronchial epithelial ALI cultures from severe asthmatic (SA) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 hours 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 6 donors. Scale bar = 25µm.

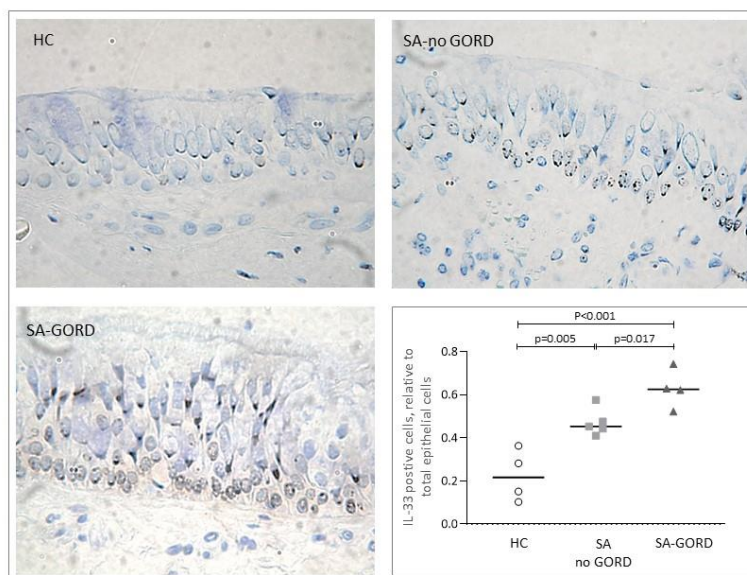


Figure 5. Epithelial IL-33 Expression is Increased Severe Asthma with GORD. Immunohistochemical staining for IL-33 in bronchial biopsies from healthy control participants without GORD (HC), severe asthmatics with no documented GORD (SA-no GORD; n=5), severe asthmatics with documented GORD who had abstained from their regular PPI treatment for 2 weeks (SA-GORD; n=4). Panel A shows typical patterns of IL-33 immunostaining and panel B shows quantitation of positive nuclei expressed as percentage of total epithelial cells.

Title: Vulnerability to acid reflux of the airway epithelium in severe asthma

Authors: Authors: Jeanne-Marie Perotin, Gabrielle Wheway, Kamran Tariq, Adnan Azim, Robert A Ridley, Jonathan A Ward, James PR Schofield, Clair Barber, Peter Howarth, Donna E. Davies*, Ratko Djukanovic*.

* joint last authors

Online Repository

METHODS

Study participants and sample collection

Healthy participants and severe asthmatics (Step 4/5 of BTS/SIGN Guidelines), aged ≥ 18 , non-smoker or ex-smoker for ≥ 12 months and < 10 pack/years history of smoking, were recruited prospectively. All participants were assessed for GORD by 24-hr pH/impedance studies. The asthmatics were further stratified into those with a documented diagnosis of GORD but not treated with PPI, severe asthmatics with documented GORD and taking PPI treatment and severe asthmatics without a diagnosis of GORD.

Demographic and clinical data as well as results of questionnaires (Asthma Control Questionnaire, Saint Georges Respiratory Questionnaire, Leicester Cough Questionnaire) were recorded on enrolment. Atopy was assessed by skin prick tests (*Dermatophagoides pteronyssinus*, mixed grass pollens, mixed tree pollens, cat dander). Pulmonary function tests, including FEV₁ reversibility to salbutamol, and routine haematology and liver and renal function blood tests were performed. Induced sputum was collected using European Respiratory Society (ERS) recommendations (1). Fiberoptic bronchoscopy was performed in accordance with the British Thoracic Society guidelines and standard operating procedures of the NIHR Wellcome Trust Clinical Research Facility and NIHR Southampton Respiratory Biomedical Research Unit at the University Hospital Southampton NHS Foundation Trust.

Epithelial cells were harvested by bronchoscopic brushings and samples were either processed into RNA later for subsequent RNAseq analysis or used in primary bronchial epithelial cells (PBEC) culture. Bronchial biopsies were also taken and embedded into a glycomethacrylate resin for immunohistochemical analyses.

Development of the refluxate mix for use in epithelial culture

Prior to cultures with epithelial cells obtained from patients and controls, dose-ranging and time-course studies were conducted with 16HBE cell line cells using varying compositions of multiple challenge protocol (MCP) containing pepsin (50 to 150 $\mu\text{g/ml}$; Sigma-Aldrich, St Louis, MO, USA), hydrochloric acid (pH 1.5 to 5) and chenodeoxycholic acid (CDC) (50 μM to 5 mM; Sigma-Aldrich, St Louis, MO, USA). Cells were maintained in minimum essential medium (MEM) with Glutamax and F12 and supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin (Life technologies, Paisley, UK) on PureCol collagen I (Advanced BioMatrix, San Diego, CA, USA) coated culture flasks. Experiments were carried out using collagen-coated Transwell® permeable supports (diameter 6.5 mm, polyester membrane with 3 μm pores, Corning Life Sciences, Amsterdam, The Netherlands). Cells were seeded at a density of 0.7×10^5 cells in 200 μL of growth medium; the basolateral compartment contained 500 μL of the same medium. Medium exchange was carried out every 2–3 days. In culture, the 16HBE cells formed a polarized epithelial sheet within seven days, as monitored by measuring the transepithelial resistance (TER). Cells with a TER $>700 \Omega/\text{cm}^2$ on day 7 were used for experiments.

Culture media containing varying concentrations of hydrochloric acid, pepsin and/or chenodeoxycholic acid were applied to the apical pole of cells for 5 to 30 minutes. Apical supernatants were collected and centrifuged (10,000 rpm 10 min at 4°C) for assessment of cytotoxicity. The epithelial cells were then washed twice and fresh control culture media was replaced. After 4hr of recovery, micro and macro-molecular permeability were measured by TER and FITC dextran 4kDa respectively. The cells were then lysed using Trizol lysis reagent (Life Technologies, Paisley, UK) and frozen at -80°C until analysed.

Exposure of 16HBE cells to hydrochloric acid was associated with dose-dependent damage of the epithelium (Figure E1). Comparison between timepoints showed no significant differences in signals (data not shown). Pepsin did not have any additional impact on cell viability at different concentrations (50 to 150 $\mu\text{g/ml}$) or pH (1.5, 2.5 and 5) after 30 minutes of exposure. Chenodeoxycholic acid exposure induced dose-dependent cytotoxicity, with no additional impact of CDC 500 μM at pH5. We, therefore, defined pH5 + pepsin 50 $\mu\text{g/ml}$ + CDC 500 μM as the working multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) at this stage of culture optimization and chose 30 minutes as the time of exposure. This MCP-PAB model induced a mean 3.5% cytotoxicity in 16HBE cells and a mean 39% decrease in ionic epithelial permeability.

Primary epithelial cultures

Having optimised the culture and AR exposure conditions for 16HBE cells, we went on to optimise the same for primary bronchial epithelial cells (PBEC). PBEC were obtained from healthy and asthmatic donors by fiberoptic bronchoscopy. After adding RPMI, 10% FBS and 2% penicillin/streptomycin, cells were centrifuged at 1200rpm for 5min at room temperature. The cell pellets were re-suspended in Airway Epithelial Cell Growth Medium (Promocell, Heidelberg, Germany) and seeded into PureCol collagen I coated culture flasks. After achieving confluence, the cells were transferred into Transwell® permeable supports and cultured at air-liquid interface (ALI). After 21 days, the epithelial cells formed a differentiated pseudostratified epithelium. Using trans-epithelial resistance (TER) as a measure of differentiation, cultures with a TER > 1000 were deemed fully differentiated and were, therefore, selected for AR experiments. The ALI cultures were starved overnight prior to start of the exposure. In these conditions CDC 500µM was found to be cytotoxic; therefore, its concentration was reduced to 250µM. The final AR was therefore defined as pH5 + pepsin 50µg/ml + CDC 250µM and this was applied for 30 min.

Analysis of the effect of multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) on epithelial cultures

Assessment of cytotoxicity

Lactate dehydrogenase (LDH) release was measured using a CytoTox 96 Nonradioactive Cytotoxicity Assay (Promega, Southampton, UK), according to the manufacturer's instructions. All samples were assayed in duplicate. Intracellular LDH in control cultures was determined by lysing cells with 1% Triton X-100 in Airway Epithelial Cell Growth Medium. LDH release in each well was calculated as a percentage of total LDH, and values for control wells were subtracted from challenged wells to give the percentage of total LDH released as a result of challenge.

Measurement of epithelial permeability

Transepithelial electrical resistance (TER) was measured using an EVOM voltohmmeter (World Precision Instruments, Hitchin, UK). Fluorescein isothiocyanate (FITC)-dextran 4 kDa (Sigma-Aldrich, Poole, United Kingdom) was applied to cells apically and incubated for 4 hours at 37°C. Basolateral dextran passage was analyzed with a Fluoroskan Ascent FL2.5 reader (Thermo Fisher, London, United Kingdom).

Immunostaining and electron microscopy

Immunostaining

ALI cultures were analysed using immunofluorescence. ALI cultures were fixed with 4% paraformaldehyde and then permeabilized with 0.1% triton X 100 for 15 min at room temperature and blocked with BSA (1% BSA, 0.1% Tween 20 in PBS) for 1 hr. Cells were stained with mouse monoclonal anti-ZO-1 (1/100; BD biosciences, San Jose, CA, USA), mouse monoclonal anti-E-cadherin (1/250; Cell Signaling, London, UK) or goat polyclonal anti-human IL-33 (1/20; R and D Systems, Abingdon, UK) diluted in 1% BSA, 0.1% Tween 20 in PBS overnight at 4°C. Cultures were then washed with 0.1% Tween 20 in PBS 4 times and incubated for 2 hrs with donkey anti-goat Alexa fluor 488 labelled secondary antibody (Life Technologies, Carlsbad, CA, USA) or goat anti-mouse Alexa fluor 647 labelled secondary antibody (Biolegend, San Diego, CA, USA). Cell nuclei were counterstained with DAPI. Images were acquired using a Leica SP8 laser-scanning confocal microscope (Leica Microsystems, UK).

Bronchial biopsies were analysed using immunohistochemistry. Bronchial biopsies were processed as previously described (3) and embedded into paraffin; 4- μ m sections were cut from all suitable biopsies and stained by immunohistochemistry using goat polyclonal anti-human IL-33 (R&S Systems, Abingdon, UK). Epithelial cells were counted and results expressed as positive cells per total epithelial cells.

Electron Microscopy

PBEC in transwells were fixed in 3% glutaraldehyde, 4% formaldehyde, 0.1M PIPES buffer pH7.2 for 30 min at RT, then rinsed with 0.1M PIPES buffer pH7.2 and subsequently post-fixative 1% Osmium tetroxide 0.1M PIPES buffer pH7.2. Transwells were stained enbloc in 2% Uranyl Acetate then dehydrated through a graded series of ethanols into acetonitrile and into embedded in Spurr resin. Ultrathin sections (90nm) were cut and stained with Reynolds lead citrate. Sections were analysed using a Hitachi HT7700 transmission electron microscope.

Cytokine analysis

Interleukin 8 (IL-8) concentrations in culture media from the apical and basolateral chambers were measured using an IL-8 DuoSet ELISA (R&D, Abingdon, UK) in accordance with manufacturer's instructions. Each sample was evaluated using 2 technical replicates and the mean value was used for subsequent statistical analysis. IL-6, TNF α , IL1 α were measured using VPLex (MSD, Maryland, USA) according to manufacturer's recommendations.

Analysis of gene expression in epithelial brushings and differentiated cells

RNA extraction

Total RNA was extracted from primary bronchial epithelial samples using the Qiagen RNeasy kit. RNA quality and concentration were measured using an RNA Nano chip on the Agilent Bioanalyser 2100. Samples with total RNA concentration $\geq 50\text{ng}/\mu\text{l}$, RIN ≥ 6.8 and OD 260/280 were taken for cDNA library preparation and sequencing.

cDNA library preparation and sequencing

cDNA libraries were prepared using NEBNext Ultra (non-stranded) mRNA library prep kit with polyA pulldown for mRNA enrichment (Novogene Inc). Library quality was assessed using a broad range DNA chip on the Agilent Bioanalyser 2100. Library concentration was assessed using Qubit and q-PCR. Libraries were pooled. Paired-end 150bp sequencing to a depth of 20M reads (epithelial brushings) or 50M reads (differentiated cells) was performed on an Illumina HiSeq2500 by Novogene Inc (Cambridge, UK).

Data processing

Quality control

Raw FASTQ reads were subjected to adapter trimming and conservative quality filtering (reads containing N > 10%, reads where >50% of read has Qscore ≤ 5) by Novogene Inc. Quality of sequence was assessed using fastqc, aggregated using multiqc.

Alignment

Paired FASTQ files were aligned to human genome build 38 using gencode v29 gene annotations and STAR v2.6.0 splice aware aligner (4), using ENCODE recommend options (3.2.2 in the STAR manual (<https://github.com/alexdobin/STAR/blob/master/doc/STARmanual.pdf>)). Samples aligned at a rate of 88.04 - 93.78% uniquely mapped reads.

Read counting

SAM files were sorted by name using SAMtools (5). Reads were counted using HTSeq (6) and gencode v29 annotations following guidelines in the HTSeq documentation (https://htseq.readthedocs.io/en/release_0.11.1/overview.html#documentation-overview)

Differential gene expression analysis

Count data from HTseq was subjected to quantile filtration to remove genes with more than 25% of samples with a read count of 0. Count data was normalised by library size to calculate counts per million (CPM). CPM data was normalised by distribution using the trimmed Mean of the M-values (TMM) approach using edgeR (7, 8).

Gene ontology was performed by AmiGO (9-11) using the top 300 upregulated differentially expressed genes (DEGs) in the group of severe asthmatic with GORD compared to the group of severe asthmatics with no GORD.

qRT-PCR

RNA was reverse transcribed to cDNA using a Precision Reverse Transcription kit (PrimerDesign, Southampton, UK) according to the manufacturer's instructions. Expression of CXCL8 (Applied Biosystems, Paisley, UK) and IL-33 (Primer Design, Chandler's Ford, UK) was determined using probe-based qPCR, whereas expression of the housekeeping genes ubiquitin C and glyceraldehyde 3-phosphate dehydrogenase was determined using a probe-based duplex primer mix (PrimerDesign). Fold change in gene expression relative to time-matched controls was determined using the $\Delta\Delta C_t$ method.

SUPPLEMENTARY TABLES

Table E1. Top DEGs fold changes relative to control in healthy subjects (HC) and severe asthmatics (SA) ALI cultures exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) from negative (blue) to increasingly positive (increasing intensity of red).

	HC	SA	Gene name
IL1RL1		11,8	Interleukin 1 Receptor Like 1
CHAC1	8,9	8,5	ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1
IL36G	2,9	6	Interleukin 36 Gamma
PLAUR	2,5	5,7	Plasminogen Activator, Urokinase Receptor
SERPINB9		5,3	Serpin Family B Member 9
DUSP5	2,6	4,7	Dual Specificity Phosphatase 5
EMP1	1,3	3,9	Epithelial Membrane Protein 1
BACH2		3,8	BTB Domain And CNC Homolog 2
FOSL1		3,6	FOS Like 1, AP-1 Transcription Factor Subunit
SEMA7A		3,4	Semaphorin 7A (John Milton Hagen Blood Group)
EHD3		2,5	EH Domain Containing 3
AKAP12		2,4	A-Kinase Anchoring Protein 12
IL1RN		2,2	Interleukin 1 Receptor Antagonist
CEACAM1		2,1	CEA Cell Adhesion Molecule 1
SLC7A11		2,1	Solute Carrier Family 7 Member 11
LIPH	1,3	2,1	Lipase H
ERRFI1	1,6	1,9	ERBB Receptor Feedback Inhibitor 1
HSPA5	1	1,6	Heat Shock Protein Family A (Hsp70) Member 5
LPIN1		1,6	Lipin 1
SOX2		-0,7	SRY-Box Transcription Factor 2
CYP26A1	11,7		Cytochrome P450 Family 26 Subfamily A Member 1
ASNS	6,6		Asparagine Synthetase (Glutamine-Hydrolyzing)
LIF	3,9		LIF Interleukin 6 Family Cytokine
BCL2A1	3,8		BCL2 Related Protein A1
DDIT3	2,7		DNA Damage Inducible Transcript 3
MAFF	2		MAF BZIP Transcription Factor F
HERPUD1	1,6		Homocysteine Inducible ER Protein With Ubiquitin Like Domain 1
SESN2	1,5		Sestrin 2
NCOA7	1,5		Nuclear Receptor Coactivator 7
CDC42EP1	1,4		CDC42 Effector Protein 1
TICAM1	1,4		Toll Like Receptor Adaptor Molecule 1
GMNC	-0,7		Geminin Coiled-Coil Domain Containing
GRPEL2			GrpE Like 2, Mitochondrial
OCA2			OCA2 Melanosomal Transmembrane Protein
ODC1			Ornithine Decarboxylase 1

HC : healthy controls ; SA : severe asthmatics

LEGENDS TO SUPPLEMENTARY FIGURES

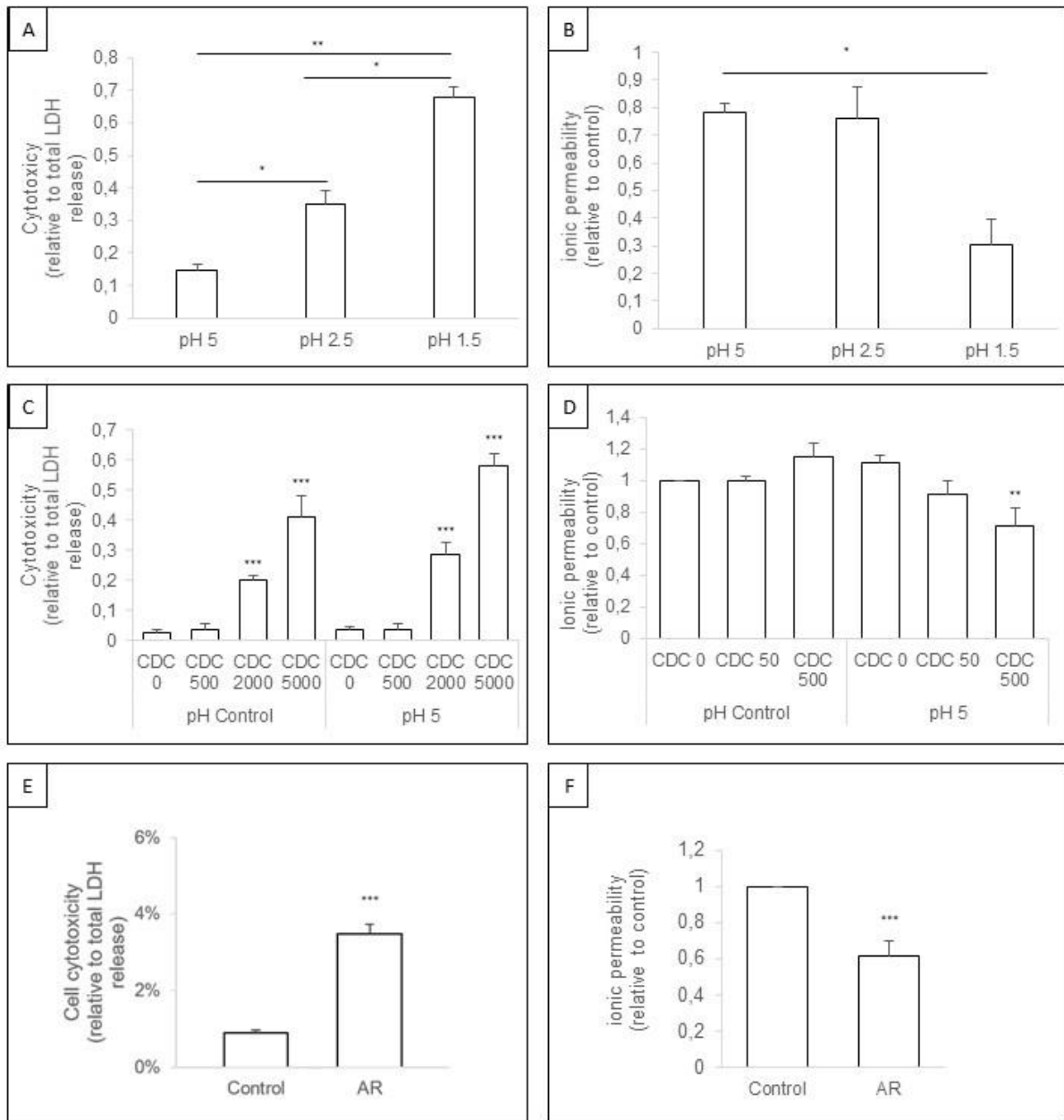


Figure E1. Development of the refluxate mix. Exposure of 16HBE cell cultures to hydrochloric acid (A, B), chenodeoxycholic acid (C, D) and/or artificial refluxate (E, F) induced cytotoxicity (A, C, E) and increase in epithelial ionic permeability (B, D, F). CDC: Chenodeoxycholic Acid (μM); MCP-PAB: multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$

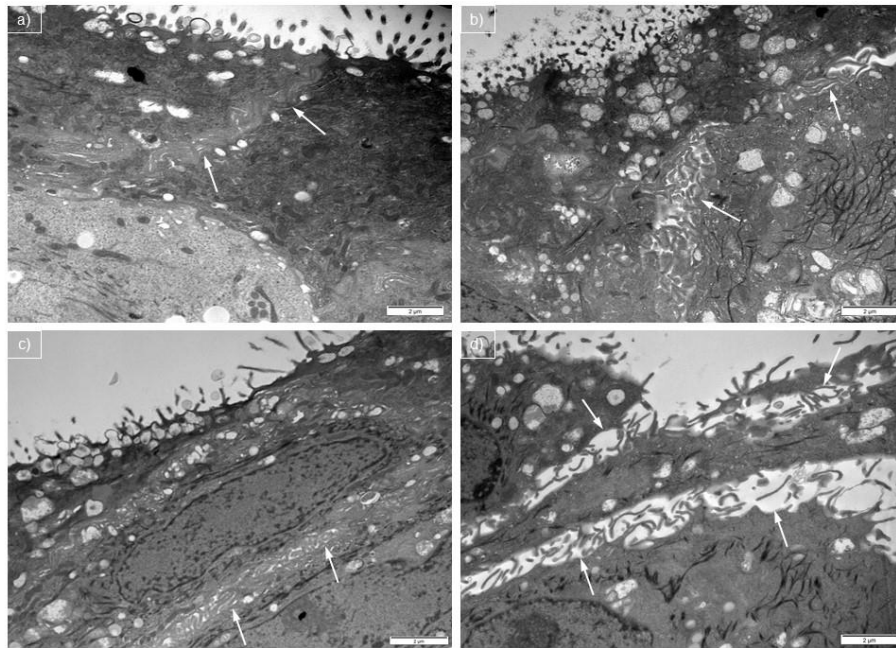


Figure E2. Enlargement of intercellular spaces by artificial refluxate. Bronchial epithelial ALI cultures from severe asthmatic donors were untreated (A, C) or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) (B, D) for 30 mins, washed and allowed to recover for 4 hours before fixing. TEM photographs of intercellular spaces (white arrows) are representative of experiments using 4 donors.

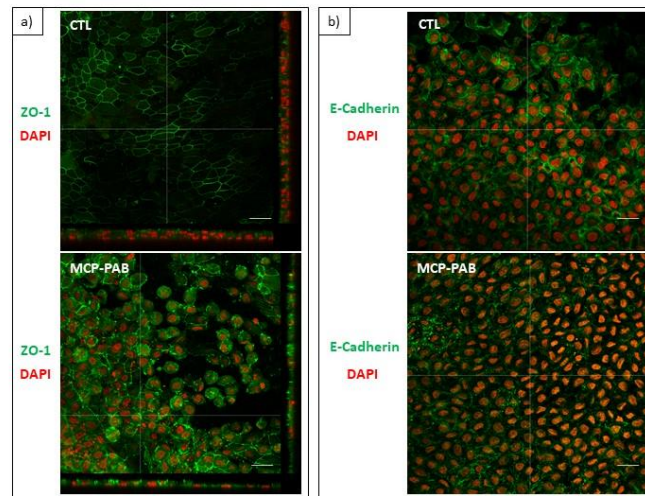


Figure E3. Disruption of Epithelial Tight junctions by Artificial Refluxate. Bronchial epithelial ALI cultures from severe asthmatic donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 hours before fixing and immunofluorescence staining. Panel A shows ZO-1 (green) and 4',6-diamidino-2-phenylindole (DAPI) (red) and Panel B shows E-cadherin (green) and 4',6-diamidino-2-phenylindole (DAPI) (red). Images are representative of experiments using 6 donors. Scale bar = 25 μ m.

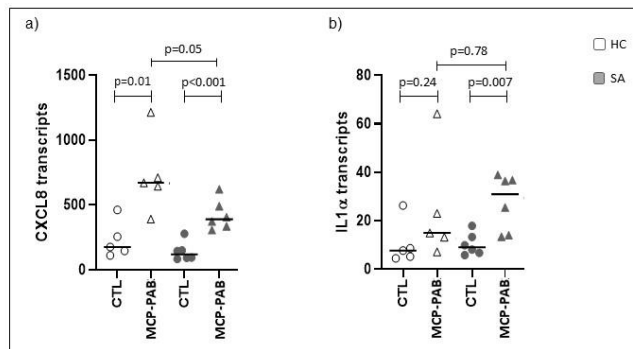


Figure E4. Stimulation of Epithelial Cytokine expression by artificial refluxate. Bronchial epithelial ALI cultures from healthy controls (HC) (N=5) and severe asthmatic (SA) (N=6) donors were untreated (CTL) or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 hours before RNA extraction for CXCL8 (A) and IL-1 α (B) gene expression analysis. * p<0.05 vs control. ** p<0.005 vs control.

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