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Early View

Research letter

Epithelial dysregulation in obese severe asthmatics with gastro-oesophageal reflux

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Title: Epithelial dysregulation in obese severe asthmatics with gastro-oesophageal reflux

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Dear Editor,

Gastro-oesophageal reflux disease (GORD) and obesity are associated with frequent exacerbations and poor quality of life in asthmatics. Multiple mechanisms have been proposed for the effect of obesity, including modification of inflammation affecting epithelial cell proliferation and wound repair, while the role of GORD is poorly understood and proton pump inhibitor (PPI) are of variable efficacy. GORD might exert a deleterious effect by inducing vagal reflex, neuroinflammation and directly (via microaspiration) triggering airway inflammation. Studies of reflux in animal models and human bronchial epithelial cell culture show varying impact on inflammation and airway remodelling. We have recently demonstrated changes in the sputum proteome in severe asthmatics with GORD, providing supportive evidence for gastric secretions exerting a direct effect on the airways (1). The epithelium plays a key role in asthma, so in this study we speculated that severe asthma in obese patients with GORD would be associated with epithelial dysfunction. Because GORD is treated with PPI, drugs associated with risk of pneumonia and exacerbations of COPD and cystic fibrosis, the impact of PPI was also assessed.

We analyzed 61 never or ex-smoker asthmatics and 44 healthy never smokers from the U-BIOPRED study *(2)* who had undergone bronchoscopy, bronchial biopsy and epithelial brushing. Patients were categorised as obese if BMI≥30kg/m², having or not a physician's diagnosis of GORD, and treated or not for GORD. Epithelial brushings were processed into RNAlater for Affymetrix U133 Plus 2.0 microarray analysis (GSE76226) and bronchial biopsies were immunostained for CD3+, CD4+ and CD8+ lymphocytes and analysed for basement membrane thickness. The study was approved by national ethics committees. All participants provided consent.

Epithelial transcriptomic data were clustered by TDA using the Ayasdi Core software (Ayasdi, MenloPark, CA), with cluster boundaries defined by density using Morse theory *(3)*. Paired t-tests were applied to log2 transformed transcriptomic data. Clinical data were analyzed by Kruskal-Wallis, Mann-Whitney U or Student t tests depending on data distribution. False discovery rate correction was

applied to the differentially expressed genes (DEGs). Pathway signatures and upstream regulators were identified by Ingenuity Pathway Analysis (IPA) (QIAGEN, Redwood City, CA). Potential drug impact on DEGs was identified by Connectivity Map (CMap) analysis of DEG signatures.

TDA analysis produced three clusters of similar size (C1, 2 and 3), comprising 21 participants (34%) in C1, 23 (38%) in C2 and 22 (36%) in C3 (Fig 1). When compared to combined C2/3 clusters, C1 had a higher incidence of obesity (76% vs 47%, p=0.02), GORD (85% vs 43%, p=0.04) and GORD treatment (81% vs 36%, p=0.004) and 48% were obese and had a diagnosis of GORD and GORD treatment (compared to 10% in C2/C3, p=0.0009); this cluster was, therefore, termed the Obesity-GORD-PPI treatment [OGP] phenotype. When compared to C2/3, the OGP cluster had lower blood eosinophil counts (p=0.007), but was similar in respect of corticosteroid treatment.

IPA identified 77 pathways dysregulated in the OGP cluster relative to health, the top being the WNT/β-catenin pathway (z-score: 2.2-fold difference vs. healthy participants). Amongst the 38 DEGs related to WNT/β-catenin signalling, *FZD3* and *WISP1* were amongst the top upregulated (Figure 1). Application of CMap analysis to these DEGs and comparison with the genes regulated by PPI and bile acids in A549 epithelial cells (Fig 1) showed that the WNT/β-catenin signalling pathway was not associated with PPI or bile acid effects. Furthermore, although evidence points to WNT/β-catenin signalling and the WNT target gene *WISP1* regulating airway remodelling and pulmonary myofibroblast proliferation in COPD and IPF *(4)*, we did not find a difference in the thickness of the sub-epithelial *lamina reticularis* between the OGP cluster and the other patients.

WISP1 has been shown to be upregulated in obese individuals but has also been identified as an inhibitor of adipocyte differentiation by blocking the induction of the adipogenic transcription factors PPARγ and C/EBPα *(5)*, both of which were downregulated in the OGP cluster. Given that PPARγ expression in bronchial epithelial cells has been shown to protect against oxidative stress and suppress MUC5AC expression, these data suggested that the OGP phenotype should be associated with airways inflammation. However, our study showed that the OGP cluster had a predominantly pauci-

granulocytic sputum cell profile (p=0.017) and fewer sub-mucosal T-cells when compared to health (CD8+ cells: 10.2 vs. 20.4 cells/mm², p=0.05; CD4+ cells: 7.2 vs 10.4, p=0.02; CD3+ cells: 23.0 vs 36.7, p=0.03). This could be explained by the finding in the OGP cluster of downregulated immune response pathways, including proliferation, activation and survival of lymphocytes, leucocyte recruitment and transepithelial migration (IL-7, TREM1 (Triggering receptor expressed on myeloid cells 1), B cell receptor and Calcium signaling; z-scores -0.69, -2.18, -2.33, -3.66 respectively). When compared to health and C2/3, the OGP cluster also exhibited downregulation of CCL5, CXCL1 and CCL11. CMap analysis identified high connectivity scores between TREM1 and effects of bile acids (CMap score 99.2), and between PPI treatment and Calcium signaling (CMap score 76.6) (Fig 1), suggesting a direct impact of bile acids and PPI treatment on immune cell accumulation.

In support of our study, recent analysis of bile acid effects on LPS-stimulated macrophages identified downregulated genes involved in differentiation and migration of immune cells, including T-cells, and decreased chemokine expression, including CCL5 and CXCL1 *(6)*. PPI-inhibition of H^+/K^+ ATPase induces intracellular acidification that inhibits immune cell proliferation, decreases heparanase activation involved in ECM remodeling and degradation *(7)*, and decreases intercellular adhesion molecule expression, resulting in reduced immune cell transmigration *(8)*. In airway epithelial cells, TLR2 activation by DAMPs releases calcium from endoplasmic reticulum stores, resulting in chemokine regulation through activation of NFκB and calpains which cleave junctional proteins and facilitate immune cell transmigration *(9)*. In our study, expression of TLR2, NFκB and NFκB-regulated chemokines (*CCL5, CXCL1, CXCL2, CCL11, CCL22, CXCL8*) was also downregulated in the OGP cluster. In contrast, expression of calreticulin and calnexin, two endoplasmic reticulum calcium storage proteins, whose expression is increased by low intracellular calcium storage *(10)*, was upregulated in the OGP cluster, suggesting dysregulated intracellular calcium influx. This could be speculated to involve PPI-induced decrease of Ca^{2+}-ATPase sensitivity as previously shown in myocytes *(11)*.

The top upstream regulator for the OGP cluster was CD24 (activation z-score 2.2; p=0.0004), a cell surface receptor involved in suppression of immune responses to DAMPs *(12)*, organization of tight

junction proteins *(13)*, regulation of adipogenesis, B-cell survival and T-cell activity *(14)*. CD24 is a direct WNT target gene *(15)*, which is consistent with our findings of upregulated WNT/β catenin signaling. Together, these associations suggest a central role for CD24 in the OGP phenotype.

In summary, this exploratory analysis of epithelial gene expression in severe asthma has identified 3 clusters, one of which is enriched for obesity, GORD and treatment with PPI, with an as yet unreported mechanism that could represent a new endotype. This potentially new endotype was shown to be pauci-granulocytic as a consequence of downregulated mechanisms of cell recruitment linked to bile acid exposure and PPI treatment. The implications of this endotype for virus-induced exacerbations, which are increased in asthmatics with GORD, remains to be elucidated.

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Figure legend

Severe asthma clusters based on epithelial transcriptomics. (a) TDA network constructed with transcripts from bronchial brushings using density analysis by Morse theory, with (b) the identified clusters 1-3 colored yellow, pink and orange. (c-e) Applying BMI, GORD treatment and blood eosinophil counts as meta data, nodes are colored by intensity from blue (low intensity) to red (high intensity). (f) Heat map of the three top upregulated and three top downregulated differentially expressed genes of WNT/β-catenin, TREM1 and Calcium signalling in the OGP cluster and in airway epithelial cells exposed to PPI (column L for Lansoprazole and column R for Rabeprazole) and bile acids (column G for Glycodeoxycholic acid and T for Taurodeoxycholic acid).

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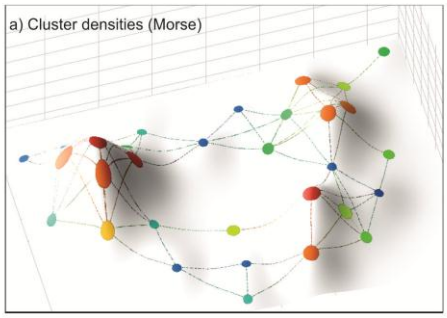

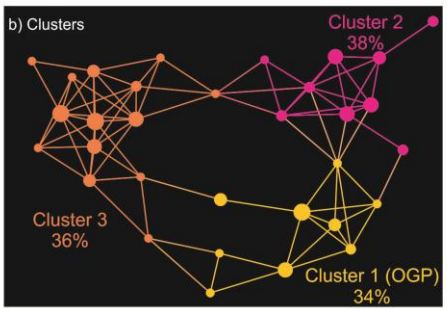

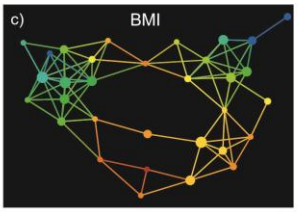

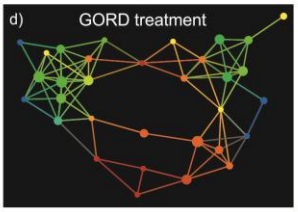

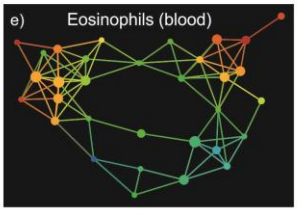

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WNT/β-catenin genes					
Cmap score		29.39	-14.77	0	-60.02
FZD3					
WISP1					
ACVR1B					
SOX11					
GNAO1					
MDM2					
TREM1 Signaling genes					
Cmap score		0	-80.47	97.51	99.23
MAPK1					
TLR7					
STAT3					
CXCL8					
TYROBP					
TREM1					
Calcium Signaling genes					
Cmap score		76.57	71.01	0	39.63
CACNA1D					
CACNB3					
TP63					
GRIN1					
CACNG5					
RAP1A					