**A multi-omics approach to delineate sputum microbiome-associated asthma inflammatory phenotypes**

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**To the editor,**

Asthma is a heterogeneous disease with multiple clinical presentations (phenotypes) [1]. Neutrophilic asthma is characterized by increased sputum neutrophils and generally has a poor response to corticosteroids and limited other therapeutic options. Neutrophilia originates from different factors including the defective resolution of inflammation or bacterial infections [2]. An association between airway bacterial imbalance (disturbance) and the neutrophilic phenotype has been reported [3], suggesting that airway microbiota composition is involved in neutrophilic asthma. Rather than being a separate entity [4], neutrophilic asthma may be in part, an alliance between innate immunity and microbiota composition that prompts protective mechanisms against invading pathogens [2].

In the Unbiased BIOmarkers in PREDiction of respiratory disease outcomes (U-BIOPRED) study, adults with severe asthma were classified into two clusters (phenotypes) based on the sputum microbiome [3]. The microbiome-driven cluster 2 (C2) showed relatively worse asthma outcomes, sputum neutrophilia, and microbial imbalance (reduced microbial diversity and commensals and enrichment of pathogenic bacteria particularly *Haemophilus influenzae* and *Moraxella catarrhalis*) compared with cluster 1 (C1). We investigated the underlying molecular mechanisms of microbiome-driven phenotypes by investigating differences in sputum eicosanoids, transcriptomics, and proteomics to reveal potential targets for diagnosis and treatment.

Sputum omics, including eicosanoids, transcriptomics, and SomaScan® proteomics, were assayed as described previously [3, 5, 6]. C2 was compared with C1 patients, mild-moderate asthma patients (MM), and healthy controls (H) according to a panel of 11-eicosanoids and Shannon metagenomics α-diversity using Kruskal-Wallis H test. C2 and C1 were compared by sputum transcriptomics and proteomics using Linear Models for Microarray Data (limma) analysis after adjusting for age, sex, and oral corticosteroid intake. Pathway enrichment analysis for the sputum differential transcriptome was performed by gene set enrichment analysis (Gage R package) using two databases for homo sapiens; the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the Molecular Signatures Database (MSigDB) Hallmark gene sets. A similar analysis was also performed comparing C2 against MM and H groups. Multiple testing was adjusted for using Benjamini Hochberg false discovery rate (FDR) correction and an α (q-value) <0.05 was considered significant.

The sputum 11-dehdro-TXB2 and PGE2 (but not LTB4) levels were significantly elevated in C2 compared to C1 subjects (q<0.05, Figure A), consistent with sputum PTGS2 gene upregulation in C2 (Log2 fold change (FC)=1.1, *q*<0.05). Furthermore, the levels of 15-HETE, LTE4, and PGD2 were elevated in C2 compared with MM and/or H groups. These findings were associated with decreased Shannon alpha-diversity in C2 compared to other groups (*P*<0.001, Figure B), consistent with the increase in the inflammatory signal with the increased severity level in the groups.

A total of 2578 differentially expressed genes (DEGs, *q*<0.05) were found different between C2 and C1, of which 194 genes had a least a two-FC (Figure C). Several up-regulated DEGs were related to immune regulation and inflammation particularly tumor necrosis factor (TNF)α and related regulatory genes (*TNFAIP-6*, *TNFAIP3*, *TNFSF10*, *TNFRSF10,* and *TNFSF14*); interleukins (IL) and related regulatory genes (*IL18R1*, *IL18RAP*, *IL1R1*, *IL1R2*, *IL1B*, *IRAK2*, *IRAK3*, *IL6,* and *IL6R*); Toll-like receptors (TLRs e.g. *TLR2*, *TLR4,* and *TLR10*), inflammasomes (e.g. *NLRP3*, *NLRP12*, and *NLRC5*) in C2 relative to C1. Similar DEGs were significantly up-regulated in C2 compared to MM and H groups.

Upregulated KEGG pathways in C2 mainly related to pathogen fighting, inflammation, and innate immune-regulation mechanisms such as natural killer cell-mediated cytotoxicity, chemokine, NOD-, Toll-, and RIG-I-like receptor signaling pathways (*q*<0.05, Figure E). Down-regulated KEGG pathways in C2 were related to cell proliferation and growth including ribosome biogenesis, DNA replication, and repair; fatty acid, amino acid, nucleotide, and carbohydrate metabolism; as well as cellular degradation and catabolism (lysosome, peroxisome, and proteasome). The Hallmark gene sets supporting KEGG analysis including up-regulation of immuno- and inflammatory pathways in C2 such as TNF-α signaling via NFκB, IFN-α, IFN-γ, inflammatory response, IL-6 JAK-STAT3 signaling, and apoptosis (Figure F). Down-regulated pathways included oxidative phosphorylation (OXPHOS), reactive oxygen species (ROS), DNA-repair, cell cycle, proliferation and growth, peroxisome, and metabolism. Similar up-regulated and down-regulated pathways were significant in C2 compared to MM and H groups. Additional KEGG/Hallmark upregulated pathways in C2 relative to MM and H groups were hypoxia, Jak-STAT, MAPK-, T-cell-, KRAS-, and IL2 STAT5 signaling (data not shown).

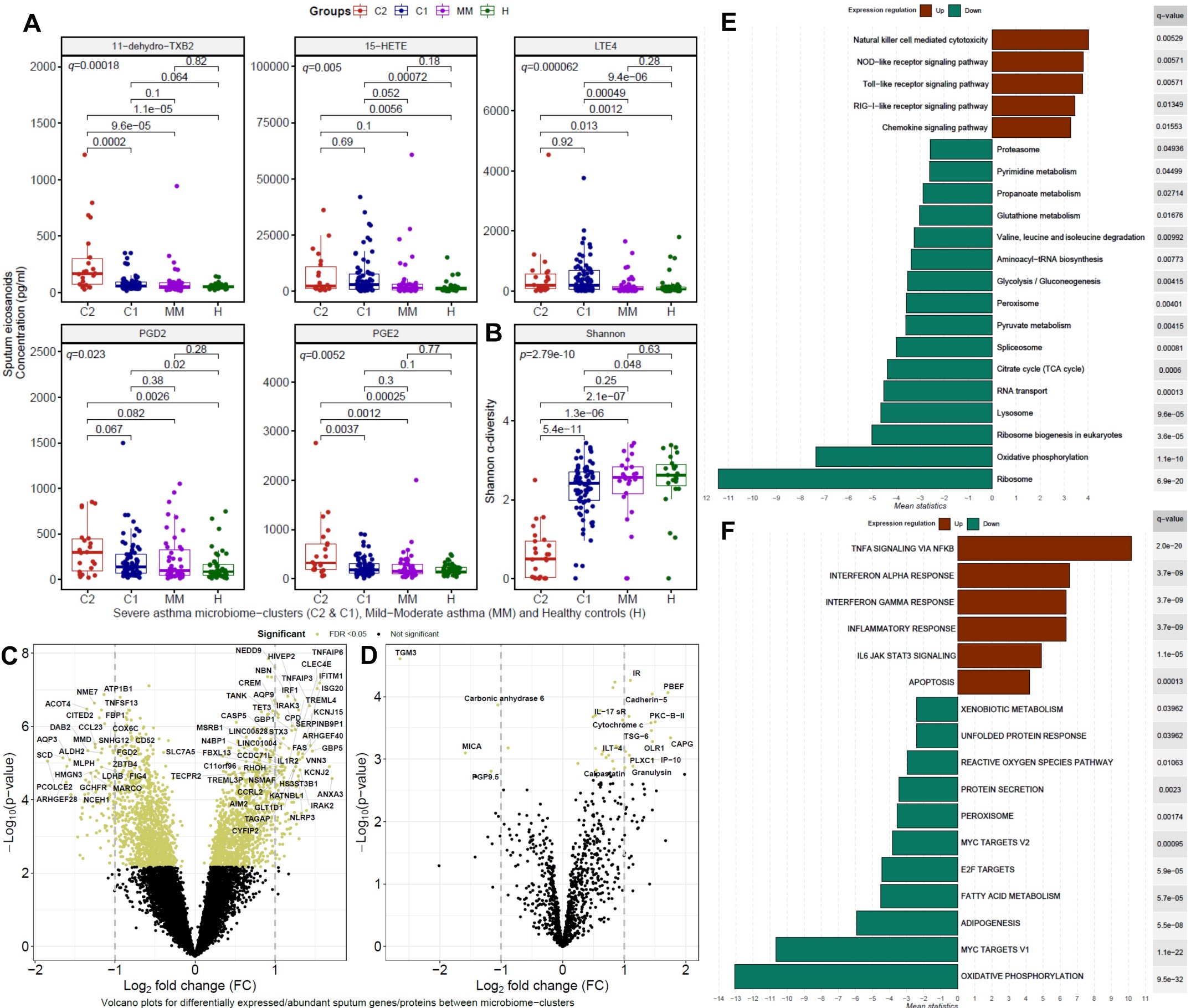
36 differentially abundant proteins (DAPs) were significant (*q*<0.05) between C2 and C1, of whom 14 proteins had at least a two-FC (Figure D). Up-regulated DAPs in C2 (TSG-6, IL-17RA, IP-10, Granulysin, ILT-4, PBEF, TLR4:MD-2 complex, MMP-10, NKp46, and IGFIR) were related to neutrophilia, inflammation, and/or TH17 and Th1 mediated pathways. Similar DAPs were significant when C2 patients were compared to MM and H groups. Additional enriched (pro)inflammatory DAPs were found in C2 relative to MM and/or H groups (*q*<0.05) including CRP, Light, Protein-S, Azurocidin, PGRP-S, TIMP-2, IL-8, Notch1, MMP1, MMP2, and C5a.

The bacterial imbalance in C2 was associated with sputum neutrophilia suggesting an immune-modulatory response to fight clinical/subclinical infections, potentially resulting in more inflamed/obstructed airways [7] with worse lung function parameters as observed in C2 patients. Interestingly, sputum macrophages were significantly decreased in C2 compared to C1, consistent with a defective immune capacity to adequately eliminate pathogenic micro-organisms [8]. Most of the elevated signaling eicosanoids molecules in C2 are proinflammatory and/or induce bronchoconstriction [9], which may partly explain the reduced lung function in the relatively more severe C2 cluster. In addition, sputum PGE2 and PGD2 regulate neutrophils and/or Th17 and Th1 pathways [10], suggesting that their elevated levels in C2 patients might be driving these clinical features. PGE2 suppresses phagocytosis by airway macrophages in asthma [11] consistent with our sputum pathway enrichment analysis showing that C2 patients had down-regulated lysosome and ROS pathways, which are critical in pathogen fighting [12], relative to C1.

Many of the observed DEGs, DAPs, and pathways are implicated in immune regulation and/or characteristic of the neutrophilic asthma signature [7] distinguishing C2 patients. TNFα was markedly upregulated in C2, consistent with what was found in the *Haemophilus*-enriched cluster in patients with asthma and COPD [13]. Upregulation of the interferon (IFN) pathways was observed in patients with C2 and IFN-γ has been shown to decrease epithelial barrier function and enhance neutrophil transmigration [14]. Impaired barrier function could render the airway mucosa of C2 subjects vulnerable to pathogenic bacterial infections. Our metagenomics analysis did not detect key RNA viruses such as RV16 whose presence at a subclinical level could enhance RIG-I and IFN-γ pathways.

In contrast, down-regulated pathways showed defective cell growth, proliferation, metabolism, and DNA repair suggesting an impaired capacity to repair inflamed cells and maintain homeostasis. Moreover, C2 exhibited down-regulation of OXPHOS, tricarboxylic acid (TCA) cycle, and ROS pathways suggesting mitochondrial dysfunction [15]. These results support previous data where neutrophilic inflammation was associated with reduced OXPHOS gene expression and mitochondrial dysfunction in bronchial epithelial cells [16].

These findings reveal potential targets for the C2 microbiome-driven asthma phenotype, which might constitute a selected subtype within corticosteroid-resistant asthmatics. Therapeutic options for this cluster might be antibiotics (e.g. macrolides), or phage therapy. In addition, the dysregulated neutrophilic and cytokines/chemokines might be diagnostic/therapeutic targets in C2 patients [7]. Differing biological pathways and mediators involved in these patients suggest that pharmacotherapies targeting multiple mechanisms might be required. This also suggests neutrophilic asthma is not a single phenotype [4] and underlying mechanisms should be considered to optimize treatment.



**Figure: A;** Box-whisker plots showing differencesin sputum eicosanoids lipidomics (pg/ml) between severe asthma microbiome-driven Cluster 2 (C2, n =22), severe asthma microbiome-driven Cluster 1 (C1, n=75), mild-moderate asthma (MM, n=46), and healthy controls (H, n=47). A panel of 11-eicosanoids was tested (namely; 11-dehydro Thromboxane (TX) B2, 12-Hydroxyeicosatetraenoic acid (HETE), 15-HETE, 5-HETE, 6-keto prostaglandin (PG) F1α, Leukotriene (LT) B4, LTE4, PGD2, PGE2, and Tetranor PGDM and PGEM). *q*-values were computed using the Kruskal-Wallis H test followed by Benjamini Hochberg false discovery rate (FDR) correction, and only eicosanoids with *q*-values<0.05 were shown. Pairwise differences between the groups (*P*-values) were computed using the Mann-Whitney U test. **B;** Box-whisker plot showing differencesin metagenomics Shannon α-diversity between C2 (n=25), C1 (n=75), MM (n=24), and H (n=23) groups. *P*-value was computed using the Kruskal-Wallis H test and pairwise differences between the groups (*P*-values) were computed using the Mann-Whitney U test. **C;** Differentially expressed genes (DEGs) between C2 (n=23) and C1 (n=61). Positive log2 fold change (FC) represents over-expressed sputum genes in C2 patients relative to C1, while negative log2 fold change represents down-expression in C2. DEGs with a q-value <0.05 are highlighted in red olive green color. Only the top 75 DEGs, with at least twofold change, were labeled. Similar DEGs (q-values<0.05) were observed when C2 was compared independently to MM (n=20), and H (n=16) groups (not shown). **D;** Differentially abundant proteins (DAPs) between C2 (n=13) and C1 (n=61). Positive log2 FC represents over-abundant sputum proteins in C2 patients relative to C1, while negative log2 fold change represents down-abundance in C2. DAPs with a q-value <0.05 are highlighted in red olive green color. Only the top DAPs, with at least twofold change, were labeled. Similar DAPs (q-values<0.05) were observed when C2 was compared independently to MM (n=20), and H (n=21) groups (not shown). **E;** Up- and down-regulated KEGG gene expression pathways in C2 relative to C1 based on sputum gene differential expression. Mean statistics: mean individual statistics from multiple single array-based gene set tests calculated using the gage R package, where its absolute value estimates the magnitude changes of gene-set level, and its sign indicates the direction of the changes. The figure only depicts the statistically significant pathways, in which the ones that passed the multiple testing correction (*q*-values<0.05) are depicted. **F;** Up- and down-regulated Hallmark gene expression pathways in C2 relative to C1 based on sputum gene differential expression. The figure only depicts the statistically significant pathways, in which the ones that passed the multiple testing correction (*q*-values<0.05) are depicted.

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MIA has performed the analysis and drafted the initial version of the manuscript. MIA, AHM, IMA, FC and PJS have contributed to the design of the analysis plan. All co-authors have contributed to the acquisition of data, interpretation of the analysis, revision, drafting, critical appraisal and ensuring accuracy and integrity of the analysis. All co-authors have provided final approval of the version to be published. AHM is the corresponding author. In addition, the U-BIOPRED is a consortium effort and we wish to acknowledge the help and expertise of the individuals and groups whose names are mentioned in the U-BIOPRED study group list available in the online repository.

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**Conflict of interests:**

**MIA**, **SJHV**, **AHN**, **PB**, **AHW**, **ARS**, **AMW**, **ATB**, **MC**, **PS**, **JC**, **NK**, **JM**, **KS**, **DES**, **PM**, **RL**, **PHH**, **MS**, **IMA** and **ADK** have no conflicts of interest to disclose. **JHR** was an employee by and a share-holder of GlaxoSmithKline. **SB** is an employee of Johnson & Johnson and a former employee of GlaxoSmithKline and holds stock in both companies. **SSW** reports consulting fees from Kings College Hospital NHS Foundation Trust, Academic Medical Research, Aladdin Healthcare Technologies Ltd, AMC Medical Research BV, AMC Medical Research BV P402, Asthma UK, AstraZeneca AB, Athens Medical School, Autobedrijf Verfaillie V-CO BVBA, Boehringer Ingelheim International GmbH, Breathomix BV, Chiesi Farmaceutici S.p.A., CHU de Toulouse, CIRO, Consorcio Centro de Investigación Biomédica en Red, Dawi Iman Automobile, DS Biologicls Ltd, ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE, EFA- European Federation of Allergy, European Respiratory Society, European Society for Swallowing Disorders, F. 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