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Early View

Research letter

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ERJ Research letter

Instability of sputum molecular phenotypes in U-BIOPRED severe asthma

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Figure 1

The Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED) project has described phenotypic differences of severe asthma using a systems biology approach. We obtained three molecular phenotypes termed transcription-associated clusters (TACs) using hierarchical clustering of differentially-expressed transcripts between T2-high and T2-low (1). TAC1 was characterized by receptors IL33R, CCR3 and TSLPR, with the highest enrichment of gene signatures for IL-13/Type-2 (T2) inflammation with sputum eosinophilia, while TAC2 by inflammasome-associated genes, interferon-α (IFN-α) and tumour necrosis factor-α(TNF-α)-associated genes with sputum neutrophilia and TAC3, by metabolic and mitochondrial function genes with pauci-granulocytic inflammation. Given that sputum eosinophilia may vary with time in many asthmatic subjects (2, 3), we hypothesised that TAC status may also change with time.

Of 421 U-BIOPRED subjects with severe asthma at the baseline visit, 321 returned for a second visit at one year and whole sputum samples were obtained by induction with hypertonic saline for differential cell count and for transcriptomic analysis at both visits. All visits were made at a time when the subjects had been free of an exacerbation in the past 4 weeks. Expression profiling was performed using Affymetrix U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) microarray with RNA extracted from sputum cells. In 38 patients who returned for the second visit, we obtained good quality transcriptomic data from sputum cells (4). Hierarchical clustering based on Euclidean distance was performed on the transcriptomic data using the reduced 77 gene-set that defined the original 3 TACs (1). This led to the definition of the same 3 TACs as previously defined with the characteristics of each TAC being preserved. The distribution of sputum eosinophils (EOS) and sputum neutrophils (NEU) counts (reported as mean with 25-75% interquartile range and number) between the 3 TACs remained unchanged compared to that found at their first visit (**TAC1** *first visit*: 44% (24.2-53.4, n=6) EOS, 45.1% (30.2-65.3, n=6) NEU & *second visit*: 28% (15.3-44.5, n=12)

EOS, 41.6% (31.7-65.1, n=12) NEU; **TAC2** *first visit*: 1.4% (0.5-5.0, n=16) EOS , 78.2% (65.9-84.8, n=16) NEU & *second visit:* 2% (0.3-2.8, n=8) EOS, 92.3% (84.9-94.3, n=8) NEU; **TAC3** *first visit*: 2.7% (1.1-10.8, n=16) EOS, 52.3% (40.5-59.4, n=16) NEU & *second visit*: 1.5% (0.2-3.9, n=18) EOS, 50.6% (44.5-62.4, n=18) NEU). Thus, the pattern of sputum granulocytic-defined inflammation within the TACs remained unchanged (**Figure 1A**). Therefore, for the whole group, there was no significant differences in sputum granulocytic composition between baseline and the one-year follow-up.

However, when the data was analysed in terms of individual shifts of TAC between the first and the second visit, as shown on the Sankey flow plot which shows the movement of the granulocytic inflammatory status in relation to the TAC cluster between the baseline and follow-up visit, with the width of the flow proportional to the number of subjects (**Figure 1B**), 21 out of 38 patients remained in the same TAC at one year (5 of 12 in TAC1, 5 of 8 in TAC2 and 11 of 18 in TAC3). In the remainder, 7 TAC1 patients changed to TAC2 (n=4, 33% of baseline TAC1) or TAC3 (n=3, 25% of baseline TAC1), 3 TAC2 subjects moved to either TAC1(n=1, 12% of baseline TAC2) or TAC3 (n=2, 25% of baseline TAC2) while 7 TAC3 subjects changed to TAC2 (61% of baseline TAC3). The kappa statistic (95% confidence interval) was 0.24 (-0.08-0.56), indicating a fair to minimal agreement between TACs at baseline compared to follow-up TACs for matched samples.

We determined whether there were any characteristics measured at the first visit that could distinguish those that remained stable and those that changed TAC status within each of the 3 TAC classes. There were no significant differences in blood or sputum markers of granulocytic inflammation within each TAC shift between those that remained stable and those that moved. For those that shifted from TAC1, there was less allergic rhinitis ($p<0.001$) and eczema ($p=0.0005$), but no differences in inflammatory markers compared to those stable TAC1. For those that changed from TAC2 compared to stable TAC2, they were non-smokers

($p<0.001$) and had less nasal polyps ($p<0.001$) and eczema ($p<0.001$), but more were on oral corticosteroid therapy ($p<0.001$) with higher total serum IgE ($p=0.05$) and a less likelihood of a previous history of pneumonia. For those that changed from TAC3, they had more allergic rhinitis ($p<0.001$), more eczema ($p<0.001$), more oral corticosteroid usage ($p<0.001$) and less history of pneumonia ($p<0.01$) compared to stable TAC3. Therefore, there may be factors that determine the molecular instability of each of the 3 TACs over the one year period.

We used gene set variation analysis (GSVA) to determine the relative expression scores of specific pathways (5) in relation to TAC status using signatures indicative of IL-13-Th2 (6), innate lymphoid Type 2 (ILC2) (7), neutrophil (8), and inflammasome activation (9), and oxidative phosphorylation (10) and senescence signatures (11). We confirmed that TAC2 subjects continued to have the highest expression score for neutrophil and inflammasome activation and TAC3 subjects for OXPHOS and ageing signatures (1) (**Figure 1C**), similar to that described at the baseline visit. However, the IL13/Th2 and ILC2 signature enrichment were reduced in TAC1 subjects at follow-up. Thus, GSVA indicates that with the shift in TAC assignment, the relative importance of certain specific pathways characterising each TAC has changed from baseline.

Although only representative of a small proportion of the original U-BIOPRED severe asthma cohort, we have shown that the molecular phenotypes of severe asthma derived from an analysis of the sputum transcriptome can be unstable at one year in nearly half of the patients. Although TAC assignment was stable in the majority of 55% of patients at one year, but in 45% of the patients, there was a change in the TAC status mainly from TAC1 or TAC3 to TAC2 status. In the 7 subjects who were in TAC1 and who changed into TAC2 or TAC3, the sputum eosinophil count fell from 25.2% to 16.3% ($p<0.003$) indicating that this shift in TAC may be determined by factors that influence eosinophilic inflammation such as

adherence to or use of corticosteroid treatment. Within the TAC2 or TAC3 subjects at baseline, those who were on oral corticosteroids were more likely to switch to other TAC categories. There were no significant differences in asthma therapy between those who remain stable and those who changed TAC status in terms of antibiotic, biologic (anti-IgE antibody) and bursts of systemic corticosteroid use.

The major drawback in our study is the relatively small numbers of subjects studied cross-sectionally. A much larger longitudinal study with a larger number of follow-up patients done at multiple time-points is needed to confirm these findings of instability of transcriptomic clusters. However, this study supports the report of instability of clinical clusters based on clinical, physiologic and biomarker inflammatory data over time (12-15). An unbiased cluster analysis of exhaled metabolomic fingerprint in 78 patients with severe asthma of the U-BIOPRED cohort led to definition of 3 distinct clusters, and the follow-up clustering at one year also showed that 41% of the cluster was stable while 59% moved to other 2 clusters (16). As shown here, these shifts in phenotype were accompanied by appropriate shifts in granulocytic inflammation. Thus, although an asthmatic subject may change TAC status, the composition of each TAC class remains stable with similar inflammatory and transcript profiles. The mechanism(s) driving the molecular switch in some patients remains uncertain from this study, but this may involve the presence of sub-clinical infection, defective resolving mechanisms or perhaps changes in environmental or chronic treatment conditions. Our limited amount of data indicates that patient factors such as the presence of co-morbidities and use of oral corticosteroid therapy may potentially determine the stability of the TAC status. We did not find that the instability was associated with frequent exacerbations. The instability of TACs indicate that targeted biologic therapies that block Type-2 inflammation that would be appropriate for TAC1 phenotype may become less effective in those that shift from TAC1 to TAC2 or TAC3.

Message of study:

At one year, 45% of severe asthma change molecular phenotype as determined by sputum transcriptomic analysis. Together with concomitant shift in sputum granulocytic markers, this may indicate variability of driving mechanisms in this unstable group.

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Conflict of Interest

D. E. Shaw reports personal fees from Novartis, GlaxoSmithKline, and TEVA. S. J. Fowler reports personal fees from AstraZeneca, Boehringer Ingelheim, Novartis, and TEVA outside the submitted work. P. Howarth reports personal fees from GlaxoSmithKline and grants from Boehringer Ingelheim outside the submitted work. Ratko Djukanović has consulted and presented at symposia organised by TEVA, Novartis, GlaxoSmithKline and AstraZeneca, has shares in and consults for Synairgen; Charles Auffray reports grants from Innovative Medicine Initiative; Kian Fan Chung has received honoraria for participating in Advisory Board meetings of the pharmaceutical industry regarding treatments for asthma and chronic obstructive pulmonary disease and has also been remuneratedfor speaking engagements; Ian Adcock has received grants from Advisory Board meetings with pharmaceutical companies GSK, A-Z, Novartis, Boeringher Ingelheim and Vectura, and grants on asthma and COPD from Pfizer, GSK, MRC, EU, BI and IMI; Peter Sterk reports grants from IMI Innovative Medicines Initiative, during the conduct of the study; Matthew Loza and Frederic Baribaud are Employees and Shareholders of Janssen Research and Development, a Johnson and Johnson company; Ana R Sousa are employees of GSK;

Mohib Uddin is an employee of AstraZeneca and holds shares in the company; the rest of the authors have nothing to disclose.

Legend to figure

Figure 1. A. Pie charts showing the distribution of granulocytic inflammation measured in sputum cells in 38 patients with severe asthma at first visit and at follow-up at one year. Eosinophilia defined as eosinophil count ≥ 1.5% and neutrophilia as neutrophil count ≥ 74% ; mixed: neutrophilic and eosinophilic, and paucigranulocytic, neither. **B**. Sankey plot showing the flow of transcription-associated cluster (TAC) membership and sputum granulocyte inflammation at baseline and at one year. Eos: eosinophilic; neu: neutrophilic; mix: mixed eosinophilic and neutrophilic; pauci: paucigranulocytic. **C.** Dot plot relative enrichment scores with box and whisker plots showing median and interquartile range for 6 pathway signatures assessed at baseline (upper panels), and at one year follow-up (lower panels) on sputum transcriptomics using gene set variation analysis. Patient samples are color-based according to baseline cluster membership in the TACs. ILC2: Innate lymphoid cell Type 2; IL-13/Th2: Interleukin-13/T-helper Type 2; OXPHOS: Oxidative phosphorylation.

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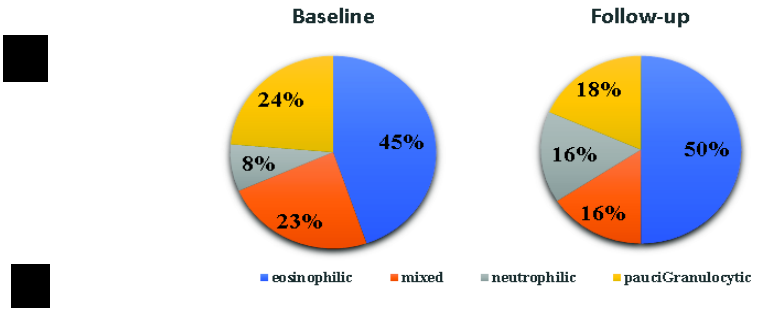
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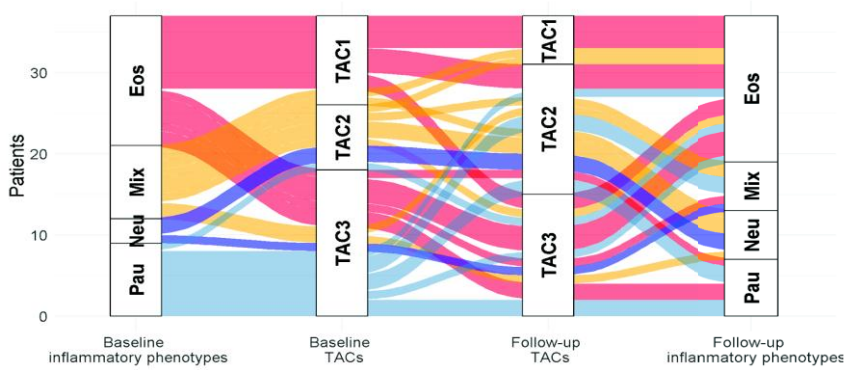
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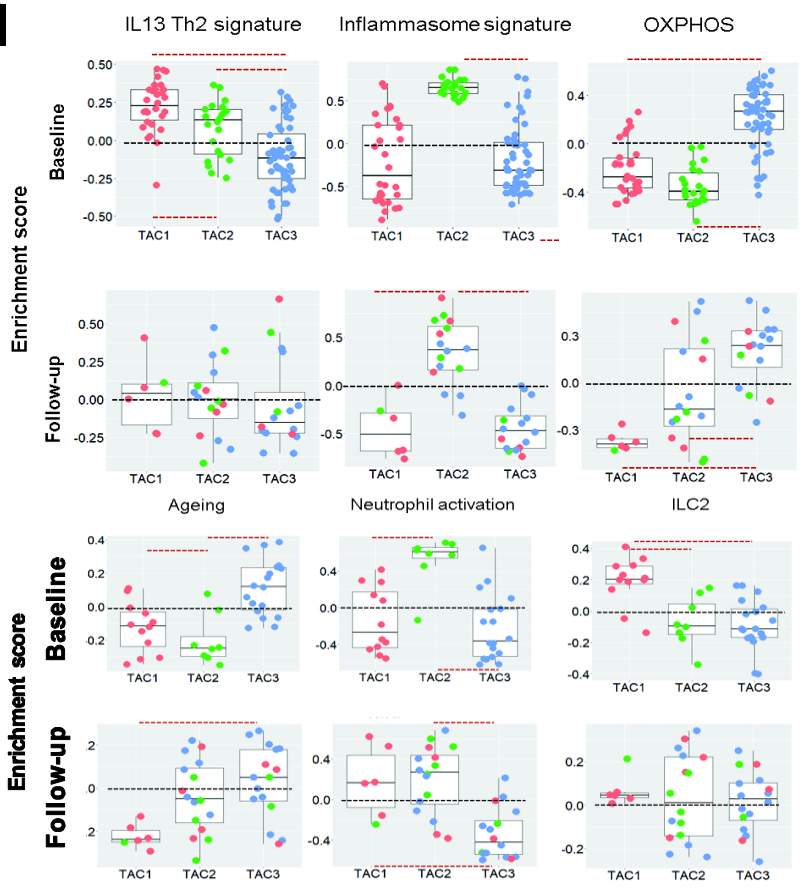
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