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Immune signatures distinguish frequent from non-frequent exacerbators among children with severe asthma.

To the editor,

Asthma is a heterogeneous condition with multiple phenotypes.[1,2] Almost 5% of children with severe asthma (SA) remain symptomatic despite high doses of inhaled corticosteroids (ICS) and other controllers.[3] Asthma has long been regarded as a type-2 (T2) disorder, but a dominant Th1 signature, with Th17 and Th2 cells in a mixed cytokine milieu, was recently described in bronchoalveolar lavages (BAL) from children with SA.[4] Moreover, among children with SA, cellular components such as IL-17-producing mucosal-associated invariant T cells may distinguish frequent (FE) from non-frequent exacerbators (nFE).[5] The inflammatory profiles of children with SA thus appear to be highly complex. We aimed to provide preliminary results to identify immune signatures associated with clinical phenotypes of SA.

Twenty children with SA were included (***supplementary material & table 1***) and their blood and BAL collected. FE (n=13) were defined as children with at least three severe exacerbations (SE) in the previous year and nFE (n=7) as those having one or two.[5] The two groups did not differ, except for the number of steroid bursts. Samples were also collected from control subjects with non-asthmatic (NA) severe respiratory conditions (n=10). Preliminary analysis i) confirmed a mixed T1/T2-type cellular profile in BAL (***figure 1A***), associated with blood eosinophilia (***table 1***), in children with SA relative to NA, and ii) showed the immune profile of FE to differ from that of nFE (T2 *versus* T1 phenotype; respectively, ***figure 1B&C***). We then performed a comprehensive non-targeted analysis of cytokines and cells in blood and BAL from children with SA (***supplementary material***) and then constructed a model to distinguish FE from nFE through supervised analysis (PLS-DA) (***figure 2A***). The model classified patients with a good predictive value (R^2Y=0.799), with both sensitivity and specificity of 100%. Although all data were used to construct the model, we identified a set of constituents that mostly supported the differences between the patient groups (***supplementary table 2,*** VIP>1). In parallel, we performed univariate comparisons of each constituent (***supplementary table 2***). Finally, we identified 11 immune constituents to be the most discriminative (PxVIP plotting, VIP>1 and p<0.1, ***figure 2B***). A higher frequency of ILC1 and Th1-associated chemokines in BAL (CCL2/8, CXCL9-11, ***figure 2C&D***) confirmed the more pronounced T1 phenotype in nFE. Conversely, the local T2 phenotype of FE was associated with a higher frequency of activated Th17 ($IL22^+$) cells in the blood (***figure 2C***) that tended to correlate with the number of exacerbations (ρ=0.41, p=0.08). Previous studies found that the number of circulating Th17 cells was higher in children with moderate-to-severe than mild asthma,[6]

that these cells may be steroid resistant,[7] and that PBMC IL-17 secretion was induced by dexamethasone.[8] Th17 IL-22^{+} cells may therefore emerge after repeated systemic steroid administration received to treat exacerbations, leading to a mixed T2/T17 phenotype. FE also exhibited lower concentrations of total IgA and higher concentrations of TWEAK, TNFSF14, and TSLP in plasma (***figure 2E***), and TLSP levels significantly correlated with the number of exacerbations (ρ=0.45, p=0.045). This is consistent with previous studies showing the involvement of these individual constituents in the pathogenesis and severity of asthma in children. Plasma constituents, such as sTNFR1, CCL26, Pentraxin3, CXCL10, IL-32, and sIL16RB, were also highly discriminant, despite high p-values. All these constituents thus contributed to characterize the FE phenotype and univariate analysis alone would not have allowed their identification. For example, the T2 chemokine CCL26 tended to be higher in FE and significantly correlated with blood eosinophil counts (ρ=0.432, p=0.025). CCL26 is a potent chemoattractant for eosinophils and elevated concentrations of CCL26 in plasma have been shown to be related to mucosal eosinophilia and the severity of various eosinophilic disorders (e.g.[9]), which may suggest tissue eosinophilia in FE.

In conclusion, despite a small sample size and ICS that may affect the immune response, our study shows the potential interest of high-dimensional/non-targeted multivariate analysis to identify specific biological signatures of children with different clinical phenotypes of SA. Although confirmation in an independent cohort is needed, our study provides new leads for delineating asthma pathogenesis and identifying new set of targets for diagnosis and personalized treatment.

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GL, KAP, and MLM: designed the research.

KAP, MG, BG, CDi, TM, MB, and FM: performed the research.

GL, RAT, NG, HF, AN, and CD: were responsible for patient recruitment or establishing the patient database.

KAP and GL: analyzed the data.

KAP, GL, and MLM: wrote the manuscript.

Abbreviations: BAL: bronchoalveolar lavage fluids, ICS: inhaled corticosteroids, NA: non-asthmatic, PLS-DA: partial least squares-discriminant analysis, SA: severe asthma, FE: frequent exacerbators, nFE: non-frequent exacerbators.

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Conflict of interests

The authors have no conflict of interests to declare.

Figure legends

Figure 1. Complex immune profile of children with different SA phenotypes. **A**. Higher frequency of Th1 cells (trend for Th2) in BAL from children with SA (grey bars) than in that from non-asthmatic children with severe respiratory disorders (NA, white bars). **B**. The higher frequency of Th1 cells in BAL of children with SA relative to that in NA children (white bars) was significant only in non-frequent exacerbators (nFE, n=7; light grey). Conversely, the higher number of Th2 cells in BAL (**B**) and eosinophils in blood (**C**) was significant only in frequent exacerbators (FE, n=13; dark grey). Data are shown as box and Tukey whisker plots. P values were obtained using the Kruskal-Wallis test comparing all groups together.

Figure 2. **Identification of immune constituents that discriminate between SA children with frequent (FE) and non-frequent (nFE) exacerbations**. **A.** Graph of the individuals provided by PLS-DA modelling. **B.** VIP x p values plot of all measured immune constituents and selection of the most discriminating and significant (red rectangle: VIP>1 and $p<0.1$; because of the small number of patients in each group, we tolerated $p<0.1$ as a cut-off). Identified discriminant immune constituents within cellular (**C**) or soluble constituents in BAL (**D**) or plasma (**E**), represented as box and Tukey whisker plots. Exact p values (Mann Whitney test) are indicated.

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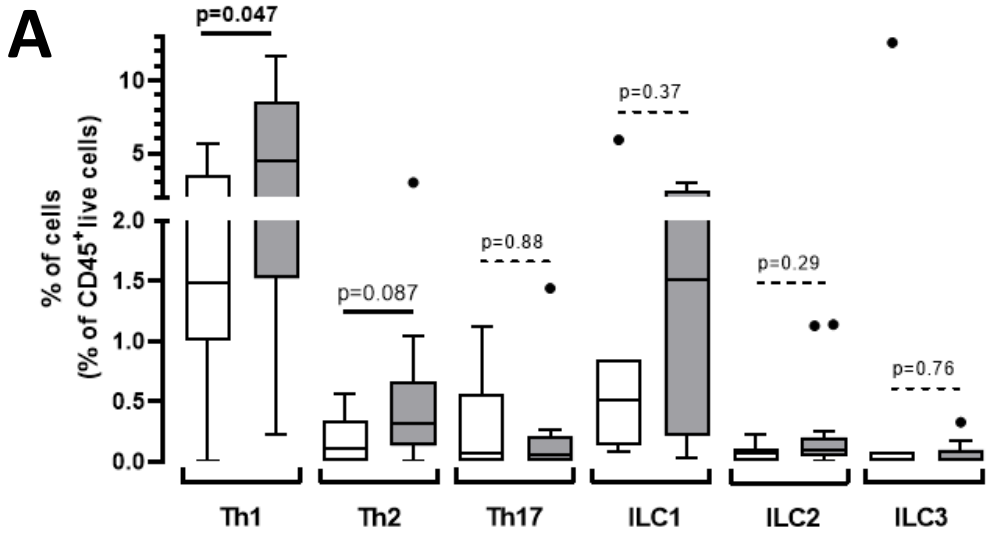
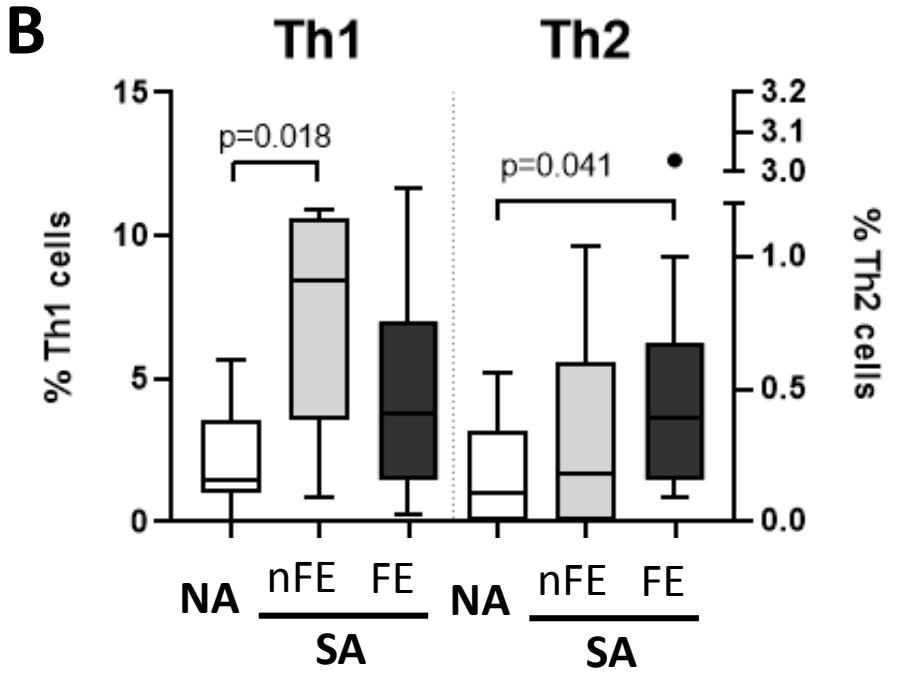

A
p=0.047
p=0.087
p=0.88
p=0.37
p=0.29
p=0.76
% of cells
(% of CD45+ live cells)
10
5
2.0
1.5
1.0
0.5
0.0
Th1
Th2
Th17
ILC1
ILC2
ILC3


B
Th1
Th2
p=0.018
p=0.041
% Th1 cells
% Th2 cells
15
10
5
0
3.2
3.1
3.0
1.0
0.5
0.0
NA
nFE
FE
SA
NA
nFE
FE
SA

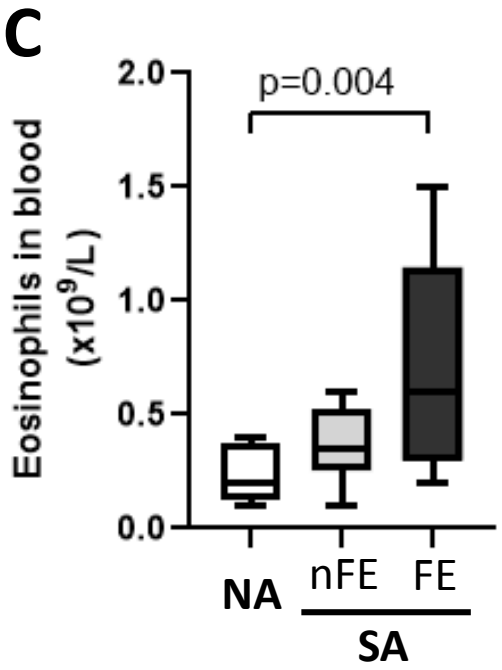
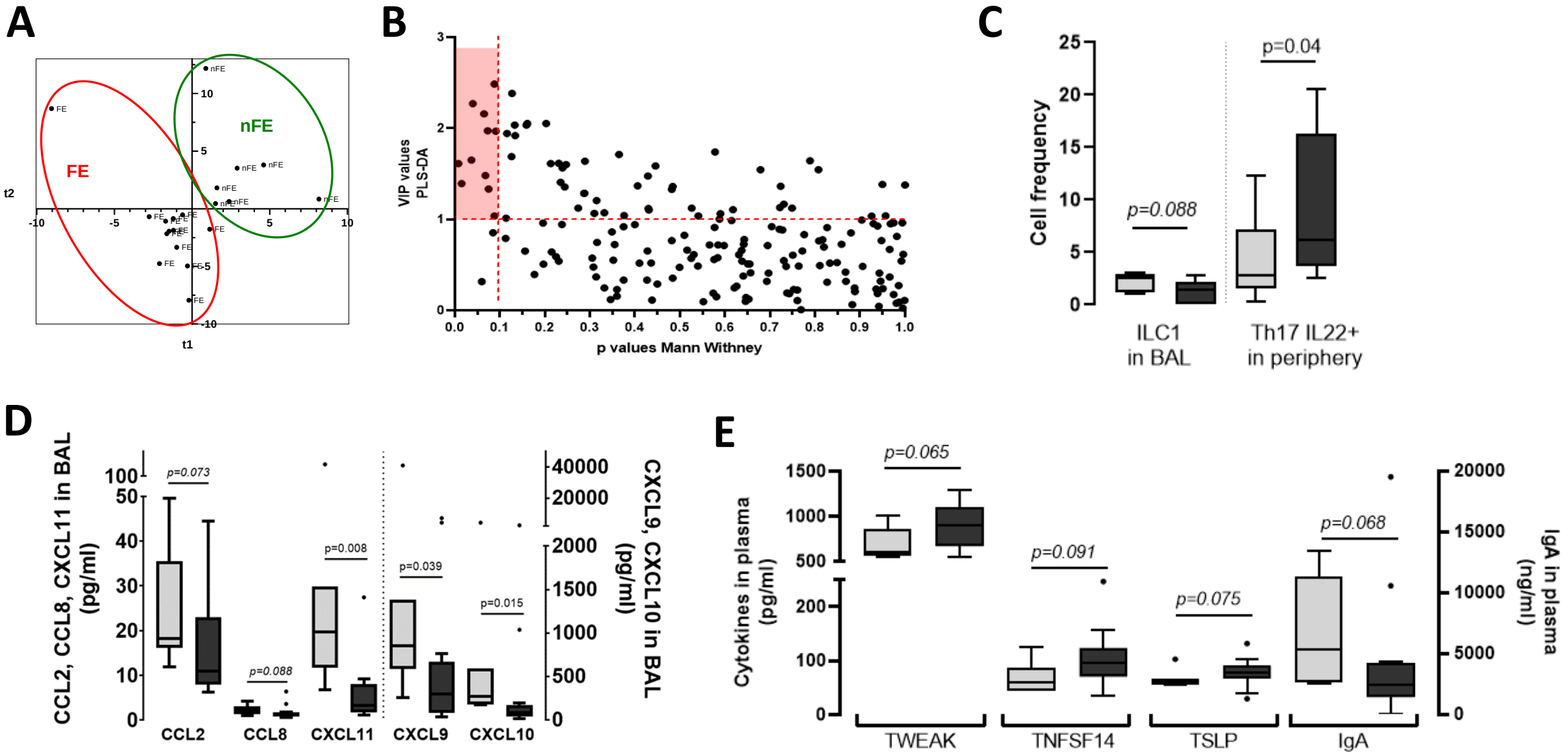

C
p=0.004
Eosinophils in blood
(x10^9/L)
2.0
1.5
1.0
0.5
0.0
NA
nFE
FE
SA

Figure 2


A
FE
nFE
t1
t2
B
VIP values PLS-DA
p values Mann Withney
C
Cell frequency
p=0.088
p=0.04
ILC1 in BAL
Th17 IL22+ in periphery
D
CCL2, CCL8, CXCL11 in BAL (pg/ml)
CXCL9, CXCL10 in BAL (pg/ml)
p=0.073
p=0.088
p=0.008
p=0.039
p=0.015
CCL2
CCL8
CXCL11
CXCL9
CXCL10
E
Cytokines in plasma (pg/ml)
IgA in plasma (ng/ml)
p=0.065
p=0.091
p=0.075
p=0.068
TWEAK
TNFSF14
TSLP
IgA