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RESEARCH LETTER

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Blood cytokines in atopic and non-atopic eosinophilic moderate to severe asthmatics

To the Editor,

The atopic eosinophilic (AT-EOS) and non-atopic eosinophilic (NA-EOS) asthma have a Type 2 high response, with different cell types acting on each phenotype. In AT-EOS asthma, there is a greater participation IL-4, IL-5, IL-13-producing T helper 2 cells along with antibody class switching to IgE, while NA-EOS asthma is linked to innate lymphoid cells 2 producing IL-5 and IL-13.[1] However, there are few studies evaluating the signature of cytokines being produced in serum of individuals with both phenotypes. Thus, the aim of this study is to evaluate the serum levels of cytokines and chemokines in individuals with atopic and non-atopic eosinophilic moderate to severe asthma (MSA). This study was planned to help understanding the immunopathogenesis of MSA in both phenotypes, as well as informing strategies for a more personalised treatment.

For this, we evaluated individuals with MSA and mild asthma (MA), all aged over 18 years. Neither smokers nor pregnant women were included in this study. Individuals with MSA were recruited at the reference centre for severe asthma in Salvador, Bahia, Brazil, called Programa para o Controle de Asma na Bahia (ProAR). The individuals with MA were invited to volunteer by advertising in health facilities and public transportation. All individuals diagnosed with difficult-to-treat severe asthma or treatment-resistant severe asthma according to the criteria adapted from the World Health Organization[2] are part of the MSA group in this study. In addition, only individuals with MSA were being treated with a combination of medium or high dose of inhaled corticosteroids and long-acting beta 2 agonists. The study was approved by Ethics Committee of Maternity Climério of Oliveira, Federal University of Bahia (Licence Number: CONEP n°15,782, CEP 095/2009 and 095/2012).

The atopic subjects were identified by the presence of specific IgE in the serum (≥0.70 KU/L) by the solid-phase fluorescence immunoassay (ImmunoCAP™) (Thermo Fisher Scientific, Uppsala, Sweden) and/or by the positive skin prick test (SPT) (papule ≥3 mm) for at least one aeroallergen. Peripheral blood eosinophilia was considered with eosinophil counts above 260 mm^3.[3] Thus, those that were eosinophilic and atopic simultaneously were called atopic eosinophilic (AT-EOS), and those that were eosinophilic and that were negative for both atopy tests, SPT and specific IgE, were called non-atopic eosinophilic (NA-EOS).

The mensuration of serum cytokines and chemokines was performed using Luminex technology to: EOTAXIN-1, IFN-γ, IL-12 (p70), IL-17A, IL-5, IL-8 and TNF using the HCYTOMAG-60K Assay kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. We defined as high producers of cytokine those individuals with cytokine concentrations above the median, which was based on the concentrations obtained by each asthma group (MA and MSA).

Regarding the characteristics of the study population, we observed that in both phenotypes the median age as well as the frequency of comorbidities was higher in the MSA group compared to the MA group ($p < .05$; data not shown). The percentage of pre-BD FEV1 was lower in the MSA group in both phenotypes (AT-EOS: median 62.2%; NA-EOS: median 59.1%). We did not observe a significant difference in the frequency of females, age at onset of symptoms and eosinophil count in peripheral blood between the groups ($p > .05$; data not shown).

In relation to the serum levels of cytokines/chemokines, we observed, firstly, no significant difference in serum levels of IL-5 (Figure 1A), IL-12 (Figure 1C), IL-17A (Figure 1E) and eotaxin-1 (Figure 1G) between the groups. However, we noticed that among AT-EOS asthmatics, IFN-γ and IL-8 levels were higher in the MSA group [1.4 (0.9–1.9) pg/ml; 2.4 (1.7–3.3) pg/ml, respectively] than in the MA group [1.1 (0.8–1.6) pg/ml; 1.7 (1.0–2.6) pg/ml, respectively] (Figure 1B,F). Furthermore, among NA-EOS asthmatics, TNF levels were lower in the MSA group [4.5 (2.9–5.6) pg/ml] compared to the MA group [6.2 (3.8–9.4) pg/ml] (Figure 1D).

Additionally, we performed sub-analyses to see the chance of finding high levels of cytokines among AT-EOS and NA-EOS MSA in comparison to individuals with MA. We observed that individuals with MSA who have the AT-EOS phenotype are more likely to have high levels of IL-5, IFN-γ and IL-8 (OR 3.5, 1.7, and 1.8, respectively) (Table 1) in comparison to individuals with MA who have the same phenotype. On the other hand, individuals with MSA who exhibit the NA-EOS phenotype are 2 fold more likely to have high levels of IL-5, surprisingly, they are 47% less likely to have high levels of TNF comparing to individuals with MA.

This study aimed to understand which cytokines would be participating in the pathogenesis of asthma in individuals with the atopic eosinophilic and non-atopic eosinophilic phenotype. When we evaluated molecules associated with a Type 2 response, such as eotaxin-1 and IL-5, we did not notice significant differences in the serum levels of either one between the groups. This is expected, since we are comparing asthmatics with eosinophilic phenotypes, and both molecules act mainly on eosinophils. However, we observed that the severity of asthma in both phenotypes (AT-EOS and NA-EOS) is associated with high serum levels of IL-5, but not eotaxin-1. One

study observed a significant reduction in blood eosinophil counts, an increase in the Asthma Control Test score and improvement in lung function parameters in patients with allergic eosinophilic asthma (EA) after use of benralizumab.[4] Given this finding, patients in our study with MSA of both phenotypes should probably benefit from the use of a monoclonal antibody targeted to IL-5 or its receptor, especially those with the AT-EOS phenotype. As for eotaxin-1, it does not seem to play a relevant role in asthma severity in these phenotypes.

Regarding Th1-type cytokine, we observed elevated levels of IFN-γ in subjects with AT-EOS severe asthma. We also noticed an association between asthma severity in AT-EOS and elevated levels of this cytokine. In a study published by our group, in which we evaluated a subsample of these same subjects, we observed that the frequency of $TCD4^{+}IFN\text{-}\gamma^{+}$ cells was higher in individuals with severe asthma refractory who are also atopic, but we did not evaluate for the eosinophilic phenotype.[5] In view of these findings, we can infer that this cytokine may contribute to the severity of asthma in individuals who are atopic eosinophilic.

As for the IL-8 chemokine, we noticed results similar to those observed for IFN-γ. This chemokine has been associated with neutrophilic inflammation and steroid resistance. However, Brooks et al.[6] observed elevated levels of IL-8 in the sputum supernatant in children with EA, and that this phenotype (EA) was also associated with atopy. These data may justify our results, since we also found high levels of IL-8 in MSA with the AT-EOS phenotype.

Key points

- IL-5 is associated with asthma severity in both AT-EOS and NA-EOS subjects.
- IFN-γ and IL-8 contribute to asthma severity in AT-EOS subjects.
- TNF is not associated with asthma severity in NA-EOS subjects.

When we evaluated the cytokine IL-17A, we did not notice any significant differences in our analyses. We found no significant difference in the frequency of TCD4 lymphocytes expressing this cytokine, between atopic and non-atopic asthmatics in a previous study.[5] Despite IL-17 being a cytokine associated with asthma severity, the use of brodalumab, an anti-IL-17 receptor human monoclonal antibody, had no effect on clinical symptoms in patients with MSA.[7] Thus, the role of IL-17A in asthma remains controversial, or it may even be a cytokine that does not play an important role in the pathogenesis of this disease.

There are few studies on the role of IL-12 in asthma, mainly correlating this cytokine with the phenotypes of the disease. In our work, we did not observe any significant differences in the levels of this cytokine in asthmatic subjects. However, we found elevated levels of IFN-γ in individuals with MSA who are AT-EOS, and IL-12

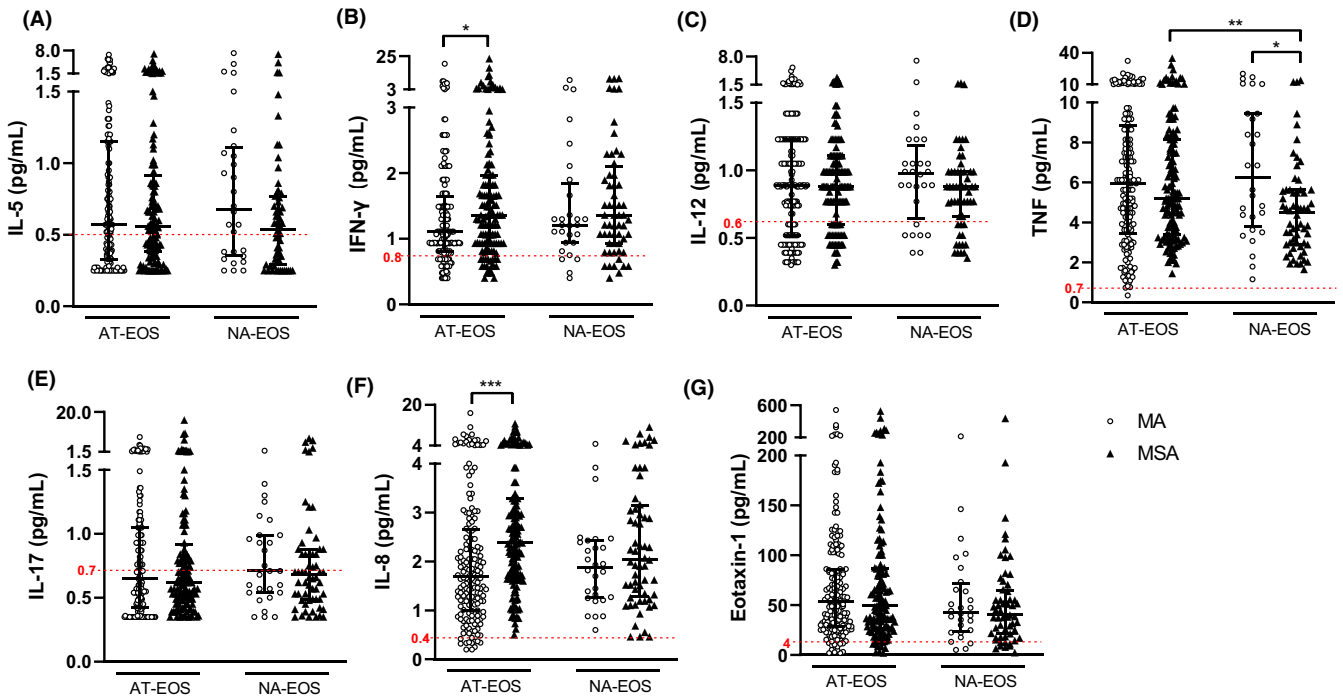

FIGURE 1 Serum levels of the cytokines IL-5 (A), IFN-γ (B), IL-12 (C), TNF (D) and IL-17 (E) and of the chemokines IL-8 and eotaxin-1 (F and G, respectively) in individuals with MA and MSA who present the atopic-eosinophilic (AT-EOS) and non-atopic-eosinophilic (NA-EOS) phenotypes. The bar represents the interquartile range and the red dashed line corresponds to minimum detectable concentrations. AT-EOS: MA ($n = 172$) and MSA ($n = 132$); NA-EOS: MA ($n = 28$) and MSA ($n = 60$). $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .0001$ (Mann Whitney)

TABLE 1 Associations between cytokines levels and asthma phenotypes

	High eotaxin-1		High IL-5		High IL-12		High IFN-γ		High IL-17		High IL-8		High TNF	
	Unadjusted OR [95% CI]	Adjusted[a] OR [95% CI]	Unadjusted OR [95% CI]	Adjusted[a] OR [95% CI]	Unadjusted OR [95% CI]	Adjusted[a] OR [95% CI]	Unadjusted OR [95% CI]	Adjusted[a] OR [95% CI]	Unadjusted OR [95% CI]	Adjusted[a] OR [95% CI]	Unadjusted OR [95% CI]	Adjusted[a] OR [95% CI]	Unadjusted OR [95% CI]	Adjusted[a] OR [95% CI]
AT-EOS severe asthma	1.00 (0.69–1.45)	0.93 (0.64–1.36)	**3.29 (2.18–4.97)**	**3.53 (2.32–5.38)**	0.86 (0.59–1.25)	0.90 (0.62–1.31)	**1.72 (1.18–2.50)**	**1.76 (1.20–2.58)**	0.80 (0.55–1.16)	0.82 (0.56–1.19)	**2.00 (1.36–2.95)**	**1.87 (1.27–2.77)**	1.13 (0.78–1.64)	1.11 (0.76–1.62)
NA-EOS severe asthma	0.74 (0.44–1.27)	0.70 (0.40–1.20)	**1.79 (1.04–3.09)**	**2.13 (1.22–3.70)**	0.66 (0.39–1.13)	0.70 (0.40–1.20)	1.48 (0.87–2.52)	1.43 (0.83–2.46)	1.10 (0.65–1.86)	1.11 (0.65–1.89)	1.15 (0.68–1.95)	0.96 (0.56–1.65)	**0.52 (0.30–0.90)**	**0.53 (0.30–0.93)**

Note: High level of cytokine/chemokine was considered values above the median. Atopic individuals were defined by skin prick test and/or specific IgE for at least one aeroallergen. Bolded results are statistically significant at $p < .05$.

Abbreviations: AT-EOS, Atopic eosinophilic; NA-EOS, Non-atopic eosinophilic.

[a]OR [95% CI] was adjusted for age, sex and comorbidities (systemic arterial hypertension, diabetes mellitus and dyslipidemia).

is an important cytokine that acts in the production of this cytokine. Some studies, mainly involving children, have not observed a difference in IL-12 levels between children with MSA and healthy controls.[8]

Regarding the TNF cytokine, we observed decreased levels in individuals with severe asthma NA-EOS, and these individuals are almost 50% less likely to have elevated levels of this cytokine. TNF has been mainly associated with neutrophilic asthma, and Manise et al.[9] observed elevated levels of TNF in the sputum supernatant in atopic asthmatics with high levels of IgE. Together, these data reinforce the finding of our work, as we noticed low levels of TNF in NA-EOS severe asthmatics and similar levels between AT-EOS MA and MSA.

Most studies that have evaluated the population of asthmatics with a certain phenotype have not compared the severity of the disease. Our study brings this analysis, however, studies using mainly other more representative samples, such as sputum or BAL, are needed to further clarify our findings.

Thus, we conclude that asthma severity in both phenotypes (AT-EOS and NA-EOS) is associated with high levels of IL-5. Likewise, the cytokines IFN-γ and IL-8 also seem to contribute to the severity of the disease in individuals with the AT-EOS phenotype.

AUTHOR CONTRIBUTIONS

J.S.F., L.S.C, C.A.F. and A.A.C. contributed equally to this work; J.S.F., C.A.F., E.M.C. and A.A.C. designed the study; J.S.F, G.P.P.C. and C.V.N.S recruited the population of study; J.S.F., N.V.Q.C., A.M.A. and I.S.O. performed the experiments and/or analysed the data; J.S.F., L.S.C., C.A.F. and A.A.C. wrote the manuscript.

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CONFLICT OF INTEREST

All authors declare that there are no conflicts of interests to report in relation to this manuscript.

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