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ORIGINAL RESEARCH

Early Detection of Asthma: Exploring Inflammatory Biomarkers in Symptomatic Adults with Normal Spirometry

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Introduction: We previously showed that individuals without a prior history of asthma, presenting with unexplained respiratory symptoms and normal spirometry, may exhibit airway hyperresponsiveness and underlying eosinophilic (T2) inflammation, features suggestive of undiagnosed early-stage asthma. Improving our understanding of the inflammatory processes that contribute to asthma onset is essential, as it may ultimately lead to earlier detection, timely intervention and improved long-term outcomes.

Purpose: This study aimed to evaluate several key inflammatory biomarkers in this well-characterized population and examine their associations with clinical presentation to identify early signs of asthma.

Patients and Methods: This retrospective, observational cohort sub-study included Canadian adults with respiratory symptoms and normal pre- and post-bronchodilator spirometry. Demographics and clinical data were extracted from study files. Plasma and serum levels of biomarkers associated with T2 airway inflammation and epithelial shedding, including IL-4, IL-5, IL-13, IL-25, IL-33, eotaxin, eotaxin-3, TARC, periostin and TNF-α, were measured using ELISA and multiplex electrochemiluminescent assays. Airway hyperresponsiveness was defined as a PC_{20} <16 mg/mL, and T2 airway inflammation as sputum eosinophils >2% and/or FeNO >25 ppb.

Results: Among 128 adults (mean age ±SD: 58.0 ±13.9 years, 52% women), 45 (35%) had T2 airway inflammation. Most biomarker levels were low or undetectable, with substantial inter-individual variability. No significant differences in biomarker levels were observed between individuals with and without airway hyperresponsiveness or T2 airway inflammation. Eotaxin levels negatively correlated with post-bronchodilator FEV_1/FVC ratio (r=−0.18, P=0.0433), and eotaxin-3 positively correlated with FeNO (r=0.18, P=0.0482).

Conclusion: This panel of clinically accessible T2 biomarkers may not reliably reflect early pathophysiological signs of asthma in symptomatic adults with normal spirometry. Longitudinal follow-up of this cohort, along with the integration of airway sampling, may provide further insight into the role of these biomarkers in asthma development and progression.

Plain Language Summary: Asthma is a common lung disease characterized by inflammation in the airways. Although inflammation is the body's natural response to harmful triggers, in asthma it becomes exaggerated and persistent. Over time, this can lead to structural and functional changes in the airways, causing symptoms like coughing, wheezing, and shortness of breath. Diagnosing asthma in its early stages can be difficult, as symptoms are often mild and lung function tests may appear normal. As a result, asthma may go undetected, delaying treatment, and increasing the risk of severe respiratory events and hospitalizations. Certain molecules in the blood, called "biomarkers", can reflect the biological processes involved in asthma, especially in more advanced stages. However, it is unclear whether these same biomarkers can help detect asthma earlier in the process—before it becomes detectable through standard lung function tests. In this study, we measured several inflammatory biomarkers in the blood, including key molecules like IL-4, IL-5, eotaxin, eotaxin-3, and others, and examined their relationship with clinical presentation in adults with unexplained

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respiratory symptoms and normal lung function tests results. Most biomarkers were either found at low levels or not detected at all. We found no clear differences in biomarker levels between individuals with and without signs of airway inflammation. Only two biomarkers, eotaxin and eotaxin-3, showed weak but statistically significant associations with certain clinical parameters. These findings suggest that blood tests using this panel of biomarkers may not reliably detect early signs of asthma in this at-risk population.

Keywords: asthma, diagnosis, airway inflammation, biomarkers, cytokines, chemokines

Introduction

Asthma is a common respiratory disease characterized by chronic airway inflammation.[1] Although its prevalence varies widely worldwide, ranging from 0.2% to 21.0%, population-based studies show that 20–70% of cases remain undiagnosed and untreated.[2] Underdiagnosis is a major health problem associated with poor quality of life, loss of work productivity and increased risk of hospitalizations.

Asthma is diagnosed based on respiratory symptoms together with physiological evidence of variable expiratory airflow limitation.[1] Spirometry is the gold standard test for assessing airway obstruction, but its access is often limited.[3] Bronchial challenge testing is an alternative, though even less accessible. Interpretation of spirometry can be challenging, especially in mild cases, as lung function may appear normal during asymptomatic periods.[4]

Airway inflammation is considered the cornerstone of asthma pathophysiology. It involves complex and interactive cascades mediated by various proinflammatory cytokines and chemokines, including a type 2 (T2, eosinophilic) pattern of injury, driving airway hyperresponsiveness (AHR) and obstruction that lead to its clinical characteristics.[5]

T2 airway inflammation is found at an early, potentially pre-clinical stage, and can contribute to asthma development. Jansen et al previously reported that AHR is associated with a greater risk of developing asthma, particularly if accompanied with airway eosinophilia.[6] Longitudinal follow-up of patients with non-asthmatic eosinophilic bronchitis, a condition characterized by eosinophilic airway inflammation without AHR, showed that about 6–10% tend to develop airway obstruction and symptoms consistent with an asthma diagnosis over time.[7–9]

We previously showed that T2 airway inflammation and AHR are underdiagnosed in a significant proportion of symptomatic adults in the community with normal pre- and post-bronchodilator spirometry.[10] In this population, 36% had AHR, with or without coexistent T2 airway inflammation, and 19% showed clinical signs of non-asthmatic eosinophilic bronchitis. Given that some patients presenting with AHR and/or T2 airway inflammation may have underlying mild asthma or another condition that predisposes to its development, early detection should be a priority.

Non-invasive biomarkers of T2 airway inflammation, including eosinophil count (blood and sputum), total and specific immunoglobulin E (IgE) and fractional exhaled nitric oxide (FeNO) have a well-established role in severe asthma and may be clinically useful in early-stage disease.[11,12] However, a broader panel of T2 biomarkers is needed to more accurately detect the early cellular and molecular signals of disease onset.

T2 cytokines and chemokines, including IL-4, IL-5, IL-13, IL-25, IL-33, eotaxin, eotaxin-3 and TARC are key drivers of asthma pathophysiology, and are frequently detected in serum of individuals with uncontrolled or severe disease.[5,13] As such, these mediators have been used as both biomarkers and therapeutic targets in severe asthma populations.[14–16] Evaluating these biomarkers in individuals with milder disease offers a unique opportunity to explore whether systemic inflammation is already present early in disease development. Improving our understanding of the inflammatory and physiological changes preceding asthma onset is crucial as it may ultimately lead to earlier intervention and improved long-term outcomes.

Therefore, this study aimed to characterize blood levels of several key biomarkers associated with T2 airway inflammation and examine their associations with clinical presentation in symptomatic adults with normal spirometry and no previous diagnosis of asthma.

Materials and Methods

Study Design

This was a multicenter, retrospective, observational cohort sub-study, using data and samples from the Underdiagnosed COPD and Asthma Population (UCAP) study.[17] Approval from each local ethics committee was obtained, including the Institut universitaire de cardiologie et de pneumologie de Québec – Université Laval ethics committee (CER 21710). All subjects provided written informed consent. This study complies with the Declaration of Helsinki.

Study Population

In the UCAP study, adults ≥18 years of age with respiratory symptoms and without any history of diagnosed lung disease were recruited across Canada, between June 2017 and March 2020, in a two-step case-finding process using random digit-dialing of landlines and cell phones within a 90-min radius of each participant site. In the first study visit, participants were assessed for COPD, asthma, or no airway obstruction using pre- and post-bronchodilator spirometry. Subjects who showed no evidence of airway obstruction were invited to participate in the UCAP sub-study and return for a second visit where they underwent measurement of FeNO, methacholine challenge testing, and blood and induced sputum collection. Measurement of biomarkers was included in the consent form signed by each study participant and ethics approval.

The present study included collected data and blood samples of all subjects from the UCAP sub-study cohort who presented with respiratory symptoms and normal pre- and post-bronchodilator spirometry, and for whom a sample was available for biomarkers measurement. Detailed inclusion and exclusion criteria were previously described.[10]

Collection of Data

Data were collected at each site following standard operating procedures, including demographics, smoking history, family history of asthma, atopy and comorbid conditions.

Respiratory symptoms were assessed using the Asthma Screening Questionnaire (ASQ).[18] Atopy was identified from skin prick tests and FeNO was measured following the American Thoracic Society recommendations.[19]

Lung function was assessed using spirometry. Airway obstruction was defined as any of the following criteria: a) pre-bronchodilator forced expiratory volume in one second (FEV_1) ≤80% predicted, or b) pre-bronchodilator FEV_1/forced vital capacity (FVC) ≤0.7 or ≤LLN, or c) FEV_1 response to 400 mcg inhaled salbutamol ≥12% or ≥200 mL.

Airway responsiveness was determined from methacholine challenge test and results were expressed as the provocative concentration causing a 20% fall in forced expiratory volume in one second (FEV_1) from baseline (PC_{20}).[20] AHR was defined as a PC_{20} methacholine <16 mg/mL.

Blood eosinophils and total IgE were assessed. Sputum was induced and differential cell count including eosinophils, neutrophils, macrophages, lymphocytes and bronchial epithelial cells was performed by an experienced lab technician using modified methods adapted from Pizzichini et al.[21] T2 airway inflammation was defined as sputum eosinophils >2%[22] and/or FeNO >25 ppb.[19]

Biomarker Assessments

Biomarkers were measured from plasma and serum samples. Plasma was collected from anticoagulant (EDTA) tubes following centrifugation, while serum was obtained from clot activator tubes after coagulation and centrifugation. All samples were aliquoted and immediately frozen at −80°C after collection. Sample handling and storage adhered to standard biobanking protocols to preserve analytes integrity and minimize degradation. Biomarker assays were performed 24–36 months after collection.

Biomarkers associated with T2 airway inflammation and epithelial shedding, including IL-4 (Abcam, Cambridge, UK), IL-5 (Abnova, Taipei, Taiwan), IL-13 (ThermoFisher, Waltham, USA), IL-25 (Abnova, Cambridge, UK), IL-33 (ThermoFisher, Waltham, USA), eotaxin, eotaxin-3, TARC, periostin, and TNF-α (all from R&D Systems, Minneapolis, USA), were initially measured in plasma samples using classic enzyme-linked immunosorbent assays (ELISA). With a majority of values falling below the lower limit of detection (LLOD), a second series of measurements was performed from serum samples using multiplex electrochemiluminescent

assays (Meso Scale Discovery, Rockville, USA) to achieve greater detection sensitivity. All measurements were performed in duplicate according to the manufacturer's recommendations. Values below the LLOD were considered "not detected" and therefore excluded from the analysis to avoid bias in parameter estimates.

Statistical Analysis

Baseline demographics, clinical characteristics and biomarker concentrations were analyzed using descriptive statistics. Continuous variables were reported as means (95% CI) and dichotomous or categorical variables as numbers (%). Log transformed data were presented as geometric means with 95% CIs of the estimates. Subjects were divided in three groups according to PC_{20} categories: 1) PC_{20} <4 mg/mL, 2) PC_{20} 4–15.9 and 3) PC_{20} ≥16 mg/mL. Clinical characteristics and biomarker levels were compared between subjects with AHR and/or T2 airway inflammation and those without. For comparison of multiple groups characterized by continuous parametric and non-parametric variables, the one-way analysis of variance (ANOVA) or Kruskal–Wallis test was used, followed by the Tukey-Kramer's multiple comparison test when appropriate. The χ^2 and Fisher's exact tests were used to compare proportions. The Pearson correlation was used to explore correlations between the data. All analyses were conducted by an experienced biostatistician using SAS version 9.4 (SAS Institute, Cary, NC, USA). A biomarker detection rate of at least 50% was required for inclusion in the analysis.

Results

Population

Of the one hundred thirty-two subjects identified from the UCAP cohort, blood samples were available in 130. Among these, two subjects were excluded as their blood sample were unsuitable for biomarker measurement. Hence, a total of 128 subjects were included in the analyses, and their clinical characteristics are shown in Table 1.

Table 1 Characteristics of subjects according to PC_{20} category

	All Subjects (n=128)	**PC_{20}**			**P-value**
		<4 mg/mL (n=20)	**4-15.9 mg/mL (n=27)**	**≥16 mg/mL (n=85)**	
Sex (female)	67 (52)	13 (65)	20 (74)	34 (42)	*0.008
Age (years)	58 (55–60)	56 (49–63)	56 (51–61)	59 (56–62)	0.546
BMI (kg/m^2)	30.2 (29.1–31.3)	31.3 (28.4–34.2)	30.9 (28.2–33.6)	29.6 (28.4–30.8)	0.426
Smoking status					
Never-smoker	62 (49)	8 (40)	13 (48)	41 (51)	
Ex-smoker	47 (37)	6 (30)	11 (41)	30 (37)	
Current smoker	19 (15)	6 (30)	3 (11)	10 (12)	
Comorbidities					
Atopic dermatitis	21 (16)	4 (20)	2 (7)	15 (19)	0.397
Allergic rhinitis	20 (16)	6 (30)	2 (7)	12 (15)	0.122
Nasal polyposis	4 (3)	0 (0)	0 (0)	4 (5)	0.460
GERD	46 (36)	6 (30)	9 (33)	31 (38)	0.803
Atopy (n=116)	64 (55)	13 (72)	14 (54)	37 (51)	0.306
Total IgE (KU/L)	32.1 (31.8–32.4)	65.7 (65.0–66.3)	39.4 (38.5–40.3)	26.1 (25.7–26.5)	0.068

(*Continued*)

Table 1 (Continued).

	All Subjects (n=128)	PC_{20}			P-value
		<4 mg/mL (n=20)	4-15.9 mg/mL (n=27)	≥16 mg/mL (n=85)	
FeNO (ppb)	15.4 (15.3–15.5)	15.4 (15.0–15.8)	15.4 (15.0–15.8)	17.2 (17.0–17.4)	0.957
Sputum neutrophils (%)	46.6 (40.8–52.4)	40.1 (27.8–52.4)	32.1 (18.3–45.9)	52.8 (45.7–59.9)	*0.014
Sputum eosinophils (%)	0.9 (0.6–1.2)	2.1 (0.7–4.9)	0.7 (0.3–1.4)	0.7 (0.6–1.4)	*0.035
Blood eosinophils (cells×10^9/L)	120.5 (120.3–120.7)	199.3 (199.0–199.6)	108.9 (108.3–109.5)	108.9 (108.6–109.2)	*0.014
FEV_1 pre-BD (% pred)	98.4 (96.1–100.5)	93.0 (89.3–96.7)	96.4 (93.2–99.6)	100.3 (97.2–103.4)	*0.012
FVC pre-BD (% pred)	100.0 (97.7–102.3)	97.5 (89.1–105.8)	100.6 (93.6–107.5)	100.4 (97.2–103.5)	0.741
FEV_1/FVC pre-BD	77.8 (77.1–78.5)	76.9 (75.2–78.6)	77.8 (75.9–79.7)	78.1 (77.3–79.0)	0.505
ΔFEV_1 (%)	2.7 (1.9–3.6)	3.9 (2.4–5.5)	4.1 (0.7–7.6)	2.0 (1.2–3.6)	0.174
ASQ Score	8.9 (8.3–9.5)	8.8 (7.0–10.6)	8.7 (7.6–9.8)	9.0 (8.2–9.8)	0.936

Notes: Results are presented as mean (95% confidence interval) for continuous variables and as N (%) for dichotomic variables. Comparisons between groups were performed using ANOVA (continuous variables) or Fisher's exact test (dichotomic variables). *P-value <0.05 was considered statistically significant.

Abbreviations: ASQ, asthma screening questionnaire; BD, bronchodilator; BMI, body mass index; FEV_1, forced expiratory volume in one second; ΔFEV_1, Percentage change in FEV_1 after bronchodilator; FVC, forced vital capacity; FeNO, fractional exhaled nitric oxide; GERD, gastro-esophageal reflux disease; IgE, immunoglobulin E; PC_{20}, provocation concentration causing a 20% fall in FEV_1; Ppb, parts per billions; T2, type 2 inflammation.

Biomarker Levels

Overall, biomarker levels were found in low concentrations in serum (or plasma), with a considerable number of values below the LLOD for IL-4, IL-5, IL-13, IL-25 and IL-33 (58% to 85% of samples, as demonstrated in Table 2). Only five biomarkers met the minimum detection rate for statistical analysis: eotaxin, eotaxin-3, TARC, TNF-α (all measured with MSD) and periostin (measured with ELISA). A color matrix was generated to better visualize the distribution of biomarker profiles in relation to different clinical characteristics (Figure 1)

Differences in Biomarker Levels According to Clinical Presentation

We first compared biomarker levels according to PC_{20} category. As shown in Figure 2, there were no statistically significant differences in the levels of eotaxin, eotaxin-3, TARC, periostin and TNF-α between any of the groups.

Table 2 T2 biomarkers and % of detected samples in serum (or plasma)

Analyte (LLOD in pg/mL)	% Detected samples (n=128)
IL-4 (0.08)	30%
IL-5 (0.24)	15%
IL-13 (3.10)	35%
IL-25 (1.26)	42%
IL-33 (0.90)	33%

(*Continued*)

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Table 2 (Continued).

Analyte (LLOD in pg/mL)	% Detected samples (n=128)
Eotaxin (3.20)	100%
Eotaxin-3 (7.30)	100%
TARC (0.32)	100%
Periostin (10.00)	100%
TNF-α (0.39)	98%

Notes: IL-33 and periostin were measured in plasma samples using ELISA.
Abbreviations: LLOD, lower limit of detection; T2, Type 2 inflammation, defined as sputum eosinophils ≥2% or FeNO ≥25 ppb.

Then, we subdivided the three groups of subjects according to their inflammatory profile (T2 vs non-T2) and compared their biomarker levels. Once again, no statistically significant differences were found between any of the subgroups (Figure 2). Considerable inter-individual variation in biomarker levels was observed within the subgroups of subjects as shown in Table 3.

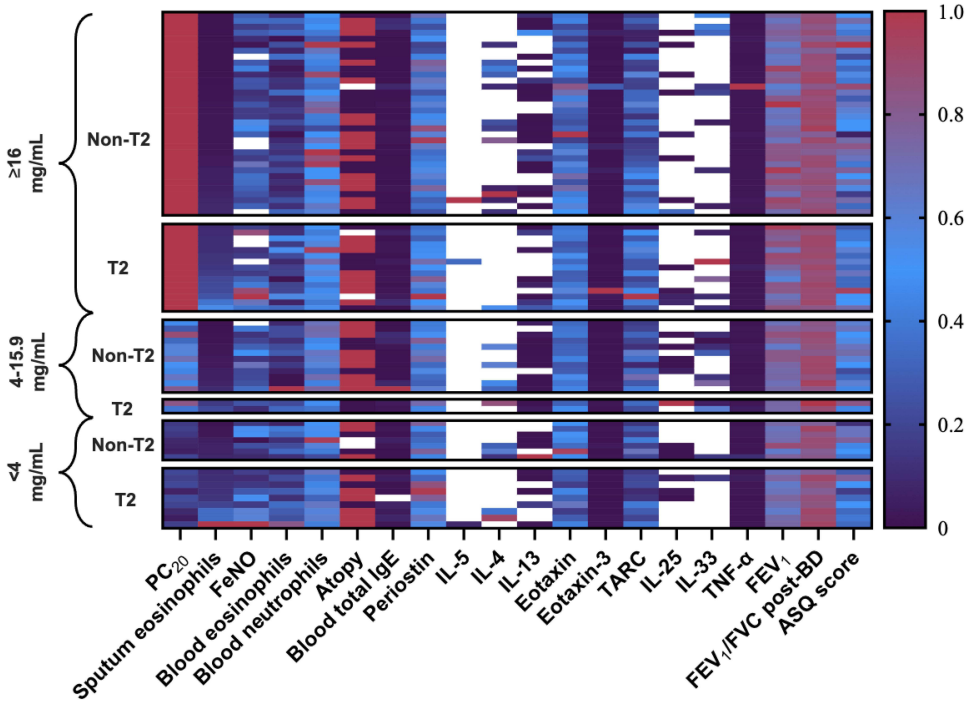

Figure 1 Heatmap of subjects' biomarker levels and clinical characteristics stratified by PC_{20} and inflammatory profile. Each row represents an individual subject, and each column represents a variable. Subjects were stratified by PC_{20} values (PC_{20} <4, 4–15.9, and ≥16 mg/mL) and inflammatory profile (T2 or non-T2). Variables include sputum eosinophils (%), sputum neutrophils (%), FeNO (ppb), blood eosinophils (cells×10^9/L), blood neutrophils (cells×10^9/L), atopy, blood total IgE (KU), plasma periostin (pg/mL), serum IL-5 (pg/mL), serum IL-4 (pg/mL), serum IL-13 (pg/mL), serum eotaxin (pg/mL), serum eotaxin-3 (pg/mL), serum TARC (pg/mL), serum IL-25 (pg/mL), plasma IL-33 (pg/mL), serum TNF-α (pg/mL), FEV_1 (% predicted), post-bronchodilator FEV_1/FVC ratio, and Asthma Screening Questionnaire (ASQ) score. Each variable has been normalized to its maximum value, ranging from 0 (dark blue) to 1 (bold red).
Abbreviations: PC_{20}, provocative concentration of methacholine causing a 20% fall in forced expiratory volume; T2, Type 2 inflammation, defined as sputum eosinophils ≥2% or FeNO ≥25 ppb

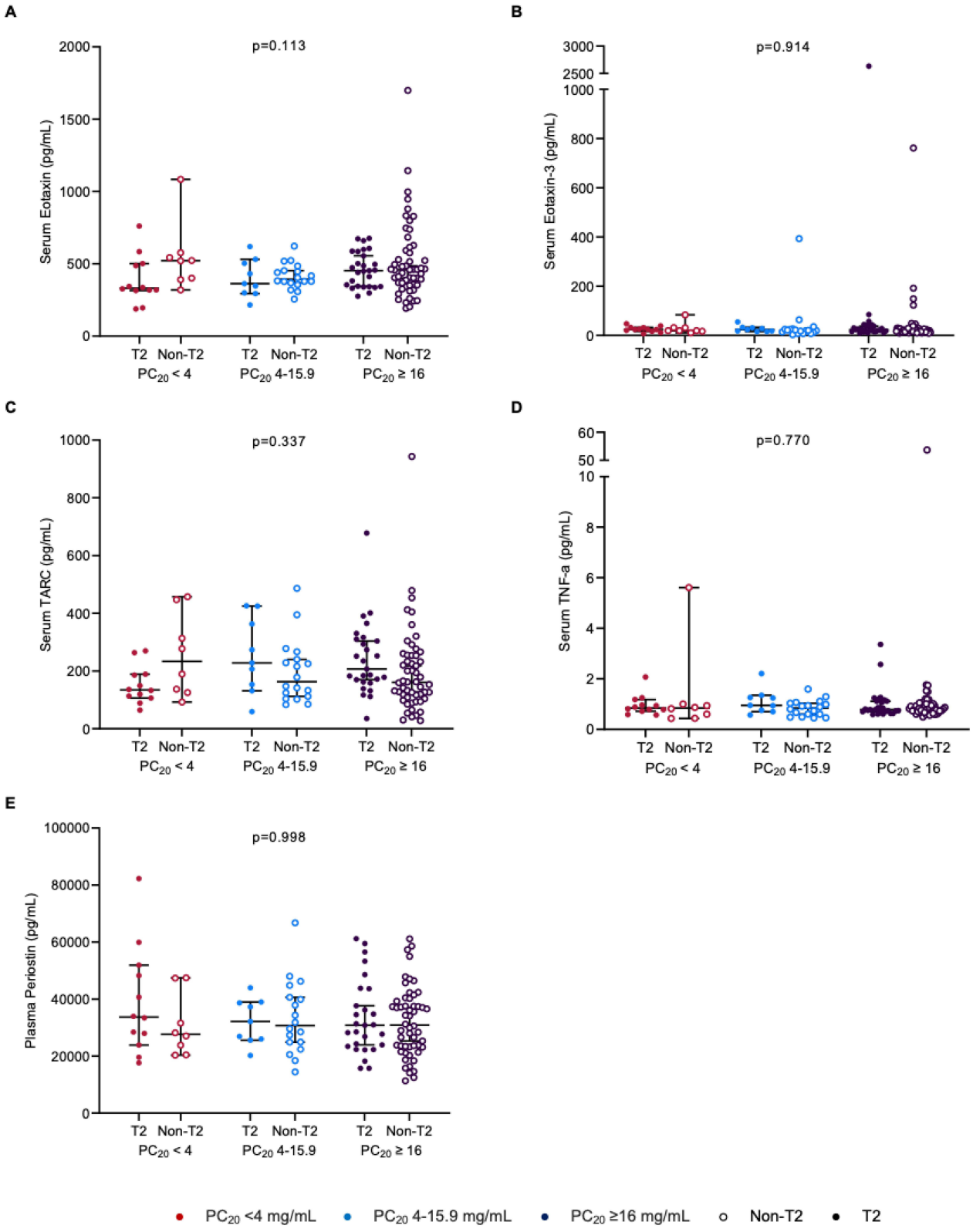

Figure 2 Serum or plasma levels of (**A**) eotaxin, (**B**) eotaxin-3, (**C**) TARC, (**D**) TNF-α and (**E**) periostin according to PC_{20} and inflammatory profile. Subjects were stratified by PC_{20} values (<4, 4–15.9, and ≥16 mg/mL) and by inflammatory profile (T2 or non-T2). T2 subjects are represented by closed circles; non-T2 subjects by open circles. Data are expressed as median ± interquartile range. Comparisons were performed using one-way ANOVA or Kruskal–Wallis tests as appropriate; p-values for each biomarker comparison are shown in the top of each panel.

Abbreviations: PC_{20}, provocative concentration of methacholine causing a 20% fall in forced expiratory volume; T2, Type 2 inflammation, defined as sputum eosinophils ≥2% or FeNO ≥25 ppb.

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Table 3 Biomarker levels according to PC_{20} category and inflammatory profile

Analyte (pg/mL)	PC_{20} <4 mg/mL		PC_{20} 4–15.9 mg/mL		PC_{20} ≥16 mg/mL		P-value
	T2 (n=12)	Non-T2 (n=8)	T2 (n=7)	Non-T2 (n=20)	T2 (n=26)	Non-T2 (n=55)	
Eotaxin	361.4 (278.7–468.7)	512.9 (376.2–692.3)	350.7 (257.2–473.4)	415.7 (376.2–459.4)	437.0 (391.5–492.8)	464.0 (411.6–523.22)	0.113
Eotaxin-3	23.1 (16.8–32.1)	24.3 (14.7–40.5)	23.1 (15.2–35.2)	21.1 (13.5–32.8)	28.5 (17.5–46.6)	24.5 (20.1–30.3)	0.914
TARC	140.3 (106.7–183.4)	219.6 (132.8–360.5)	219.6 (112.2–423.5)	175.9 (140.0–221.4)	204.2 (159.0–262.9)	162.6 (134.3–196.5)	0.337
Periostin	35242.2 (26370.5–47572.0)	29143.9 (22026.5–38561.1)	31257.0 (24100.8–40538.2)	31257.0 (26370.5–37049.1)	32532.7 (27722.5–38177.4)	29732.6 (26903.2–33189.9)	0.770
TNF-α	0.9 (0.7–1.1)	0.9 (0.5–1.8)	0.9 (0.7–1.2)	0.9 (0.7–1.0)	0.9 (0.7–1.1)	0.9 (0.8–1.1)	0.998

Notes: Log transformed data are presented as geometric means with 95% CIs of the estimates. Comparisons between groups were performed using ANOVA.
Abbreviations: PC_{20}, provocation concentration causing a 20% fall in FEV_1; T2, type 2 inflammation.

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Correlations Between Biomarker Levels and Clinical Characteristics

We further explored the associations between biomarker levels and different clinical characteristics, including PC_{20}, lung function parameters, FeNO, total IgE levels, sputum eosinophils and neutrophils, blood eosinophils and neutrophils. Results were reported in a Pearson's correlation matrix (Figure 3). Serum eotaxin level was negatively correlated with post-bronchodilator FEV_1/FVC ratio (r=−0.18, P=0.0433), while eotaxin-3 showed a positive correlation with FeNO (r=0.18, P=0.0482). No other associations were found statistically significant.

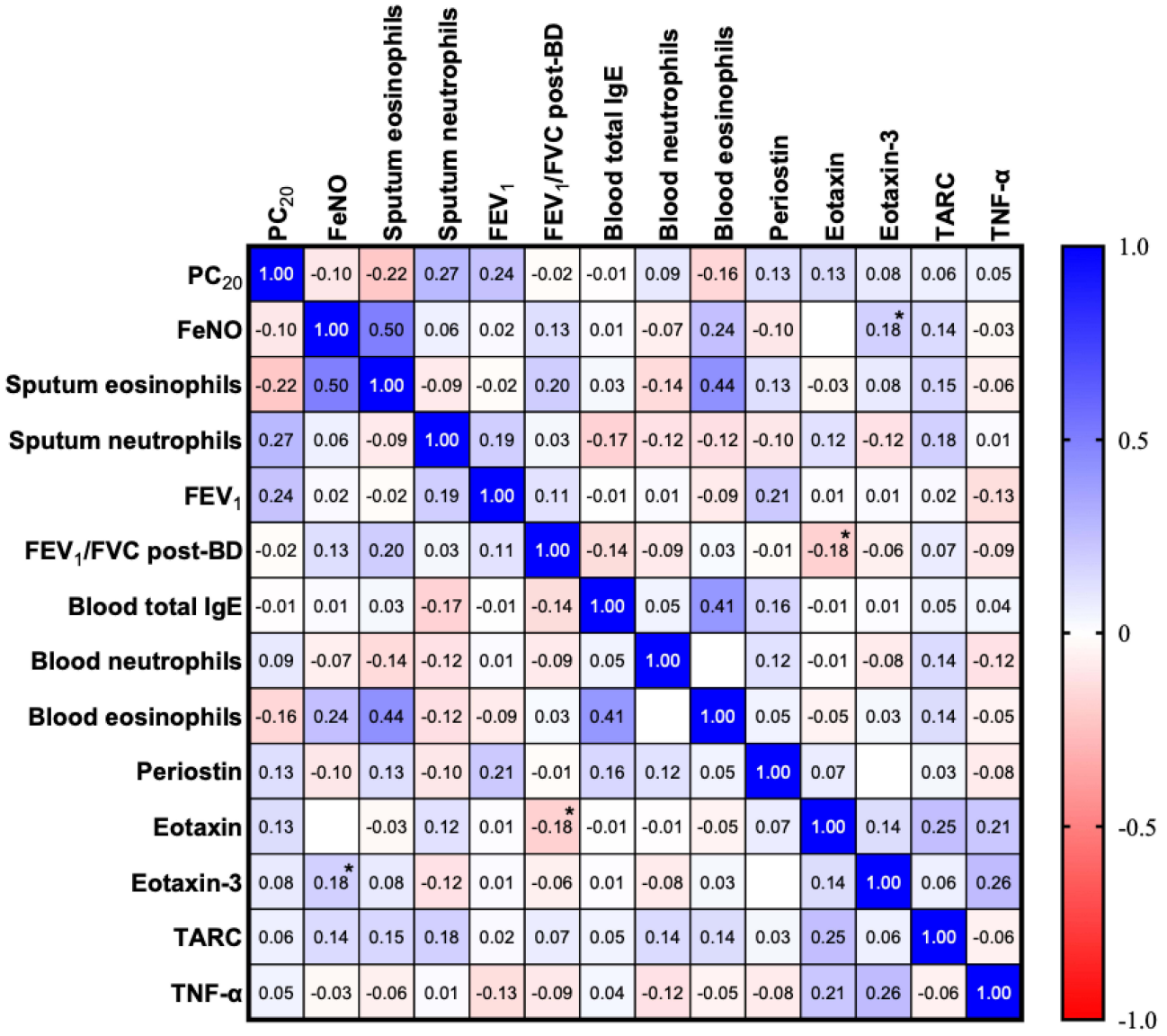

Figure 3 Pearson's correlation matrix of clinical characteristics and biomarker levels. Positive correlations are shown in blue and negative correlations in red. The intensity of the color reflects the strength of the correlation (scale from −1 to +1). Variables include PC_{20} (mg/mL), FeNO (ppb), sputum eosinophils (%), sputum neutrophils (%), FEV_1 (% predicted), post-bronchodilator FEV_1/FVC ratio, blood total IgE (KU), blood neutrophils (cells×10^9/L), blood eosinophils (cells×10^9/L), plasma periostin (pg/mL), serum eotaxin (pg/mL), serum eotaxin-3 (pg/mL), serum TARC (pg/mL) and serum TNF-α (pg/mL). Statistically significant correlations ($p<0.05$) are indicated by an asterisk (*).

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Discussion

In symptomatic adults with no previous history of airway disease and normal spirometry, only eotaxin, eotaxin-3, TARC, periostin and TNF-a were consistently detectable in serum (or plasma), with generally low concentrations. We found no significant differences in biomarker levels between groups of subjects stratified by PC_{20} or inflammatory profile (T2 or non-T2). Serum eotaxin levels were negatively correlated with post-bronchodilator FEV_1/FVC ratio, while eotaxin-3 showed a positive correlation with FeNO. To our knowledge, this is the first study to comprehensively assess blood levels of T2 cytokines and chemokines in this specific population, providing new insights into early asthma pathophysiology and highlighting the limitations of systemic biomarkers for early disease detection.

Since our study population mostly consisted of individuals without previous clinical diagnosis of asthma and preserved lung function, the low detectability of IL-4, IL-5, IL-13, IL-25 and IL-33 was not surprising. These mediators are typically absent from circulation unless chronic or severe inflammation is present. Elevated blood levels of IL-4, IL-5 and IL-13 have indeed been reported in severe asthma, particularly in eosinophilic and Th2 high phenotypes.[13] The epithelial-derived alarmins IL-25 and IL-33 have also been detected in blood of individuals with severe disease and are known to be upregulated during asthma exacerbations.[23,24] In contrast, studies in mild asthma frequently failed to detect these cytokines in serum (or plasma),[25,26] suggesting that Th2-driven immune activation in mild or early-stage disease remains confined to the airways with minimal spillover into the bloodstream. Taken together, our results indicate that, unlike confirmed asthma cohorts where circulating IL-4, IL-5, IL-13 and IL-33 are readily detectable and correlate with disease activity, systemic levels of these mediators remain largely undetectable in symptomatic adults with normal spirometry. This raises important considerations regarding the choice of sampling method for biomarker detection in early-stage disease.

With evidence of T2 cytokines in sputum supernatant from non-asthmatic eosinophilic bronchitis and mild asthma populations,[27,28] one could wonder if airway sampling would be a more suitable approach for assessing biomarkers in early-stage disease. While this approach could potentially provide a more direct reflection of local airway inflammation, the decision not to extend biomarkers analysis to sputum supernatant in our study was based on both technical and practical considerations. Technically, many sputum samples had limited volume and variable supernatant quality, precluding reliable biomarker quantification. From a practical standpoint, sputum induction remains challenging in clinical settings, as approximately 20% of patients fail to produce high-quality samples.[29] Moreover, access to sputum analysis is limited only to a few tertiary health centers in Canada, limiting its widespread use in clinical practice. For these reasons, we chose to focus on blood-based biomarkers, which are more practical for routine clinical use. Airway sampling remains nonetheless a promising avenue for detecting local inflammatory signals in early asthma and could complement systemic assessments in future studies.

Despite T2 airway inflammation being present in 35% of our subjects based on sputum eosinophils >2% and/or FeNO >25 ppb, we found no significant differences in biomarker levels between T2 and non-T2 groups. While our definition of T2 airway inflammation aligns with existing classifications,[1,19] these are largely based on studies performed in severe asthma, potentially limiting applicability to our population. The lack of standardized biomarker cut-offs across asthma severities makes it challenging to assess whether using blood eosinophils instead of sputum or adjusting thresholds could have allowed for more conclusive results in our study. However, findings from large cohorts such as U-BIOPRED, ADEPT and SIENA indicate that T2 inflammation in mild asthma is probably associated with a low inflammatory state, characterized by lower blood eosinophil counts (mean 150 cells/μL) and neutrophils, sputum eosinophils (<2%), but elevated FeNO (mean 35 ppb).[30–32] These findings imply that FeNO may be a more sensitive marker for detecting underlying T2 airway inflammation in early-stage disease, although further validation is needed. Moreover, we observed considerable variability in biomarker levels expression among subjects with similar clinical presentations, suggesting the influence of diverse underlying inflammatory pathways. This heterogeneity reflects the complex interplay of immune pathways, genetic predispositions, and environmental exposures driving individual inflammatory responses in asthma.

Eotaxin (CCL11) and eotaxin-3 (CCL26) are key mediators of T2 inflammation, driving eosinophil recruitment and activation into the airways, processes that are central to asthma pathophysiology.[33] In line with previous studies in asthma, we found a negative correlation between serum eotaxin levels and post-bronchodilator FEV_1/FVC ratio, reinforcing its contribution to development of airflow limitation, potentially through enhanced eosinophil migration and tissue infiltration.[34] The absence of correlation with sputum eosinophils however indicates that systemic eotaxin

levels may not reliably reflect airway eosinophilia in our population. The positive correlation between eotaxin-3 levels and FeNO is interesting, given that both are mediated by IL-4 and IL-13,[33] but none of these cytokines were detected in serum. Once again, this suggests that T2 airway inflammation, in early-stage disease, is probably more localized into the airways. Given the weakness of these associations, further studies are necessary to confirm their validity and clarify their clinical significance in high-risk populations.

A subset of patients in our study exhibited both AHR and T2 airway inflammation, raising the question of whether they may have undiagnosed mild asthma. These individuals often experience respiratory symptoms similar to patients with pre-post bronchodilator reversibility, leading clinicians to consider them as asthma.[35] Asthma being an obstructive airway disease in nature, its diagnosis should however include demonstrable airflow limitation on spirometry according to current guidelines.[1,22] While individuals with both AHR and T2 airway inflammation may not have yet developed airway obstruction, presence of these clinical signs may suggest increased susceptibility to later developing clinically significant disease.[6–9] It has indeed previously been shown that patients with asymptomatic airway hyperresponsiveness could evolve towards symptomatic asthma, and this may be particularly true in those with underlying T2 airway inflammation.[36] Undiagnosed mild asthmatics remain at risk for severe respiratory events, including exacerbations, hospitalizations, and even fatal outcomes.[37] Further studies on blood and sputum biomarkers in this specific sub-population are needed to enable earlier asthma detection and ultimately reduce disease progression and morbidity.

Although ultrasensitive platforms like Meso Scale Discovery have considerably improved cytokine detection in blood, biomarker measurement remains a significant challenge in both health and disease. Factors such as cytokine half-life, degradation, seasonal variability and interactions with circulating proteins can complicate measurement and interpretation of data.[38] Inconsistencies across studies may also arise from differences in patient populations, disease severity, sample processing, and detection methodologies. A common approach to address detection limitations is imputing values below the LLOD for statistical analyses; however, this practice can introduce bias and overestimate biomarker significance.[39] To ensure a more accurate representation of inflammatory biomarker distribution, our study exclusively included values above the LLOD, thereby enhancing data reliability. We also used both ELISA and MSD platforms to rigorously assess systemic biomarker levels, exposing the challenges with their measurement. Moving forward, standardizing study protocols and methodologies will be crucial to improve the reliability of systemic biomarker assessment and increase our understanding of T2 cytokine dynamics in asthma development.

Our study has some limitations that should be acknowledged. First, although our initial sample size (N=128) was adequate, the division into subgroups have significantly reduced statistical power, potentially masking significant associations. This limitation is particularly important for the subgroups with a PC_{20} <4 mg/mL and PC_{20} 4–15.9 mg/mL, which are presumed to include individuals at highest risk for underlying mild asthma, yet involving only 20 and 27 participants respectively. Second, the presence of comorbidities (eg, allergic rhinitis) in some of our subjects could have contributed to their respiratory symptoms and influenced methacholine challenge test results, confounding the interpretation of biomarker levels. Moreover, despite following optimal storage protocols and taking manipulations precautions, the time interval between biomarker collection and analysis could have contributed to biomarker degradation, contributing to low detectability. These considerations highlight the complexity of systemic biomarker analysis and the need for methodological adjustments in future studies.

Conclusion

These findings suggest that our selected panel of blood-based T2 biomarkers may not be suitable for detecting early asthma-related changes in symptomatic adults with normal spirometry. Longitudinal follow-up of this cohort across Canada may however provide valuable insights into how these inflammatory mediators evolve over time and whether they contribute to airway obstruction. Future research should focus on optimizing biomarker panels, integrating airway-specific sampling, and developing novel non-invasive techniques to improve early detection and guide targeted intervention strategies in high-risk populations.

Abbreviations

CCL, Chemokine C-C motif ligand; ELISA, Enzyme-linked immunosorbent assays; FEV1, Forced expiratory volume in one second; FVC, Forced vital capacity; FeNO, Fractional exhaled nitric oxide; IL, Interleukin; MSD, Meso scale

discovery; LLOD, Lower limit of detection; PC20, Provocation concentration causing a 20% fall in FEV1; Ppb, Parts per billions; T2, Type 2 inflammation.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

JL, MEB, AL, JG and LPB have nothing to disclose.

CB reports participation on advisory boards for Sanofi-Regeneron, AstraZeneca, Takeda, and ValeoPharma; consultancy for Areteia; honoraria for presentations from AstraZeneca/Amgen, GlaxoSmithKline, Grifols, Sanofi-Regeneron, and ValeoPharma; and research grants paid to the University of British Columbia from BioHaven, Sanofi-Regeneron, AstraZeneca, Areteia, Jasper Therapeutics, and GlaxoSmithKline.

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CL, KLV, MM, SDA have nothing to declare.

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