Beyond type 2 asthma biomarkers: risk stratification for NSAID-exacerbated respiratory disease

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Analysis of *ALOX15*, *CLC*, *CYSLTR2*, *HRH4* and *SMPD3* gene expression in different T2 asthma patient groups reveals possible associations in disease mechanisms and biomarkers, and a valuable algorithm for T2 asthma diagnosis and N-ERD risk assessment https://bit.ly/3vu6PTv

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Abstract

Introduction Type 2 (T2) asthma is often associated with chronic rhinosinusitis with nasal polyposis (CRSwNP). Additionally, nonsteroidal anti-inflammatory drug (NSAID) intolerance leads to NSAID-exacerbated respiratory disease (N-ERD). Previous transcriptomic data in non-CRSwNP T2 asthma patients showed differentially expressed genes. We focused on *ALOX15*, *CLC*, *CYSLTR2*, *HRH4* and *SMPD3* to investigate their role in T2 asthma.

Methods The study included 100 healthy controls and 103 T2 asthma patients, divided into patients with asthma (n=54), patients with asthma and CRSwNP (n=29) and patients with N-ERD (n=20). Quantitative PCR analysis was performed on blood-derived RNA samples first to validate the five differentially expressed genes. The data were further analysed to find potential associations and biomarkers.

Results Patients, regardless of stratification, exhibited significantly higher gene expression than healthy controls. The patterns of association revealed that *ALOX15* was exclusively present in the non-comorbidity group, *SMPD3* and *CLC* in the comorbidity groups, and *HRH4* in all patient groups. *ALOX15*, *CYSLTR2* and *SMPD3* expression showed potential as biomarkers to confirm the diagnosis of T2 asthma using peripheral blood eosinophils as the initial criterion. Peripheral blood eosinophils combined with gene expression, especially *SMPD3*, may improve the diagnosis. *CLC* and *CYSLTR2* expression play a specific role in discriminating N-ERD.

Discussion We validated the transcriptomic data of five differentially expressed genes in T2 asthma. Different patterns of association were identified in patient stratification, suggesting that different molecular mechanisms underlie the spectrum of T2 asthma. Potential biomarkers were also found and used to design an algorithm with practical diagnostic utility for T2 asthma, including risk stratification for N-ERD.

Introduction

Asthma is a major global health burden affecting 1–18% of the population [1]. It is a heterogeneous disease characterised by chronic airway inflammation resulting from complex immunological processes. According to inflammation, asthma is classified as type 2 (T2) and non-T2 [2, 3]. Typically, T2 asthma is

characterised by increased levels of type 2 cytokines and eosinophils in the blood and/or airways. In addition, in the case of allergic asthma, sensitisation to aeroallergens will be present [2, 4].

In addition to the inflammatory type, the complexity of asthma is increased by comorbidities. Chronic rhinosinusitis with nasal polyposis (CRSwNP) is a comorbidity with a 7% prevalence in asthma patients, increasing up to 40% in nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (N-ERD) [5, 6]. This specific clinical group is associated with the highest rates of polyp recurrence, even with systemic corticosteroid treatment and endoscopic sinus surgery [1, 7].

Recently, a significant effort has been made to find specific asthma biomarkers to aid in more accurate diagnosis and disease management [4]. In this sense, precision medicine is becoming more commonly applied. Nevertheless, for asthma to be approached in this way, its heterogeneity must be considered an essential variable in scientific studies. In a previous transcriptomic study on a cohort of patients with allergic asthma (T2 asthma phenotype), we described several genes that were highly differentially expressed in patients compared to healthy subjects. Among the most differentially expressed genes (DEGs) (|fold-change|$\geqslant$1.5), only *IL5RA* and *PTGDR2* were validated and postulated as potential biomarkers [8, 9]. *PTGDR2* also discriminated between asthma groups [9]. Based on these findings and transcriptome scoring [9], we focused on other DEGs with similar fold-change and p-adjusted values, selecting *ALOX15*, *CLC*, *CYSLTR2*, *HRH4* and *SMPD3*. We aimed to study their expression to confirm this differential upregulation in asthma patients and to determine their potential as possible biomarkers, individually or as a set, and their role in the molecular mechanisms of T2 asthma, considering the heterogeneity of disease in asthma patients in terms of CRSwNP and N-ERD.

Methods

Further details are given in the supplementary material.

Study population

Individuals were recruited from the Allergy Department of Salamanca University Hospital. The local Clinical Research Ethics Committee approved the study (PI 2020-02-433) and all subjects provided written informed consent.

Gene expression analysis

A transcriptomic RNA-sequencing (RNA-seq) analysis was performed as previously reported (repository NCBI-PRJNA686899) [8, 9]. A heatmap was generated from the data using Morpheus (https://software.broadinstitute.org/morpheus). The quantitative PCR (qPCR) expression validation analysis of our selected genes was performed on RNA samples from the peripheral blood of the subjects, as previously described [9].

Statistical analysis

Data were analysed using SPSS software, version 26 (IBM, Armonk, NY, USA).

Results

Study population

The study population comprised 203 subjects (table 1); 100 were healthy controls (HCs) and 103 were patients diagnosed with T2 asthma. The asthma patient population was stratified into three mutually exclusive groups: patients with asthma and without CRSwNP or NSAID intolerance (asthma group, n=54); patients with asthma and CRSwNP and without NSAID intolerance (asthma+CRSwNP group, n=29); and patients with asthma, CRSwNP and NSAID intolerance (N-ERD group, n=20). Both peripheral blood eosinophil (PBE) levels and total IgE levels were significantly higher ($p<0.05$) in the total asthma population compared to HCs. When stratifying the patients, we found a significant increase in atopy in the asthma group compared to the other groups. However, we did not observe significant differences in total IgE levels among asthma groups. PBE levels were significantly higher in all patient groups compared to HCs; in particular, the asthma+CRSwNP group had significantly higher PBE levels than the asthma group (table 1).

Gene expression of ALOX15, CLC, CYSLTR2, HRH4 *and* SMPD3

The heatmap generated from previous RNA-seq [8, 9] showed *ALOX15*, *CLC*, *CYSLTR2*, *HRH4* and *SMPD3* as the most upregulated genes (supplementary figure S1). The relative gene expression of these five DEGs, determined by qPCR analysis in our validation study, is shown in table 2. All patient groups showed significantly higher gene expression levels than HCs. This finding is consistent with our previous transcriptomic study, which showed upregulation of these genes in the asthma population compared to controls [8, 9]. Comparing the patient groups, we found that expression levels of *CLC* in the N-ERD group and *HRH4* in the asthma+CRSwNP group were significantly higher than in the asthma group.

TABLE 1 Clinical and phenotypic characteristics of the study population

	HCs	Patients			
		Total	Asthma	Asthma+CRSwNP	N-ERD
Subjects (n)	100	103	54	29	20
Age (years)	59.1 ±18.0	44.2±19.7*	36.9±18.7*	49.5±18.9*,#	56.6±14.9#
Female sex	65.7	59.2	57.4	58.6	65.0
Atopy	0	70.9*	87.0*	58.6*,#	45.0*,#
PBE (cells·µL^{-1})	113.0±78.47	486.5±335.4*	395.2±254.3*	626.2±429.2*,#	530.6±310.6*
Total IgE (kU·L^{-1})	67.7±103.5	420.7±591.7*	500.1±684.3*	338.7±504.8*	309.7±363.3*

Values are expressed as mean±SD or %, unless otherwise indicated. Only statistically significant differences are indicated. HC: healthy control; CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: nonsteroidal anti-inflammatory drug-exacerbated respiratory disease; PBE: peripheral blood eosinophil. *: p<0.05 compared to HCs; #: p<0.05 compared to the asthma group.

Correlations among ALOX15, CLC, CYSLTR2, HRH4 *and* SMPD3

We analysed gene expression for possible associations using correlation analysis (table 3) and created a graph to visualise the correlations with r>0.7 (*i.e.* highly correlated) (figure 1). *HRH4* was highly correlated with different genes in different groups, *i.e. ALOX15* and *CYSLTR2* in the asthma group, *SMPD3* in the asthma+CRSwNP group and *CLC* in the N-ERD group. *ALOX15* had highly significant correlations with several genes in the asthma group. By contrast, *SMPD3* showed poorer correlation with the other genes in the asthma group but was highly correlated with *HRH4* in the asthma+CRSwNP group and with *CYSLTR2* in the N-ERD group. *CYSLTR2* showed high correlations, especially with *ALOX15* and *HRH4* in the asthma group and with *SMPD3* in the N-ERD group. Finally, *CLC* only showed high correlations with other genes in the N-ERD group. Overall, our data showed various association patterns among the patient groups. Although we cannot be sure of the nature of these associations, they suggest that our five genes may be involved in different molecular mechanisms of T2 asthma, depending on the type of patient.

ALOX15, CLC, CYSLTR2, HRH4 *and* SMPD3 *as potential biomarkers*

The differential gene expression and correlation analyses showed that *ALOX15*, *CLC*, *CYSLTR2*, *HRH4* and *SMPD3* could be potential biomarkers for identifying T2 asthma. Furthermore, they could help discriminate between different asthma populations. To further explore the potential of these genes as biomarkers, we performed a receiver operating characteristic (ROC) curve analysis to determine the discriminatory capacity of these genes (table 4). PBE, a clinically accepted biomarker for T2 asthma [4], was also included in the analysis for comparison with gene expressions. We evaluated the ability of the potential biomarkers to discriminate between HCs and the general population of T2 asthma patients or between the patient groups (asthma, asthma+CRSwNP and N-ERD) and HCs (reference group). We also evaluated whether the potential biomarkers could differentiate patient groups, using the asthma group or the asthma+CRSwNP group as a reference to compare with asthma+CRSwNP and N-ERD patients, respectively. Our results showed that PBE levels had the highest area under the curve (AUC) for differentiating T2 asthma patients from HCs in all comparisons (table 4).

TABLE 2 Gene expression analysis of *ALOX15*, *CLC*, *CYSLTR2*, *HRH4* and *SMPD3*

	HCs	Patients			
		Total	Asthma	Asthma +CRSwNP	N-ERD
ALOX15	1.88±1.51	10.95±9.04*	10.35±8.63*	13.22±10.60*	9.28±7.29*
CLC	2.21±1.43	10.87±12.06*	8.49±11.72*	11.13±10.86*	16.92±13.02*,#
CYSLTR2	2.83±1.13	8.05±4.44*	7.59±4.58*	8.41±4.28*	8.75±4.33*
HRH4	1.78±0.98	4.43±2.63*	3.83±2.68*	5.28±2.33*,#	4.81±2.61*
SMPD3	2.44±1.34	11.99±13.58*	12.38±16.83*	11.63±7.70*	11.44±10.59*

Gene expression values (mean±SD) determined by quantitative PCR ($2^{-\Delta\Delta Ct}$). Data were analysed by ANOVA and *post hoc* test or Kruskal–Wallis analysis. Only statistically significant differences are indicated after adjustment by sex, age and atopy. HC: healthy control; CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: nonsteroidal anti-inflammatory drug-exacerbated respiratory disease. *: p<0.05 compared to HCs; #: p<0.05 compared to the asthma group.

TABLE 3 Gene expression correlation matrix

	ALOX15	*CLC*	*CYSLTR2*	*HRH4*	*SMPD3*
Healthy controls					
ALOX15	1	0.190	0.388*	0.556*	0.214
CLC	0.190	1	0.090	0.340*	0.245*
CYSLTR2	0.388*	0.090	1	0.438*	0.342*
HRH4	0.556*	0.340*	0.438*	1	0.091
SMPD3	0.214	0.245*	0.342*	0.091	1
Asthma					
ALOX15	1	0.635*	**0.755***	**0.859***	0.423*
CLC	0.635*	1	0.602*	0.585*	0.199
CYSLTR2	**0.755***	0.602*	1	**0.748***	0.415*
HRH4	**0.859***	0.585*	**0.748***	1	0.355*
SMPD3	0.423*	0.199	0.415*	0.355*	1
Asthma+CRSwNP					
ALOX15	1	−0.071	0.468*	0.491*	0.368*
CLC	−0.071	1	0.339	0.611*	0.571*
CYSLTR2	0.468*	0.339	1	0.640*	0.633*
HRH4	0.491*	0.611*	0.640*	1	**0.780***
SMPD3	0.368*	0.571*	0.633*	**0.780***	1
N-ERD					
ALOX15	1	0.209	0.459*	0.242	0.393
CLC	0.209	1	0.611*	**0.717***	0.458*
CYSLTR2	0.459*	0.611*	1	0.663*	**0.730***
HRH4	0.242	**0.717***	0.663*	1	0.314
SMPD3	0.393	0.458*	**0.730***	0.314	1

Data presented as Pearson's correlation coefficients. Bolding indicates statistically significant values higher than 0.7. Grey shaded boxes are repeated values in the bivariate matrix. CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: nonsteroidal anti-inflammatory drug-exacerbated respiratory disease. *: p<0.05.

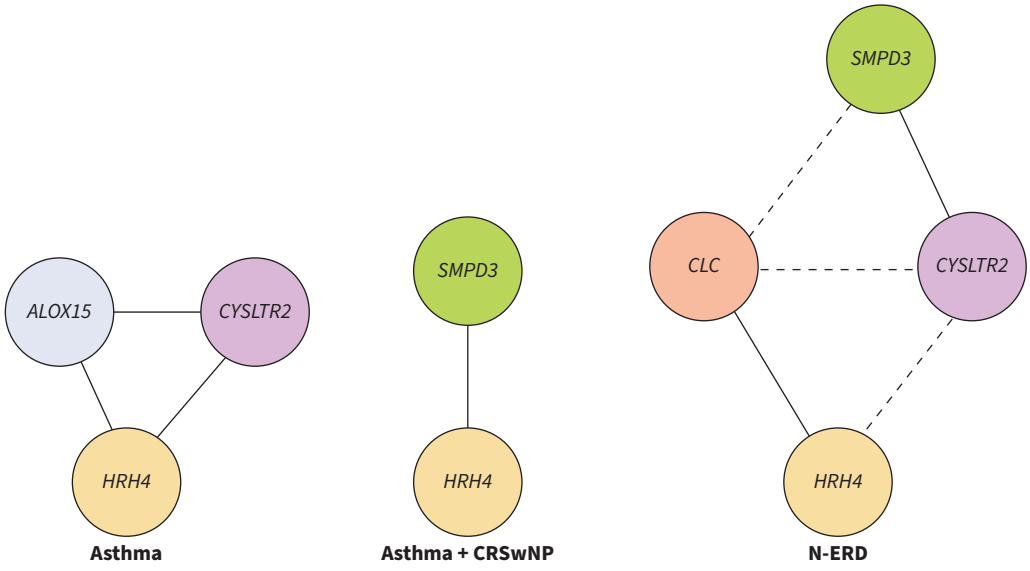

FIGURE 1 Graphical representation of gene expression associations in T2 asthma patient groups. The solid lines in the graph indicate significant correlations (p<0.05) between gene expressions with a Pearson's correlation coefficient higher than 0.7 (r>0.7). The line length is inversely proportional to the correlation coefficient, *i.e.* shorter lines in the graph indicate a higher correlation between the two gene expressions. The dashed lines represent moderate-low significant correlations (p<0.05) between two separate high correlations in the nonsteroidal anti-inflammatory drug-exacerbated respiratory disease (N-ERD) group. CRSwNP: chronic rhinosinusitis with nasal polyposis.

TABLE 4 Receiver operating characteristic curve analysis of PBE, *ALOX15*, *CLC*, *CYSLTR2*, *HRH4* and *SMPD3*

	Healthy controls *versus*				Asthma *versus* Asthma +CRSwNP	Asthma +CRSwNP *versus* N-ERD
	Total	Asthma	Asthma +CRSwNP	N-ERD		
PBE	**0.95 (0.92–0.98)**	**0.94 (0.90–0.98)**	**0.97 (0.94–1.00)**	**0.97 (0.94–1.00)**	0.68 (0.57–0.81)	0.45* (0.29–0.62)
ALOX15	0.93 (0.90–0.97)	0.93 (0.89–0.98)	0.96 (0.92–1.00)	0.91 (0.83–0.98)	0.57* (0.45–0.71)	0.38* (0.22–0.54)
CLC	0.86* (0.80–0.91)	0.79* (0.70–0.87)	0.93 (0.88–0.99)	0.93 (0.86–1.00)	0.66 (0.55–0.78)	**0.63 (0.46–0.81)**
CYSLTR2	0.92 (0.88–0.96)	0.91 (0.85–0.96)	0.93 (0.87–0.99)	0.93 (0.86–1.00)	0.57* (0.44–0.70)	0.53 (0.36–0.70)
HRH4	0.85* (0.79–0.91)	0.78* (0.70–0.87)	0.95 (0.90–1.00)	0.88* (0.79–0.97)	**0.72 (0.61–0.83)**	0.44* (0.26–0.61)
SMPD3	0.91 (0.87–0.95)	0.88 (0.82–0.94)	0.95 (0.91–1.00)	0.91 (0.84–0.98)	0.62 (0.50–0.75)	0.42* (0.25–0.60)

Data are presented as the area under the receiver operating characteristic curve (AUC) (95% confidence interval). For each patient group (columns), yellow shading indicates the highest absolute value of the AUC; grey shaded cells indicate the AUC is significantly lower than the highest AUC value; in all other cells, the AUC values are statistically equivalent (p>0.05) to the highest AUC value; CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: nonsteroidal anti-inflammatory drug-exacerbated respiratory disease; PBE: peripheral blood eosinophils. *: p<0.05.

Nevertheless, *ALOX15*, *CYSLTR2* and *SMPD3* expression also had AUCs comparable to the PBE value in all groups (p>0.05), suggesting their potential use as biomarkers for distinguishing T2 asthma patients from HCs. When analysing the discriminatory capacity of the potential biomarkers in differentiating between patient groups (table 4), PBE, *CLC* and *SMPD3* had comparable AUCs to the higher value (*HRH4*) for differentiating asthma+CRSwNP patients from the asthma group. For discriminating N-ERD patients from the asthma+CRSwNP group, the AUCs of *CLC* and *CYSLTR2* were the best. Interestingly, the AUC of *ALOX15* expression (one of the best for discriminating HCs from patients) was consistently lower (p<0.05) than the best ones in all comparisons between patient groups.

Diagnostic value of ALOX15, CLC, CYSLTR2, HRH4 *and* SMPD3

Because ROC analyses showed potential candidates for discriminating patients from HCs or between patient groups, we next used logistic regression analysis to determine which might be good predictors of a specific condition (table 5). We determined their diagnostic value in each condition using cut-offs calculated from the ROCs (table 6). Because PBE, *ALOX15*, *CYSLTR2* and *SMPD3* exhibited comparable AUCs in all patient groups compared to HCs (table 4), they were analysed to differentiate patients from HCs. In this case, they all proved good predictors for distinguishing T2 asthma (table 5). Among them, PBE had the highest diagnostic sensitivity (92.2%) (table 6, upper panels). However, the analysed gene expressions had higher specificities than PBE.

Likelihood ratios (LRs) above 10 (LR+) or below 0.1 (LR−) are considered to provide strong evidence to rule in or rule out a diagnosis, respectively [10]. Under this premise, PBE is a good biomarker to rule out disease (LR−=0.09). In contrast, gene expressions performed better to confirm the diagnosis, especially *ALOX15* (LR+=13.60). Combining PBE with gene expression resulted in higher LR+ values (and AUCs) than gene expression alone, especially the combination of PBE with *SMPD3* (LR+=78.60), but also *CYSLTR2* combined with PBE, given that the OR value of *CYSLTR2* (OR=3.12; table 5, upper-panel) was higher than for the other genes.

When discriminating between patient groups (tables 5 and 6, lower panels), the evaluated potential biomarkers were less effective than those analysed in differentiating patients from HCs. Only PBE could be considered to discriminate asthma+CRSwNP patients from the asthma group with moderate LR+ (2.53, table 6). *CLC* and *CYSLTR2* expression deserve special attention in differentiating N-ERD patients from asthma patients with CRSwNP and NSAID-tolerance. They had the highest AUC values (table 4), but only *CLC* had a significant weight in the regression analysis (table 5, lower panels). Given the aetiopathogenic role of *CYSLTR2* in N-ERD [11], we also decided to analyse the diagnostic value of *CYSLTR2*. The results

TABLE 5 Regression analysis of potential biomarkers

Healthy controls *versus* patients											
SLR	**Biomarker**	**PBE**			***ALOX15***			***CYSLTR2***			***SMPD3***
	OR (95% CI)	1.023 (1.013–1.034)			2.625 (1.724–3.996)			3.628 (1.893–6.952)			2.438 (1.651–3.600)
	p-value	**<0.05**			**<0.05**			**<0.05**			**<0.05**
MLR	**Biomarker**	**PBE**	**+**	***ALOX15***	**PBE**	**+**	***CYSLTR2***	**PBE**	**+**	***SMPD3***	
	OR (95% CI)	1.018 (1.005–1.031)		2.206 (1.285–3.788)	1.021 (1.008–1.034)		3.124 (1.379–7.074)	1.020 (1.009–1.032)		1.985 (1.169–3.371)	
	p-value	**<0.05**		**<0.05**	**<0.05**		**<0.05**	**<0.05**		**<0.05**	
Patients											
		Asthma *versus* Asthma+CRSwNP						**Asthma+CRSwNP *versus* N-ERD[#]**			
SLR	**Biomarker**	**PBE**		***HRH4***	***CLC***		***SMPD3***	***CLC***		***CYSLTR2***	
	OR (95% CI)	1.002 (1.001–1.004)		1.258 (1.043–1.517)	1.020 (0.980–1.061)		1.008 (0.973–1.044)	1.106 (1.022–1.196)		1.072 (0.921–1.248)	
	p-value	**<0.05**		**<0.05**	0.342		0.651	**<0.05**		0.368	
MLR	**Biomarker**	**PBE**	**+**	***HRH4***				***CLC***			
	OR (95% CI)	1.002 (1.001–1.004)		1.077 (0.832–1.393)				1.106 (1.022–1.196)			
	p-value	**<0.05**		0.574				**<0.05**			

Potential biomarkers with significantly higher area under the receiver operating characteristic curve values (no shading, table 3) were tested individually by simple logistic regression (SLR). Only significant biomarkers were further tested by multiple logistic regression (MLR). Bolding indicates statistically significant values. Values were adjusted for sex, age and atopy. PBE: peripheral blood eosinophils; CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: nonsteroidal inti-inflammatory drug-exacerbated respiratory disease. [#]: PBE was also used as a confounding variable.

showed that *CLC* moderately differentiated N-ERD patients (LR+=2.90; AUC=0.63). However, we found that combining *CLC* with *CYSLTR2* discriminated N-ERD patients more accurately from the asthma+CRSwNP group (LR+=8.70; AUC=0.75).

TABLE 6 Diagnostic values of potential biomarkers

Healthy controls *versus* patients							
	Biomarkers (cut-off)				**Combinations (cut-off)**		
	PBE (⩾195)	***ALOX15* (⩾4.07)**	***CYSLTR2* (⩾4.12)**	***SMPD3* (⩾3.75)**	**PBE (⩾195) and *ALOX15* (⩾4.07)**	**PBE (⩾195) and *CYSLTR2* (⩾4.12)**	**PBE (⩾195) and *SMPD3* (⩾3.75)**
S (%)	92.20	81.60	83.50	81.60	75.70	81.60	78.60
SP (%)	84.00	94.00	90.00	89.00	96.00	97.00	99.00
LR (+)	5.76	13.60	8.35	7.42	18.93	27.20	78.60
LR (−)	0.09	0.20	0.18	0.21	0.25	0.19	0.22
AUC (95% CI)	0.95 (0.92–0.98)	0.93 (0.90–0.97)	0.92 (0.88–0.96)	0.91 (0.87–0.95)	0.99 (0.99–1.000)	0.99 (0.99–1.000)	0.99 (0.98–1.000)
Patients							
	Asthma *versus* Asthma+CRSwNP		**Asthma+CRSwNP *versus* N-ERD**				
	Biomarkers (cut-off)		**Biomarkers (cut-off)**		**Combination (cut-off)**		
	PBE (⩾455)		***CLC* (⩾14.30)**	***CYSLTR2* (⩾11.58)**	***CLC* (⩾14.30) & *CYSLTR2* (⩾11.58)**		
S (%)	65.52		60.00	35.00	30.00		
SP (%)	74.10		79.31	82.76	96.55		
LR (+)	2.53		2.90	2.03	8.70		
LR (−)	0.47		0.50	0.79	0.73		
AUC (95% CI)	0.68 (0.57–0.81)		0.63 (0.46–0.81)	0.53 (0.36–0.70)	0.75 (0.61–0.89)		

Cut-off values for each potential biomarker were calculated from receiver operating characteristic curve data by the Jouden index. PBE is cells·μL^{-1} and gene expression from quantitative PCR as $2^{-\Delta\Delta Ct}$. PBE: peripheral blood eosinophils; S: sensitivity; SP: specificity; LR: likelihood ratio; AUC: area under the receiver operating characteristic curve; CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: nonsteroidal anti-inflammatory drug-exacerbated respiratory disease.

Based on all the diagnostic value data, we propose an algorithm using PBE as a starting point to diagnose T2 asthma and N-ERD (figure 2).

Discussion

This study aimed to validate the differential expression of *ALOX15*, *CLC*, *CYSLTR2*, *HRH4* and *SPMD3*, previously identified in a transcriptomic analysis [8, 9], in a similar independent T2 asthma population and to extend this validation to two other T2 asthma plus comorbidity populations (asthma+CRSwNP and N-ERD).

Our results showed upregulation of the five genes in all groups compared to HCs and some significant differences in the expression among the patient groups. Additionally, we found several patterns of potential associations between gene expressions that depended on patient stratification.

CYSLTR2, *SMPD3* and especially *ALOX15* expression showed a good discriminatory capacity, statistically comparable to PBE, in confirming T2 asthma, the phenotype common to all patients, and could be proposed as disease-specific biomarkers. Furthermore, combining them with PBE improved the confirmatory diagnosis. In this case, PBE with *SMPD3* expression was the best confirmatory combination for diagnosing T2 asthma and was included in our diagnostic algorithm (figure 2). Indeed, Global Initiative for Asthma guidelines indicate a cut-off of ⩾150 cells·μL^{-1} to suspect T2 asthma [1]. However, this level is also associated with the lower level of response to biologicals in clinical trials [12] and is intended to avoid misclassifying patients as having non-T2 asthma [1]. So, our threshold could have a

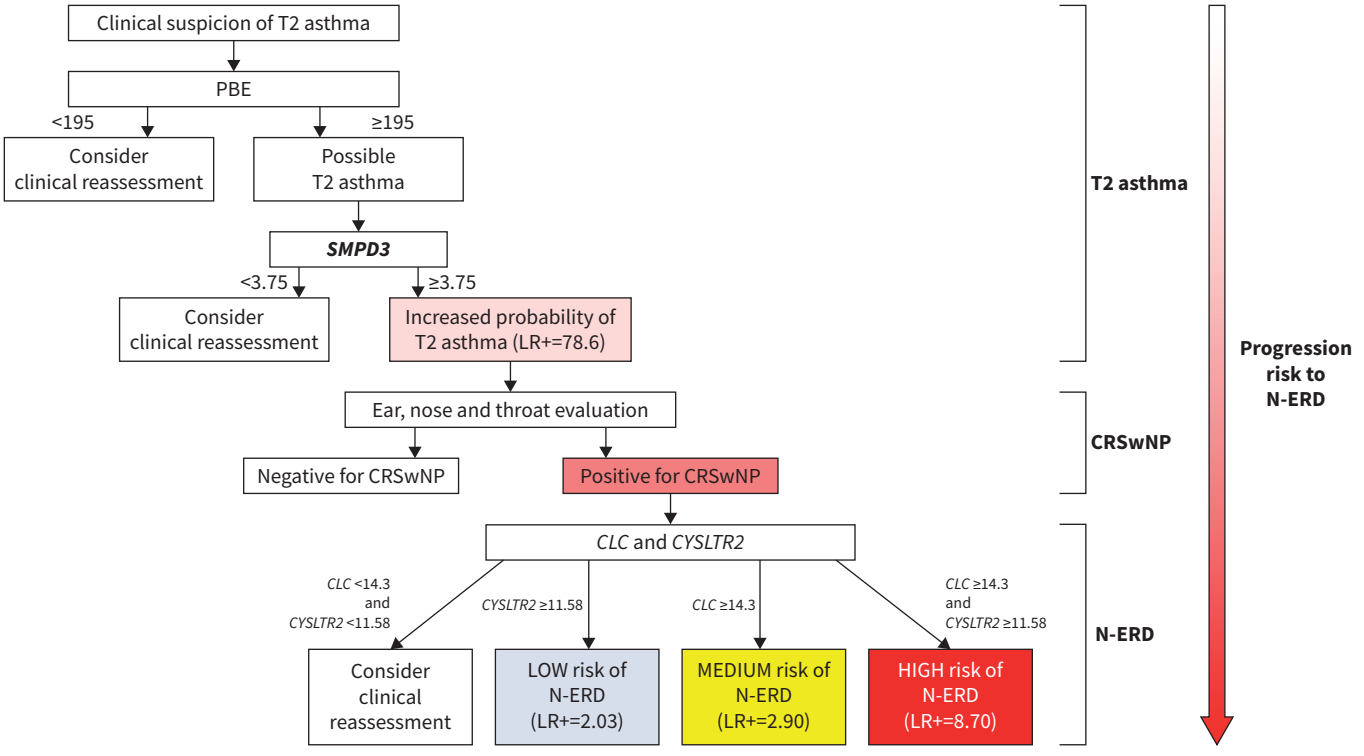

FIGURE 2 Algorithm to assist in the diagnosis of T2 asthma and nonsteroidal anti-inflammatory drug-exacerbated respiratory disease (N-ERD). The proposed algorithm starts with the peripheral blood eosinophil (PBE) counts as part of the T2 asthma diagnostic criteria. Our PBE cut-off (<195 cells·μL^{-1}) can exclude the disease (likelihood ratio (LR)−=0.09). However, as other cut-offs exist (Global Initiative for Asthma guidelines [1]), negative results are left to the clinician's discretion (clinical reassessment is suggested). Also, obtaining airway samples for these patients would be advisable. If positive (⩾195 cells·μL^{-1}), the test should be confirmed. *SMPD3* expression (cut-off >3.75 as per $2^{-\Delta\Delta Ct}$) in combination with PBE can accurately confirm the positive result (LR+=78.60), thus diagnosing T2 asthma. If the *SMPD3* cut-off is <3.75, a negative result cannot be accurately given and a clinical reassessment should be considered. The diagnosis of chronic rhinosinusitis with nasal polyposis (CRSwNP) is assumed to be made by physicians (ear, nose and throat evaluation). If positive, the *CLC* and/or *CYSLTR2* expression (considering the indicated cut-off points) can either indicate a low, medium or high risk for N-ERD. If both *CLC* and *CYSLTR2* expression cut-offs are below their thresholds, a negative result cannot be accurately given and clinical reassessment should be undertaken. The boxes displaying a gradient of colour from light red (T2 asthma) to dark red (N-ERD) correspond to a possible disease progression. See text and table 6 for further details of cut-off points and LRs.

better predictive value for T2 asthma, particularly with a concomitant *SMPD3* value >3.75 (as per $2^{-\Delta\Delta Ct}$). This demonstrates that combining biomarkers enhances diagnostics over individual biomarkers, as previously suggested [4].

ALOX15 encodes the arachidonate 15-lipoxygenase protein (15-LOX), which is involved in several inflammatory diseases, including asthma [13]. In addition to its diagnostic value, *ALOX15* showed major changes in the correlation analysis in the asthma group compared to HCs. Therefore, our results align with those highlighting the role of *ALOX15* in asthma [13, 14]. However, *ALOX15* expression was not a suitable biomarker for differentiating among the patient groups. These findings may be in contrast to a previous study describing significantly increased expression of *ALOX15* in nasal polyp cells from N-ERD patients which concluded that the dysregulation of arachidonic acid metabolism *via* the 15-LOX pathway contributes to increased inflammation in N-ERD disease [15]. We found no significant differences in *ALOX15* expression among patient groups (table 2). However, the data showed a decrease in gene expression in the N-ERD group compared to the asthma+CRSwNP group. Although the differences between our results and those mentioned above could be due to using different cell types (nasal polyp cells *versus* peripheral blood cells), other hypotheses could be raised. Because 15-LOX has pro- and anti-inflammatory properties [13, 16], non-increased or even reduced *ALOX15* expression, as we observed, could also be associated with the increased severity of N-ERD disease. How the balance between pro- and anti-inflammatory properties of 15-LOX, mediated by *ALOX15* expression, is regulated or represented by specific cell types could be a topic for further research.

Similar to *ALOX15*, PBE levels were irrelevant in discriminating N-ERD patients. However, *CLC* expression could be helpful for diagnosing N-ERD, a result that agrees with previous reports [17]. *CLC* encodes Charcot–Leyden crystal protein. First described in the late 1800s [18, 19], it has been proposed as a valuable biomarker of eosinophilic T2 inflammation in several diseases [20–22]. *CLC* expression was significantly higher in the N-ERD group than the asthma group (table 2), and its AUC value was the highest for differentiating N-ERD from asthma+CRSwNP patients (table 3). Given the low LR+ values (table 6), *CLC* expression alone may not be sufficient to differentiate N-ERD patients. Nevertheless, combining *CLC* and *CYSLTR2* expression discriminated N-ERD disease more accurately. Although the results of these genes are not entirely conclusive for N-ERD and further research is needed, the combination of *CLC* and *CYSLTR2* expression could be a valuable novel tool in diagnosing NSAID hypersensitivity, where the gold standard is currently the aspirin (acetylsalicylic acid) or other NSAID challenge [23], with the potential risk of severe reactions [1]. Therefore, this combination was proposed for the diagnosis and follow-up of N-ERD in our algorithm (figure 2). This result could be useful before considering an oral challenge with acetylsalicylic acid, because it allows for risk stratification.

CYSLTR2 encodes the G protein-coupled receptor (GPCR) cysteinyl leukotriene receptor 2, which binds the cysteinyl leukotrienes C_4 and D_4, potent lipid inflammatory mediators in asthma [24]. *CYSLTR2* is expressed in various cell types, including eosinophils [24, 25]. Several genetic studies have linked this receptor to asthma [26–28], and a role in the immunopathology of N-ERD disease has been reported previously [11]. We also found that *CYSLTR2* expression highly correlated with other genes in the N-ERD group (figure 1), so it may be reasonable to consider this gene a critical player in the specific molecular mechanisms driving N-ERD disease. Moreover, the usefulness of *CYSLTR2* expression as a biomarker to confirm T2 asthma diagnosis when combined with PBE levels suggests that this gene may also play a role in molecular mechanisms common to all patients. We found a high correlation between *CYSLTR2* and *ALOX15* in the asthma group, indicating some association. That is consistent with evidence that in asthma, CYSLTR2 and 15-LOX are involved in signalling pathways starting from a common precursor, arachidonic acid [13, 24].

HRH4 encodes the GPCR protein histamine receptor H4 [29], which is linked to several inflammatory processes [30]. In allergic asthma, histamine signalling *via* HRH4 is implicated in the immune-inflammatory response [31, 32]. Although *HRH4* expression was significantly higher in asthma patients with CRSwNP (table 2), our subsequent analyses suggest that its expression may not be as effective a biomarker to differentiate that patient group as PBE. Nonetheless, we observed that *HRH4* expression highly correlated with other gene expressions in all patient groups (figure 1), suggesting a transversal role in the molecular mechanisms underlying T2 asthma. That is a likely hypothesis, given the several lines of evidence linking the receptor to this disease [30–32].

Our results on *SMPD3* are exciting because there is less evidence for its association with asthma than for the other genes. *SMPD3* encodes neutral sphingomyelinase II, which is involved in sphingomyelin metabolism [33]. This protein has been proposed as a novel target in COPD [34]. In a recent study,

SMPD3 was significantly associated with atopy and/or atopic asthma in children and adolescents [35]. Although our results on *SMPD3* are based on an adult population, they are consistent with the findings of the latter study. In the asthma group (mostly allergic), *SMPD3* expression was significantly higher than in HCs. Furthermore, its AUC was among the best for discriminating this patient group (*versus* HCs; table 4). However, the importance of *SMPD3* could be extended to all T2 asthma patients, given that the combination of PBE with this gene was the best to confirm a T2 asthma diagnosis (figure 2), suggesting that future research needs to focus on *SMPD3* and its possible functional role in the underlying mechanisms of this asthma phenotype. The role of *SMPD3* in T2 asthma may be part of the lipid-mediated signalling mechanisms that are common in asthma. Our results support this hypothesis because we found that *SMPD3* was highly correlated with *CYSLTR2* (N-ERD group), which is involved in the signalling pathway mediated by the lipid mediator leukotrienes [24]. We also found that *SMPD3* expression was highly correlated with *HRH4* in the asthma+CRSwNP group. Interestingly, the correlation between *SMPD3* and genes encoding GPCR proteins (*HRH4* and *CYSLTR2*) in the CRSwNP patient groups suggests possible SMPD3-GPCR-mediated signalling mechanisms specific to this comorbidity.

Our study's strengths include the identification of potential biomarkers among the DEGs and using them to design an algorithm with practical diagnostic utility for T2 asthma and N-ERD. As shown in figure 2, our algorithm proposes PBE as a starting point because it is the most sensitive biomarker. For the subsequent confirmatory step, we opted for *SMPD3*, because PBE and *SMPD3* were the best combination of biomarkers for diagnosing T2 asthma. *CLC* and *CYSLTR2* were suggested as valuable tools for N-ERD diagnosis. Furthermore, considering the potential progression from T2 asthma to N-ERD [7], PBE, *CLC* and *CYSLTR2* could be considered to monitor this transition. In particular, combining *CLC* and *CYSLTR2* could signal a potential risk gradient (low-medium-high) for N-ERD.

In addition, identifying patterns of association between gene expressions provided data to better understand the molecular mechanisms underlying the spectrum of T2 asthma.

One study limitation is the possible differences between peripheral blood gene expression and that of the airways. Future research could include airway samples to improve the findings. Additionally, validation of N-ERD risk stratification results will require a larger sample size for broader generalisation. Finally, results cannot be extrapolated to non-T2 asthma.

In conclusion, a variety of possible relevant roles emerged for the five DEGs in our study, which could be seen as a reflection of the heterogeneity of this disease and highlights the importance of focusing research on the type of asthma patient beyond the common phenotype, bringing us closer to the concept of personalised medicine.

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