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Early View

Original research article

Beyond T2-asthma biomarkers: risk stratification for NSAID-Exacerbated Respiratory Disease

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Title Page

Beyond T2-asthma biomarkers: risk stratification for NSAID-Exacerbated Respiratory Disease.

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Take home message

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

Abstract

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

The study included 100 healthy controls (HCs) and 103 T2-asthma patients, divided into asthmatics (54), asthmatics with CRSwNP (30), and N-ERD (19). Quantitative PCR analysis was performed on blood-derived RNA samples first to validate the five DEGs. The data were further analyzed to find potential associations and biomarkers.

Patients, regardless of stratification, exhibited significantly higher gene expressions than HCs. 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 (PBE) as the initial criterion. PBE combined with gene expressions, especially *SMPD3*, may improve the diagnosis. *CLC* and *CYSLTR2* expressions play a specific role in discriminating N-ERD.

We validated the transcriptomic data of five DEGs in T2-asthma. Different patterns of association were identified in patient stratification, suggesting different molecular mechanisms underlying 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 characterized 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 characterized by increased levels of type 2 cytokines and eosinophils in the blood and/or airways. In addition, in the case of allergic asthma, sensitization to aeroallergens should 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 asthmatic patients, increasing up to 40% in non-steroidal 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, aiding in a more accurate diagnosis and disease management [4]. In this sense, precision medicine is becoming more commonly applied. Nevertheless, for asthma to be approached 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 highly differentiated expressed genes compared to healthy subjects. Among the most differentially expressed genes (DEGs, |fold-change|≥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 asthmatic 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 asthmatic patients in terms of CRSwNP and N-ERD disease.

Methods

Further details are given in the supplementary material.

Study Population

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

Gene expression analysis

The transcriptomic RNA Sequencing (RNAseq) analysis was performed as previously reported (repository NCBI-PRJNA686899) [8, 9]. A heatmap was generated from the data using Morpheus [10]. 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 analyzed using SPSS software, version 26 (IBM, Armonk, NY, USA).

Results

Study population

The study population comprised 203 subjects (Table 1). One hundred were healthy controls (HCs), and 103 were patients diagnosed with T2-asthma. The patient population was stratified into three, mutually exclusive, groups: patients with asthma and without CRSwNP nor NSAID-intolerance -asthma group-(54 subjects); patients with asthma, CRSwNP and NSAID-tolerance -asthma+CRSwNP group-(29 subjects); and patients with asthma, CRSwNP and NSAID-intolerance -N-ERD group-(20 subjects). Both peripheral blood eosinophils (PBE) and total IgE levels were significantly higher ($p<0.05$) in the total asthmatic 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 asthmatic groups. PBE levels were significantly higher in all patient groups compared to HCs, and 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 RNAseq [8, 9] showed *ALOX15*, *CLC*, *CYSLTR2*, *HRH4,* and *SMPD3* at the top of the most upregulated genes (Figure S1). The relative gene expression of these five DEGs, determined by qPCR analysis in our validation study, is shown in Table 2. All groups of patients showed significantly higher gene expression levels than HCs. This finding is consistent with our previous transcriptomic study, which showed the upregulation of these genes in the asthmatic 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.

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

We analyzed gene expressions for possible associations using correlation analysis (Table 3) and created a graph to visualize the correlations with $r>0.7$ (Figure 1). *HRH4* was highly correlated to 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 only highly significant correlations with several genes in the asthma group. By contrast, *SMPD3* was poorly correlated to the other genes in the asthma group, highly correlated to *HRH4* in the asthma+CRSwNP group, and to *CYSLTR2* in the N-ERD group. For *CYSLTR2*, it should be noted that it 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 to 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 asthmatic populations. To further explore the potential of these genes as biomarkers, we performed a 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 to discriminate between HCs and the general population of T2-asthmatic 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, left-panel, Healthy controls vs.).

Nevertheless, *ALOX15*, *CYSLTR2*, and *SMPD3* expressions also had AUC 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 analyzing the discriminatory capacity of the potential biomarkers in differentiating between patient groups (Table 4, right-panels), 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 in the HCS vs. patients contrast) 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

Since ROC analyses showed potential candidates to discriminate patients from HCs or between patient groups, we next tested them using 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). Since PBE, *ALOX15*, *CYSLTR2,* and *SMPD3* exhibited comparable

AUCs in all patient groups compared to HCs (Table 4), they were analyzed to differentiate patients from HCs. In this case, they all proved good predictors for distinguishing T2-asthma (Table 5, upper-panels). Among them, PBE had the highest sensitivity (92.2%) in the diagnostic value (Table 6, upper-panels). However, the analyzed gene expressions had higher specificities than PBE.

LR above 10 (LR+) or below 0.1 (LR-) are considered to provide strong evidence to rule in or rule out a diagnosis [11]. 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+ (also AUCs) values than individually, especially the combination of PBE with *SMPD3* (LR+=78.60), but also *CYSLTR2* combined with PBE, as the OR value of *CYSLTR2* (3.12; Table 5, upper-panel) was higher than the other genes.

When discriminating between patient groups (Table 5 and 6, lower-panels), the evaluated potential biomarkers were less effective than those analyzed 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* expressions deserve special attention in differentiating N-ERD patients from asthmatics with CRSwNP and NSAID tolerance. Both had the highest AUC values (Table 4), but only *CLC* had a significant weight in the regression analysis (Table 5, lower-panels). Given the etiopathogenic role of *CYSLTR2* in N-ERD [12], we also decided to analyze the diagnostic value of *CYSLTR2*. The results 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).

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-asthmatic 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, GINA guidelines indicate a cut-off of ≥ 150 cells/µl to suspect T2-asthma [1]. However, it is also considered that this level is associated with the lower level of response to biologicals in clinical trials [13] and is intended to avoid misclassifying patients as having non-T2-asthma [1]. So, our threshold could have a better predictive value for T2-asthma, particularly with a concomitant *SMPD3* value higher than 3.5. That demonstrates how 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 [14]. 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 [14, 15]. 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 (NP) cells from N-ERD patients and concluded that the dysregulation of arachidonic acid metabolism via the 15-LOX pathway contributes to increased inflammation in N-ERD disease [16]. 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 (NP cells vs. peripheral-blood cells), other hypotheses could be raised. As 15-LOX has pro- and anti-inflammatory properties [14, 17], 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. Nevertheless, in N-ERD patients, *CLC* expression could be helpful for their diagnosis, a result that agrees with previous reports [18]. *CLC* encodes Charcot-Leyden Crystal protein. First described in late 1800 [19, 20], it has been proposed as a valuable biomarker of eosinophilic T2-inflammation in several diseases [21–23]. *CLC* expression was significantly higher in the N-ERD group compared to asthma patients (Table 2), and its AUC value was the highest to differentiate N-ERD from asthma+CRSwNP patients (Table 3). However, 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* expressions could be a valuable novel tool in diagnosing NSAID hypersensitivity, where the gold standard is the Aspirin or other NSAID challenge [24], 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 relevant before considering an oral challenge with ASA, as it allows for risk stratification.

CYSLTR2 encodes the G protein-coupled receptor (GPCR) CYSLTR2, which binds the cysteinyl leukotrienes C_4 and D_4, potent lipid inflammatory mediators in asthma [25]. *CYSLTR2* is expressed in various cell types, including eosinophils [25, 26]. Several genetic studies have linked this receptor to asthma [27–29], and a role in the immunopathology of N-ERD disease has been reported previously [12]. We also found that *CYSLTR2* expression highly correlated to 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 signaling pathways starting from a common precursor, arachidonic acid [14, 25].

HRH4 encodes the GPCR protein HRH4 [30], which is linked to several inflammatory processes [31]. In allergic asthma, histamine signaling via HRH4 is implicated in the immune-inflammatory response [32, 33]. Although *HRH4* expression was significantly higher in asthmatic patients with CRSwNP (Table 2), our subsequent analyses suggest that its expression may not be as effective as a biomarker to differentiate that patient

group compared to 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 [31–33].

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 [34]. This protein has been proposed as a novel target in chronic obstructive pulmonary disease [35]. In a recent study, *SMPD3* was significantly associated with atopy and/or atopic asthma in children and adolescents [36]. 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 compared to HCs. Furthermore, its AUC was among the best for discriminating this patient group (vs. HCs; see Table 4). However, the importance of *SMPD3* could be extended to all T2-asthma patients, as 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 lipid-mediated signaling mechanisms that are common in asthma. Our results support this hypothesis as we found that *SMPD3* was highly correlated with *CYSLTR2* (N-ERD group), which is involved in the signaling pathway mediated by the lipid mediator leukotrienes [25]. 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 signaling 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*, as 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 understand better 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 generalization. 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 asthmatic patient beyond the common phenotype, bringing us closer to the concept of personalized medicine.

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Conflicts of interest

Miguel Estravís has received payment for lectures from SANOFI in the last three years. Asunción García-Sánchez has received payment for lectures from Leti. Ignacio Dávila has received payment for lectures, including service on speaker's bureaus from Allergy Therapeutics, Astra-Zeneca, Chiesi, Diater, GSK, Leti, Novartis, Sanofi; for a consultancy from Allergy Therapeutics, ALK-Abello, Astra-Zeneca, GSK, Merck, MSD, Novartis, Sanofi; and grants for Thermofisher Diagnostics. Jacinto Ramos-González has received payments for lectures from Astra-Zeneca, Chiesi, GSK, Novartis, Sanofi, Menarini, and Boehringer; for a consultancy from Astra-Zeneca, GSK, Novartis, Sanofi. María Gil-Melcón has received payment for lectures from Astra-Zeneca, GSK, and Sanofi. The rest of the authors declare no conflict of interest. The funders had no role in the study's design, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Table 1. Clinical and phenotypic characteristics of the study population

	HCs	PATIENTS			
		Total	Asthma	Asthma +CRSwNP	N-ERD
N	100	103	54	29	20
Age, Years (SD)	59.1 ± 18.0	44.2 ± 19.7*	36.9 ± 18.7*	49.5 ± 18.9*¥	56.6 ± 14.9¥
Sex, F (%)	65.7	59.2	57.4	58.6	65.0
Atopy (%)	0	70.9*	87.0*	58.6*¥	45.0*¥
PBE (cells/µl)	113.0 ± 78.47	486.5 ± 335.4*	395.2 ± 254.3*	626.2 ± 429.2*¥	530.6 ± 310.6*
Total IgE (kU/L)	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 ± standard deviation or percentage (%). Only statistically significant differences are indicated: (*): $p<0.05$ compared to HCs; (¥): $p<0.05$ compared to the asthma group. N: Number; HCs: healthy controls; CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: NSAID-exacerbated respiratory disease; PBE: peripheral blood eosinophils.

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 ± standard deviation) determined by qPCR ($2^{-\Delta\Delta Ct}$). Data were analyzed by ANOVA and post hoc test or K-W analysis. Only statistically significant differences ($p<0.05$) are indicated after adjustment by sex, age, and atopy: (*) compared to healthy controls (HCs); (¥) compared to the asthma group. CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: NSAID-exacerbated respiratory disease

Table 3. Gene expression correlation matrix

		ALOX15	***CLC***	***CYSLTR2***	***HRH4***	***SMPD3***
HCs	***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

Pearson's correlation coefficients of *ALOX15*, *CLC*, *CYSLTR2*, *HRH4,* and *SMPD3* gene expression in healthy controls (HCs) or patient groups (asthma, asthma+CRSwNP, or N-ERD). Only statistically significant values ($p<0.05$; *) higher than 0.7 are bolded. Shaded boxes are repeated values in the bivariate matrix. CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: NSAID-exacerbated respiratory disease.

Table 4. ROC curve analysis of PBE, *ALOX15*, *CLC*, *CYSLTR2*, *HRH4*, and *SMPD3*

Reference group	**Healthy controls vs.**				**Asthma vs.**	**Asthma +CRSwNP vs.**
Patient group	**Total**	**Asthma**	**Asthma +CRSwNP**	**N-ERD**	**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)

Values of the area under the curve (AUC) (95% confidence interval) are represented. For each patient group (columns), the AUC values are color-coded: the highest, absolute value of the AUC is highlighted in yellow cells. The AUC values in blank cells are statistically equivalent ($p>0.05$) to the highest AUC value. The AUC values in shaded cells are significantly lower (*, $p<0.05$) than the highest AUC value. PBE: peripheral blood eosinophils. CRSwNP: chronic rhinosinusitis with nasal Polyposis; N-ERD: NSAID-exacerbated respiratory disease.

Table 5. Regression analysis of potential biomarkers

HCs vs. PATIENTS

SLR	**Biomarker**	**PBE**	***ALOX15***	***CYSLTR2***	***SMPD3***
	O.R.	1.023	2.625	3.628	2.438
	(95% CI)	(1.013-1.034)	(1.724-3.996)	(1.893-6.952)	(1.651-3.600)
	p	*	*	*	*
MLR	**Biomarker**	**PBE + *ALOX15***		**PBE + *CYSLTR2***	**PBE + *SMPD3***
	O.R.	1.018	2.206	1.021 3.124	1.020 1.985
	(95% CI)	(1.005-1.031)	(1.285-3.788)	(1.008-1.034) (1.379-7.074)	(1.009-1.032) (1.169-3.371)
	p	*	*	* *	* *

PATIENTS

		Asthma vs. Asthma+CRSwNP				**Asthma+CRSwNP vs. N-ERD[(a)]**	
SLR	**Biomarker**	**PBE**	***HRH4***	***CLC***	***SMPD3***	***CLC***	***CYSLTR2***
	O.R.	1.002	1.258	1.020	1.008	1.106	1.072
	(95% CI)	(1.001-1.004)	(1.043-1.517)	(0.980-1.061)	(0.973-1.044)	(1.022-1.196)	(0.921-1.248)
	p	*	*	0.342	0.651	*	0.368
MLR	**Biomarker**	**PBE**	**+**	***HRH4***		***CLC***	
	O.R.	1.002		1.077		1.106	
	(95% CI)	(1.001-1.004)		(0.832-1.393)		(1.022-1.196)	
	p	*		0.574		*	

Potential biomarkers with significantly higher AUC values (blank cells, Table 3) were tested individually by simple logistic regression (SLR). Only significant biomarkers were further tested by multiple logistic regression (MLR). Values were adjusted for sex, age, and atopy. (a) PBE (peripheral blood eosinophils) was also used as a confounding variable. CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: NSAID-exacerbated respiratory disease; O. R. (95% CI): Odds Ratio (95% confidence interval); p: probability value. Values statistically significant ($p<0.05$) are indicated.

Table 6. Diagnostic values of potential biomarkers

HCs vs. PATIENTS

	Biomarkers (Cut-off)				**Combinations (Cut-off)**		
	PBE (≥ 195)	*ALOX15* (≥ 4.07)	*CYSLTR2* (≥ 4.12)	*SMPD3* (≥ 3.75)	PBE (≥ 195) & *ALOX15* (≥ 4.07)	PBE (≥ 195) & *CYSLTR2* (≥ 4.12)	PBE (≥ 195) & *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%C.I.)	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 vs. Asthma+CRSwNP	**Asthma+CRSwNP vs. 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%C.I.)	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 ROC curve data by the Jouden index. Peripheral blood eosinophils (PBE, cells/µl); gene expression (qPCR, $2^{-\Delta\Delta Ct}$); S: sensitivity; SP: Specificity; LR: Likelihood ratio; AUC (95% C.I.): Values of the area under the curve (95% confidence interval); HCs: healthy controls; CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: NSAID-exacerbated respiratory disease.

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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 N-ERD group. CRSwNP: chronic rhinosinusitis with nasal polyposis; N-ERD: NSAID-exacerbated respiratory disease.

Figure 2. Algorithm to assist in the diagnosis of T2-asthma and N-ERD. The proposed algorithm starts with the peripheral eosinophil counts (PBE) as part of the T2-asthma diagnostic criteria (*). Our PBE cut-off (<195) can exclude the disease (LR-=0.09). However, as other cut-offs exist (GINA 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), the test should be confirmed. *SMPD3* expression (cut-off>3.5) in combination with PBE can accurately confirm the positive result (LR+ =78.60), thus diagnosing T2-asthma. If the *SMPD3* cut-off is <3.5, 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 – 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 (NSAID-exacerbated respiratory disease). If both *CLC* and *CYSLTR2* expression cut-offs are below of their threshold, a negative result cannot be accurately given and clinical reassessment should be undertaken. The boxes displaying a gradient of color 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.

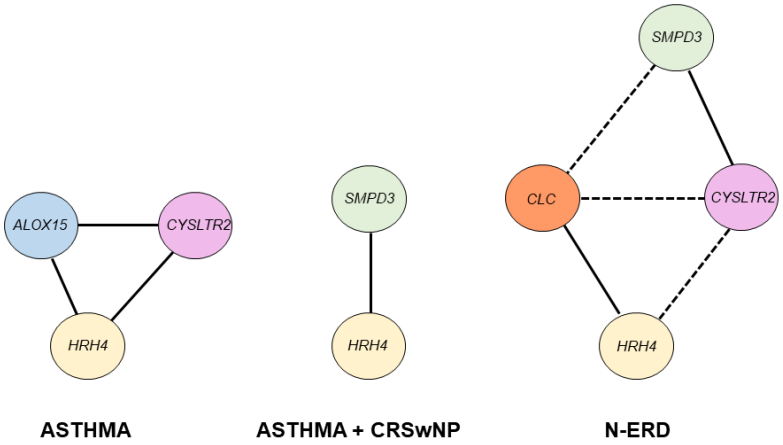

Figure 1

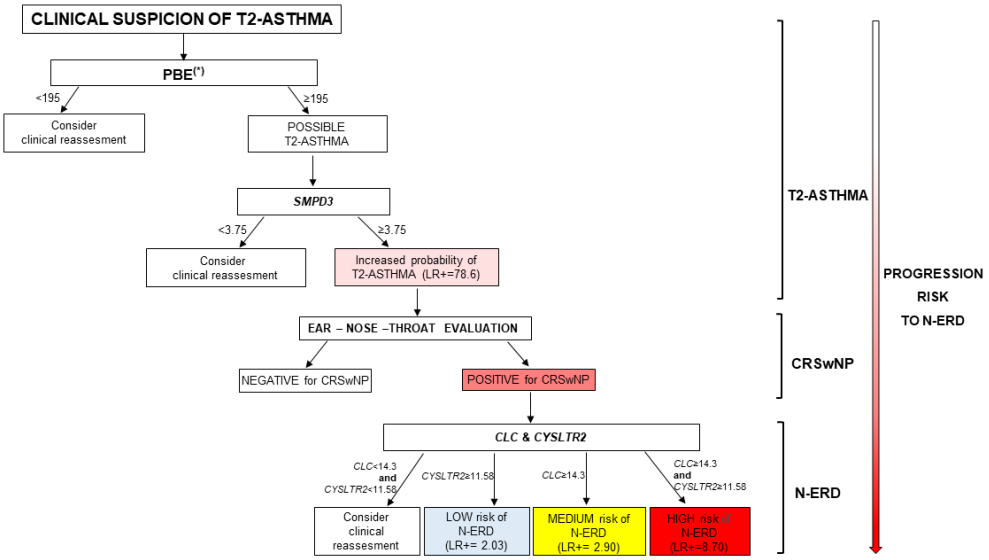

Figure 2

Methods

Study Population

Healthy controls (HCs) fulfilled the criteria previously described [1]. Briefly, HCs should have no symptoms or history of asthma, other pulmonary diseases, rhinitis, CRSwNP, NSAID intolerance, or atopy and should have negative skin tests for a standard battery of aeroallergens. T2-asthma was diagnosed according to GINA guidelines [2, 3]. CRSwNP and N-ERD were diagnosed according to EPOS criteria [4]. Skin prick tests were performed as previously described [5] according to The European Academy of Allergy and Clinical Immunology (EAACI) recommendations [6]. Patients were divided into asthmatics, asthmatics with CRSwNP, and N-ERD. Patients were treated according to GINA guidelines [2]. Nevertheless, none were receiving oral corticosteroids or biologicals thus avoiding influences on gene expression.

Gene expression analysis

Table SI. Primer pairs used in the qPCR analysis.

GENE	Primer pairs 5'-3'sequences	
	Forward	**Reverse**
ALOX15	ACCTTCCTGCTCGCCTAGTGTT	GGCTACAGAGAATGACGTTGGC
CLC	ATCAAAGGGCGACCACTTGCCT	CCCATACTCACGGCTGTTCATG
CYSLTR2	ATGTCACCAGCATCAGGAGTGC	TGTGACACTGCCGTTCTGCTCA
HRH4	CGATACCTGTCAGTCTCAAATGC	GCCCATTCACTAAGAAGGCCAG
SMPD3	AACTGCTCCTCTGACGACAA	GTGTCCAGCAGAGTACCGAT

Statistical analysis

A p-value < 0.05 indicated statistical significance. The sample size was determined using statistical power and false positive report probability considerations to minimize the risk of Type I error. The normality distribution was evaluated using the Kolmogorov-Smirnov test. Proportions were compared using the Chi-squared test. ANOVA with DMS or Games-Howell post hoc tests was used to compare continuous parametric data. Krustal-Wallis (K-W) test was used for non-parametric data. Pearson's correlation coefficient (r) was used to test possible relations between variables. Receiver operating characteristic (ROC) analysis was used to assess the biomarkers' diagnostic performance, and cut-off values were calculated from ROC data using the Jouden index [7]. Sensitivity, specificity, positive (+), or negative (-) likelihood ratio (LR) were calculated to assess the diagnostic value of a test. Logistic regression analysis was used to predict disease phenotype based on potential biomarkers individually (simple

logistic regression) or in combination (multiple logistic regression). Age, sex, and atopy were used as potential confounding variables for the adjustment of the p-values.

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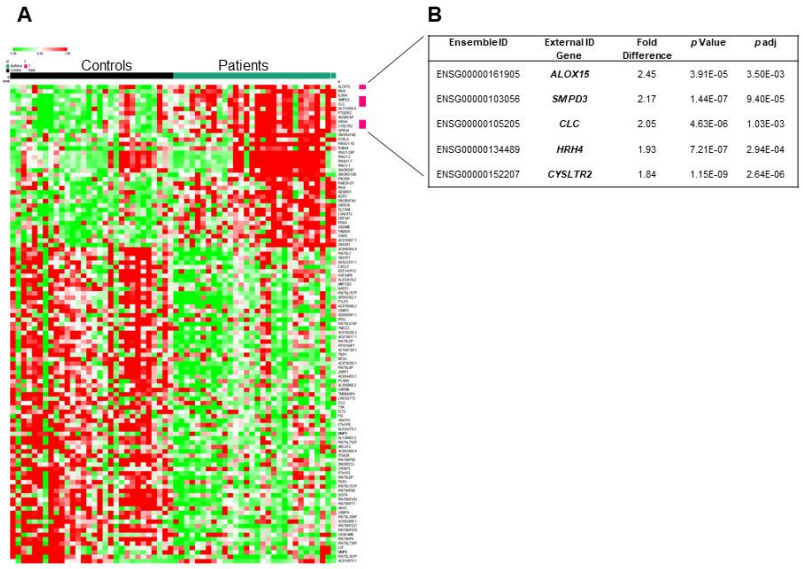
Figure legends

Figure S1. (A) Heatmap of RNAseq data [1] was plotted and computed with Morpheus [8]. Color representation was performed using expression values transformation subtraction of row median, divided by row median absolute deviation, with extreme values -1 and +1. Green represents downregulated genes, and red represents upregulated genes. **(B)** RNAseq data analysis of genes selected (pink squares in panel A) for the validation study.



EnsembleID	External ID Gene	Fold Difference	*p* Value	*p* adj
ENSG00000161905	*ALOX15*	2.45	3.91E-05	3.50E-03
ENSG00000103056	*SMPD3*	2.17	1.44E-07	9.40E-05
ENSG00000105205	*CLC*	2.05	4.63E-06	1.03E-03
ENSG00000134489	*HRH4*	1.93	7.21E-07	2.94E-04
ENSG00000152207	*CYSLTR2*	1.84	1.15E-09	2.64E-06

Figure S1