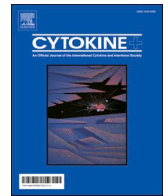




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Molecular analysis of phenotypic interactions of asthma

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ABSTRACT

Introduction: Asthma is a heterogeneous disease characterized by multiples respiratory symptoms; this is a polygenic entity that involves a complex interaction of environmental factors and inherent to the individual. To understand the development of asthma, some phenotypes have been proposed.

Objective: This work's purpose was to explore different molecules related to asthma development and to define each phenotype's specific characteristics.

Material and methods: 96 adult patients diagnosed with asthma before any treatment were enrolled in the protocol. Spirometric parameters, circulating leukocytes, serum IgE, body mass index, exhaled nitric oxide (FENO), and leukotrienes (LTB4) in urine were determined in each patient. The presence of asthma phenotypes proposed by the Global Initiative for Asthma (GINA) were explored: A) Allergic asthma, B) Non-allergic asthma, C) Late-onset asthma, D) Asthma with persistent airflow limitation, and E) Asthma with overweight and obesity.

Results: In the cohort analyzed, we found four of phenotypes proposed by GINA; however, these phenotypes overlapped, due to this, 4 groups were integrated with allergic, non-allergic and obese patients, which were the main phenotypes. The main overlap was that of patients not-obese allergic, and was characterized by earlier onset, elevated levels of IgE, LTB4 and inflammasome related cytokines. Non-allergic patients had a significant association between interleukin (IL)-18 and IL-18 binding protein (BP) with narrow ratio between these cytokines. Finally, LTB4 had remarkable capacity to discriminate between allergic and not allergic patients.

Conclusions: Asthmatic phenotypes exist as interrelated characteristics and not as discrete entities. High levels of leukotrienes and IgE are hallmarks in the allergic phenotype of asthma.

Abbreviations: FENO, Fractioned exhaled nitric oxide fraction; LTB4, Leukotrienes B4; IL, Interleukin; BP, Binding protein; GINA, Global Initiative for Asthma; ELISA, Enzyme-Linked Immunosorbent Assay; TGF- β , transforming growth factor-beta; IFN- γ , Interferon-gamma; TSLP, Thymic stromal lymphopoietin; RPM, Revolutions per minute; RT, Room temperature; ATS, American Thoracic Society; FEV1, Forced expiratory volume in 1 s; SD, Standard deviation; IQR, Interquartile range; ROC, Receiver Characteristic Operating; IU, International Units; BMI, Body mass index; Th2, T helper type 2.

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1. Introduction

Asthma is a heterogeneous disease characterized by chronic inflammation of the airways and respiratory symptoms such as wheezing, shortness of breath, chest tightness, and cough [1,2]. This is a polygenic disease that involves a complex interaction of environmental factors and inherent to the individual [3]. Nowadays there are different ways to classify asthma and some of these classifications are based on their etiology, type of induced response, or clinical control [4]. All asthma types are characterized by considerable damage to the epithelial barrier, intense inflammation of mucous membranes, and epithelia [5]. The common element in all varieties of this disease is inflammation. Usually, this phenomenon becomes so intense that it causes the remodeling of airways wall, caused by the release of soluble mediators related to tissue repair, mucus production, muscle proliferation, angiogenesis and fibrogenesis [6]. Due to the multiple factors involved in the origin and development of asthma, different classifications have been proposed. One of the simplest way to discriminate asthma types is to consider atopic and non-atopic patients, this system is based on the presence or absence of atopy, which is a reflection of multiple genetic characters that result in high levels of IgE and marked eosinophilia. However, it is currently known that the development of asthma goes beyond the allergic phenomenon.

In order to a better understanding of the development of asthma, several research groups have proposed multiple phenotypes related to different pathophysiological pathways or endotypes [7].

The Global Initiative for Asthma (GINA) has sought to standardize the criteria, and has proposed 5 general phenotypes. This classification considers immunological parameters, origin, and response to treatment in adult patients with severe asthma [8]. The phenotypes proposed by GINA are: A) Allergic asthma, B) Non-allergic asthma, C) Adult-onset asthma, D) Asthma with persistent airflow limitation, and E) Asthma with obesity [8]. GINA's proposal of phenotypes is useful to direct the diagnosis and management of patients; nevertheless, molecules that distinguish each of the phenotypes have not been established. Multiple researchers have proposed useful biomarkers to distinguish the different phenotypes [2,9,10], but the values for each of the markers often depend on population characteristics such as age or racial background. This research aims to analyze clinical, immunological, and physiological biomarkers to define GINA's phenotypes in the Mexican population's adult patients.

2. Material and Methods

2.1. Period and participants

The study was conducted between April 1, 2017, and March 31, 2018. During this period, all patients older than 18 years who first entered the Allergy and Immunology Service of the General Hospital of Mexico were examined by allergists experts, who recorded the different signs and symptoms compatible with asthma, such as wheezing, shortness of breath, chest tightness and cough; subsequently, the relevant cabinet studies were carried out to confirm the diagnosis according to the criteria established by GINA. The exclusion criteria considered were patients with parasitic and bacterial infections, hypocalcemia, lithiasis, autoimmune diseases, pregnancy, immunodeficiencies, cancer, smoking history and immunosuppressive therapy. All patients signed informed consent. The Local Research and Ethics Committee of the General Hospital of Mexico, governed by the principles established in the Helsinki Declaration for human research, approved this study.

2.2. IgE quantification

Eight mL of blood was obtained without anticoagulant. The serum was separated by centrifugation and stored at -20°C . IgE levels were determined using a commercial ELISA kit (Abcam® AB 108650, Mexico)

according to the manufacturer's recommendations.

2.3. Peripheral leukocytes quantification

The determination of peripheral leukocytes included the absolute number and the percentage of neutrophils and eosinophils from 8 mL of blood sample collected without anticoagulant. Data were generated by each cell passing through the aperture and were optically and electronically measured using the Coulter LH750 (Beckman Coulter, Fullerton, CA, USA), determining the type of cell from its impedance, volume, and conductivity. All samples were analyzed within 2 h after collection, and daily quality controls were run according to the manufacturer's instructions.

2.4. Cytokines immunoassays

Levels of cytokines in all patients' serum were determined using a commercially available ProcartaPlex 10 Plex, 1 Plate Thermo Fisher Scientific Assay ID MXRWEK2 (Thermo Fisher Scientific, Waltham, MA, USA; cat. #: PPX-10- MXRWEK2). The magnetic bead assay is based on the Luminex xMAP technology, which enables the simultaneous detection and quantitation of multiple secreted cytokines, including: IL-1 β , IL-4, IL-5, IL-8, IL-9, IL-13, IL-17, IL-18, IL-18 binding protein (BP), IL-33, transforming growth factor-beta (TGF- β), Interferon-gamma (IFN- γ) and thymic stromal lymphopoietin (TSLP). Multiplex assays were performed according to the manufacturer's instructions. Briefly, magnetic beads at working concentration were added into each well of the kit-provided black 96-well flat-bottom plates. After washing the beads and removing any residual liquid, samples, standards, and blanks were added. For serum, 25 μL of the kit-provided $1\times$ universal assay buffer was added to each well, followed by 25 μL four-fold serial diluted standards or undiluted samples. For wells designated as blanks, an additional 25 μL Buffer was added. The plate was incubated for 30 min under agitation at 300 revolutions per minute (rpm) at room temperature (RT) and protected from the light. Afterward, plate was transferred to a level surface at 4°C . After overnight incubation, the plate was again shaken at 300 rpm for 30 min at RT, and then the magnetic beads were washed. Next, 25 μL of $1\times$ detection antibody mixture was added to each well and incubated for 30 min under agitation at 300 rpm at RT and protected from light. After washing of magnetic beads, 50 μL of streptavidin-phycoerythrin (SAPE) solution was added and incubated for 30 min as described above. After another washing step, the test plate was prepared for analysis on a Luminex instrument by adding 120 μL of reading buffer into each well and incubation with agitation at 300 rpm for five minutes at RT. Finally, the plate was analyzed on the Luminex MAGPIX instrument (Software: xPonent 4.2). The cytokines concentrations were calculated using the standard curve generated by the five-parameter logistic regression method. In samples where cytokines/chemokines were undetectable, the detection limit values were used for analyses.

2.5. Leukotrienes (LTB₄) quantification

LTB₄ concentrations were measured using a specific enzyme immunoassay (Leukotriene B₄ ELISA kit, Abcam™ ab133040), with a sensitivity of 10 pg/mL. The entire test was performed following the manufacturer's instructions. To prevent adherence of fatty acid derivate (such as leukotrienes), all polypropylene tubes were coated with tween 20 [11].

2.6. Respiratory parameters

Fractioned exhaled nitric oxide fraction (F_{ENO}) was measured by using the Sievers 280i NO Analyzer (Boulder, Colorado, USA), which uses a chemiluminescence technique. According to ATS recommendations for on-line measurement of FENO in adults, the measurements

were performed at an expiratory flow of 50 mL/s [12]. The spirometry (FEV1) was performed using a MasterScreen Pneumo PC spirometer (Jaeger, Hoechberg, Germany), according to the ATS standards [13].

2.7. Statistical analysis

Categorical variables are described with frequencies and percentages and continuous variables with a mean±standard deviation (SD), or medians and interquartile range (IQR) according to the distribution of the variables. To compare cytokine serum levels among the phenotype overlapping, we used the paired t-test or Wilcoxon sign rank as appropriate. Finally, Receiver Characteristic Operating (ROC) curves were elaborated to estimate the discriminatory capacity of LTB4 being assessed, to differentiate between allergic or non-allergic patients (NPP). The Spearman's rank correlation coefficient was estimated to evaluate the strength of association between IL and 18 and IL18BP in all the overlapping stratum evaluated. All analyses were two-sided; α was set at 5% unless otherwise specified. The statistical software Stata v. 14.2 was employed to perform all analyses.

3. Results

Patients from all over Mexico were referred to the Allergy and Immunology Service at the General Hospital of Mexico, which is a national referral center; 107 patients were enrolled in the period of the study; however, 11 of them were discarded because of incomplete records. The other 96 patients were clinically diagnosed as asthmatic based on signs such as cough, paroxysmal dyspnea, rales, wheezing and cyanosis in the hands or lips.

To group patients with the different phenotypes proposed by GINA [14], parameters were determined as follows: A) Allergic asthmatic phenotype, eosinophils higher than 3%, IgE higher than 165 UI/mL in blood and positive to skin prick test [15]; B) Non-allergic asthmatic phenotype, clinical signs compatible with asthma with eosinophils less than 3%, serum IgE concentration less than 165 IU / mL and negative to skin prick test [16]; C) Late-onset asthmatic phenotype, the onset of clinical signs consistent with asthma after 65 years old [17]; D) Asthmatic with persistent airflow limitation phenotype, FEV1 less than or equal to 80% and reversibility greater than 12% or 200 mL [18]; and E) Asthma with overweight or obesity phenotype, body mass index (BMI) higher than 30 Kg/m² [19,20].

3.1. The analyzed population of asthmatic patients consisted mainly of overweight women with high levels of IgE and eosinophils

According to the determined parameters, we found that most of the patients enrolled in the protocol were overweighted women, according to national standards (25–29 BMI), and with high levels of IgE the main immunoglobulin associated with Th2-type inflammation of type Th2 (Table 1).

3.2. The greatest phenotypic overlapping was present in allergic non obese asthmatic patients

An outstanding fact was the inability to group patients into independent phenotypes, and this was due to the multiple overlap between each one's parameters. However, it was possible to observe that the two main interacting characteristics were obesity and allergy, from this observation, four overlapping groups were analyzed (Table 2). Also, obesity was a shared characteristic of both allergic and non-allergic patients in this population (Table 2).

3.3. Obstructive asthma was present in all the phenotypic overlapping analyzed

On the other hand, we observed that the parameters to be considered

Table 1

Main demographic and clinical information of the 96 patients diagnosed with asthma included in this study.

Variable	Patients (n = 96)
Age (years)	39 (29–48)
Female	74 (77.1%)
BMI kg/m ²	27.15 (24.45–30.35)
IgE IU/mL	177 (117.5–388.50)
Eosinophils (%)	4.9 (2.80–7.90)
Neutrophils (%)	53.3 (48.85–58.50)
IL-4 pg/mL	13 (10.50–16.75)
IL-5 (pg/mL)	12.9 (10.50–14.50)
IL-8 (pg/mL)	16.65 (12.75–21.00)
IL-9 (pg/mL)	17.5 (15.50–21.75)
IL-13 (pg/mL)	14.8 (12.40–17.00)
IL-17A (pg/mL)	12.5 (10.50–15.15)
IL-18 (pg/mL)	1584.5 (594.50–1656.50)
IL-18BP (pg/mL)	16,545.5 (5,165.50–17,456.50)
IL-33 (pg/mL)	14.5 (12.50–17.50)
TGFβ (pg/mL)	13.75 (11.50–22.50)
IFNγ (pg/mL)	15.5 (13.50–18.20)
TSLP (pg/mL)	17 (15.00–19.40)
FEV1%	88.3 (76.30–102.05)
FVC%	96.4 (87.05–106.50)
FEV1/FVC	95.6 (83.30–98.70)
FENO (ppb)	38 (24.50–56.50)
LTB4 (pg/mL)	399 (171.50–561.50)

Show median and interquartile range (IQR) except to female, eosinophils and neutrophils. BMI, Body mass index. ppb, parts per billion.

Table 2

Phenotypic overlapping present in the population of asthmatic patients participating in the study.

	Allergic-Obese	Not allergic-Obese	Allergic-Not obese	Not allergic-Not obese	p
N	16	9	47	24	
Sex F/M (n)	13/3	8/1	33/14	20/4	0.4
BMI (kg/m ²)	32 (30.9–34.2)	32.4 (32–37.6)	25 (21.7–27.5)	26.7 (25.1–28.2)	0.001
Onset age (years)	26 (21.5–36)	32 (9–32)	12 (6–34)	35.5 (16.44.5)	0.005
Obstructive (n)	5	5	19	8	0.5

Show median and interquartile range (IQR) except to sex and obstructive. M, male. F, female. BMI, Body mass index.

obstructive asthmatic phenotype were present in the four groups that made up the different phenotypic overlapping (Table 2).

3.4. The serum concentrations of inflammasome-related cytokines were significantly higher in allergic asthmatic patients

In order to relate the phenotypic relationships with cytokine profile, the concentration of cytokines in serum related to the development of asthma were determined. Surprisingly, the serum concentrations of IL-4, IL-5, IL-9, IL-13, IL-17A, IL-33, IFN-γ and TSLP, did not show differences between the four groups of phenotypic overlapping (Fig. 1). Nevertheless, cytokines related to the activity of the inflammasome (IL-1β and IL-18) showed significant variations between groups (Fig. 1).

The serum concentrations of IL-1β were markedly variable, and the interaction of obesity and allergy in the patients had the highest IL-1β concentrations (265 pg/mL (225–278.5 pg/mL)), followed by allergic, non-obese patients (236 pg/mL (222–279 pg/mL)), non-allergic, obese patients (142 pg/mL (132–158 pg/mL)); while the lowest concentrations were observed in patients with the non-allergic asthma and non-obese (67 pg/mL (57.5–77.5 pg/mL)) (Figs. 1, 1A). Corroborating this data, a similar pattern was observed in the serum concentrations of IL-18 and IL-18BP; the highest concentrations were observed in allergic and

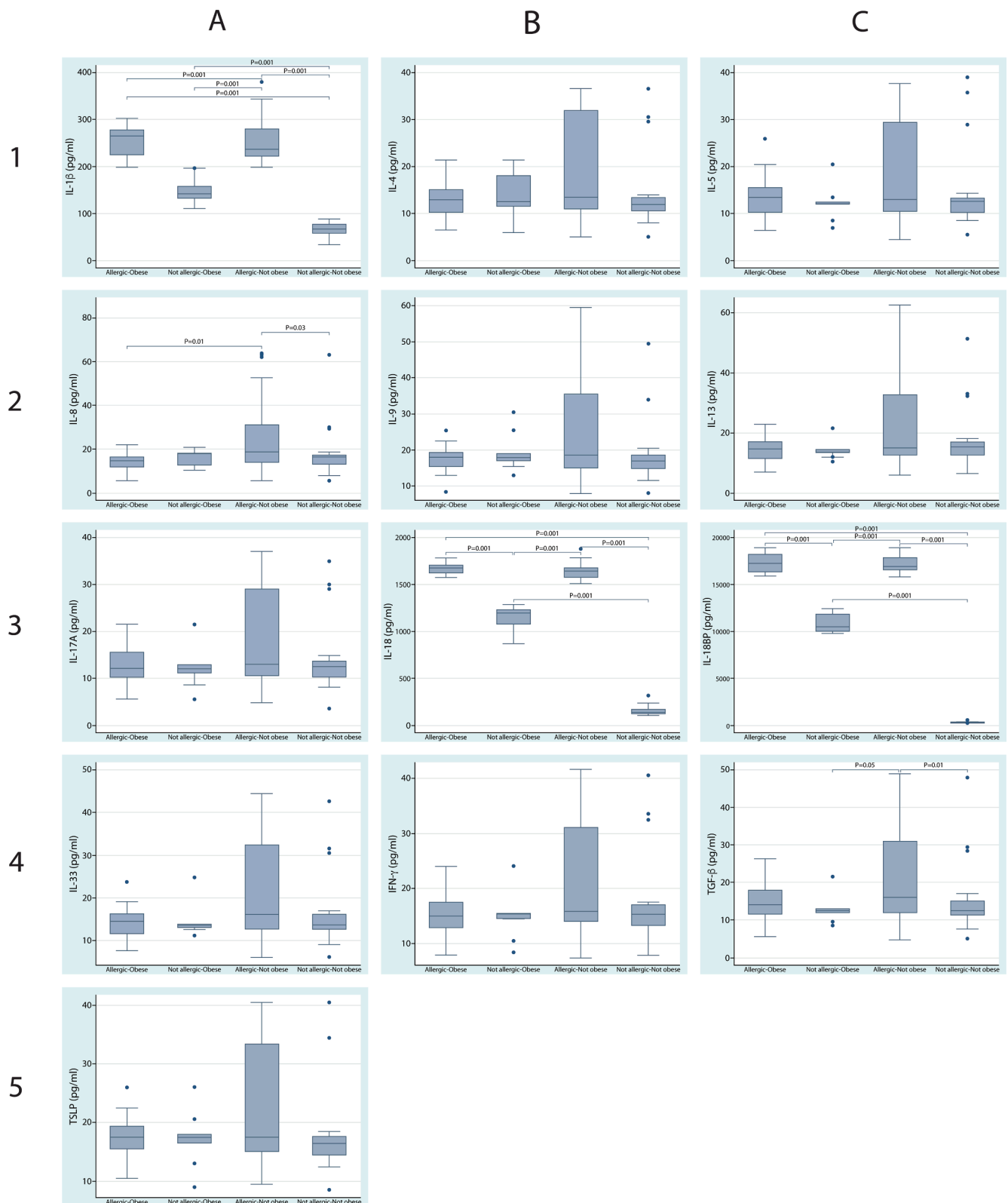


Fig. 1. Serum concentrations of cytokines in patients with a confirmed diagnosis of asthma. Each box shows a particular cytokine. Lines indicate the significant differences with the corresponding p-value.

obese patients (IL-18: 1675 pg/mL (1622.5–1712 pg/mL) IL-18BP: 17,205.5 pg/mL (16,286.5–18,112.5 pg/mL)) and allergic non-obese patients (IL-18: 1645 pg/mL (1578–1678 pg/mL) IL-18BP: 16,890 pg/mL (16,547–17,836 pg/mL)); followed by non-allergic obese patients (IL-18: 1192 pg/mL (1083–1231 pg/mL) IL-18BP: 10,435 pg/mL (9,875–11,830 pg/mL)) and non-obese non-allergic patients (IL-18: 134 pg/mL (122–171.5 pg/mL) IL-18BP: 328.5 pg/mL (301.5–354 pg/mL)) (Figure 1, 3B).

3.5. Serum IL-8 and TGF- β concentrations were significantly higher in not-obese allergic patients.

Regarding IL-8 concentrations, not-obese allergy patients (18.5 pg/mL (14–31 pg/mL)) had significantly higher levels than obese allergic patients (14.7 pg/mL (11.75–16.4 pg/mL)) and those who were not-obese not-allergic patients (16.5 pg/mL (12.9–17.25 pg/mL)); however, the difference with not-obese allergic patients (18 pg/mL (12.5–18 pg/mL)); was not significant (Figs. 1, 2A).

Finally, TGF- β serum concentrations were significantly higher in not-obese allergic patients (16 pg/mL (12–31 pg/mL)) when compared to obese and not-allergic (12.5 pg/mL (12–31 pg/mL)) and not-obese not-allergic overlapping (12.5 pg/mL (11.25–15 pg/mL)), but there was not differences with obese allergic (14 pg/mL (11.5–17.9 pg/mL)) (Figure 1, 4C).

3.6. Allergic asthmatic patients are characterized by an earlier onset of symptoms and higher percentages of circulating eosinophils

In order to find characteristics that specifically distinguish phenotypic overlapping, the age of onset of symptoms were recorded along with inflammatory markers related to the development of asthma. No significant differences were found in the percentage of circulating neutrophils or FENO when comparing the four phenotypic overlapping (Figs. 2, 1C and 2B). However, it was observed that non-obese allergic patients had significantly higher percentages of eosinophils (7.4% (4.8%–11.2%)), and together with obese non-allergic patients, had a much earlier onset of symptoms related to asthma (12 years (6 years–34 years; 32 years (9 years–32 years)) (Figs. 2, 1A and 2A). Regarding the serum IgE, it was observed that the overlapping with the presence of the allergic factor had significantly higher concentrations, whether with (276 IU/ml (164.5–546 IU/ml)) or without obesity (240 IU/ml (165 IU/ml–548 IU/ml)) (Figs. 2, 1B).

3.7. Urine leukotriene concentrations are significantly higher in allergic asthmatic patients

Leukotriene urine quantification was performed in all patients, and significant differences were observed in different groups of phenotypic overlapping (Figs. 2, 2C). No differences were observed between the allergic phenotypic overlapping with obesity (448.5 pg/mL (276.5–650

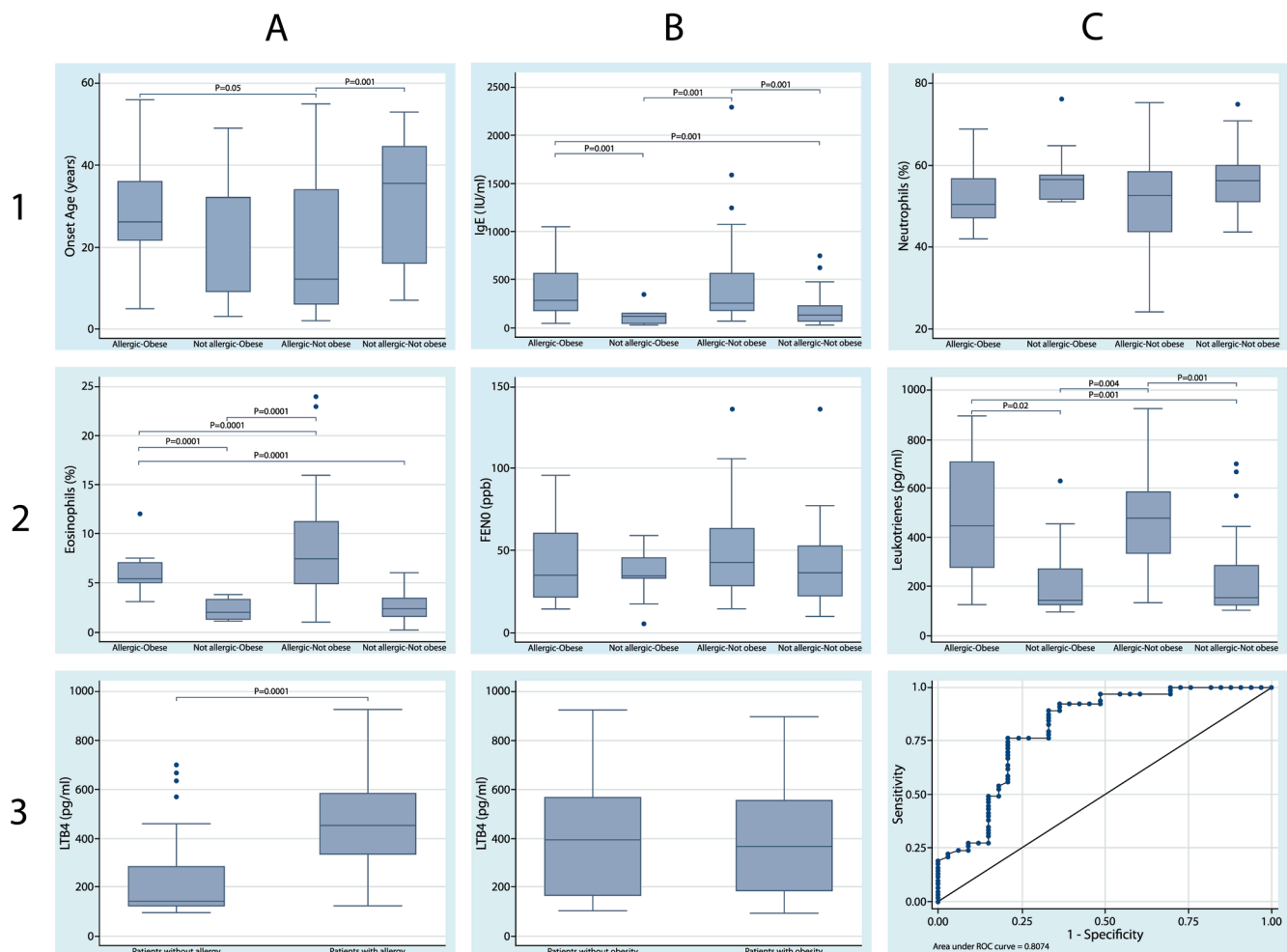


Fig. 2. Age of onset symptoms and inflammatory markers in patients with confirmed asthma diagnoses. Box 1A shows onset-age for each phenotypic interaction present in the population; box 1B shows IgE serum concentrations; boxes 1C and 2A show the percentages of circulating leukocytes; box 2C shows FeNO test; boxes 2C, 3A and 3B show LTB4 in urine; Finally, box 3C shows the discrimination capacity of LTB4 calculated using ROC curve. Lines indicate the significant differences with the corresponding p-value.

Table 3
IL-18/IL-18BP ratio in phenotypic overlapping of asthmatic patients.

	IL-18 pg/mL	IL-18BP pg/mL	Ratio
Allergic-Obese	1,675	17,205	1: 10.27
Not allergic Obese	1,192	10,435	1: 8.75
Allergic Not obese	1,645	16,890	1: 10.26
Not allergic Not obese	134	328.2	1: 2.44

Show median.

pg/mL)) or without obesity (476 pg/mL (335–587 pg/mL) (Figure 2, 3A and 3B). To explore whether this inflammatory mediator's urine concentration was able to distinguish the presence of the allergic factor specifically, LTB4 concentrations were compared between allergic and not-allergic patients and between patients with and without obesity. Indeed, urine concentrations of leukotrienes were only statistically different in the presence of allergy (allergic patients: 453 pg/mL (335–587 pg/mL); not allergic patients: 142 pg/mL (122–283 pg/mL) and not in the presence of obesity (obese patients: 367 pg/mL (187–555 pg/mL); not obese patients: 399 pg/mL (167–568 pg/mL). At ROC curve analysis, urine LTB4 had an area under a curve of 0.80 with a remarkable capacity to discriminate asthma allergic patients from not allergic asthma patients. LTB4, at a cut of value of ≥ 399 pg/ml had a sensitivity of 66.7% and a specificity of 79% for the classification of allergic asthma; however, with values higher than 670 pg/ml, specificity increase to 96% (Figure 2, 3C).

3.8. High correlation between IL and 18 and IL-18BP serum concentrations in non-allergic asthmatic patients

The production of IL-18 and its relationship with the negative regulator IL-18BP have recently been described as important in asthma development [21]. Therefore, we evaluated the correlation between these two molecules in patients with allergy and those with obesity. Interestingly, IL-18 and IL-18BP had a robust correlation in patients with obesity $\rho = 0.68$, p -value = 0.0001, but this correlation was not observed in subjects with an allergic background ($\rho = 0.09$, p -value = 0.45). We also evaluated the subjects with allergy and obesity overlapping; and in this group, the correlation between IL and 18 and IL-18BP was also observed ($\rho = 0.70$, p value = 0.0001).

On the other hand, when calculating the ratio IL-18/IL-18BP of the medians of serum concentrations of these two molecules in the 4 groups of phenotypic overlapping, it is observed that the lowest ratio is found in the group of not-obese and not-allergic patients (Table 3).

4. Discussion

This study, designed to explore different variables associated with the development of asthma in the phenotypes suggested by GINA, shows that asthmatic phenotypes do not act as independent entities but rather as overlapping involving two or more of the proposed phenotypes.

GINA defined asthma as a heterogeneous disease characterized by chronic airway inflammation and variable remodeling that results in a range of clinical presentations, treatment responses, and natural history across the patient's life course [8]. Asthma involves a history of respiratory symptoms, including wheezing, shortness of breath, chest tightness, and cough, which vary over time and in intensity, variable limitation of expiratory airflow, and hyperresponsiveness of the airways to a variety of conditions such as exercise and inhaled irritants. Asthma is not a disease that involves high or direct mortality; however, a subset of people with asthma exhibit an accelerated decline in lung function throughout their lives, consequently a progressive deterioration in their life quality [22].

The demography of the population analyzed in this trial is consistently similar to that previously documented in other populations of asthmatic patients, where a higher prevalence is observed in women in

the third decade of life [23,24]; however, a characteristic feature of the Mexican population sample that participated in this study was the high body mass index, with a marked tendency to overweight and obesity.

Obesity represents a major global health problem [25]. In Mexico, according to the National Health and Nutrition Survey, the prevalence of overweight and obesity is 33.2% in children and 72.5% in adults [26]. Therefore, the relationship between a high BMI and the development of asthma found in this study indicates that obesity has an intimate relationship to the development of degenerative pathologies.

According to the parameters established to define each of the phenotypes, we found that the main phenotypes found in this population were allergic, non-allergic, and associated with overweight or obesity and the airflow limitation phenotype was related to the overlapping of the phenotypes mentioned above. The late-onset phenotype was not found in the patients analyzed in this population.

Late-onset asthma is characterized by the onset of symptoms after 65 years of age [17], in addition to the predominance of a Th1 inflammatory response [27,28]. This particular phenotype of asthma has been associated with specific genetic variants associated with the development of allergy [29,30], so the absence of this phenotype in the sample of patients analyzed in this trial is impressive; however, more information is needed to determine if this phenotype is exclusive to a particular racial group.

Our findings confirm that the most representative phenotype of people who develop asthma is the allergy, with early onset in presenting symptoms. This phenotype has an important relationship with atopic history; indeed, together with other allergic manifestations such as eczema and allergic digestive complaints are included in pediatric pathologies of medical importance [31].

According to GINA, it is common to consider asthma with persistent airflow limitation as a particular phenotype [8]; nevertheless, we observed that this was a characteristic that was related to the different phenotypic overlapping; coinciding with previous observations reporting that airflow limitation is a clinical characteristic related to both allergic [32] and non-allergic asthma [33]. It is possible that the airflow limitation is a reflection of the intensity with which occurs the development of asthma and that this is related to factors such as the number of infections that these patients recurrently suffer [32].

Surprisingly, no differences were observed between the different phenotypic overlapping in most cytokines analyzed, even when it came to Th1 and Th2 inflammatory profiles. It has previously been reported that allergic asthma is characterized by the predominance of circulating eosinophils, while non-allergic asthma is characterized by an abundance of neutrophils [34]; however, when analyzing the cytokines involved in the development of the different asthmatic phenotypes, a combination of the different Th1, Th2, and Th17 inflammatory profiles are observed. Although Th2 cytokines are predominant in all cases, which favors airway remodeling mechanisms, elevated levels of INF- γ and IL-17A were also observed in all cases [34], which could be explained by the persistence of respiratory infections such as bacteria, viruses, and fungi to which asthmatic patients of all phenotypes are most susceptible [32,35].

On the other hand, our results strongly suggest that inflammasome activation is an inflammatory mechanism that acts differentially. Zhang et al. previously associated the production of cytokines related to the inflammasome with allergic asthma development [21]. Our results confirm that these cytokines are a distinctive feature of asthma's allergic process, but not in asthmatic patients without this characteristic.

There is evidence that the expression of IL-18 increases the presentation of many allergic conditions [36]; nevertheless, its endogenous antagonist, IL-18BP, prevents the binding of this cytokine to its receptor, neutralizing the biological effect. At a molar excess of two, IL-18BP neutralizes IL-18 to > 95% [37], and an imbalance between IL and 18 and IL-18BP expression may account for increased IL-18 activity in asthma [21].

Belonging to the IL-1 family, IL-18 is produced due to inflammasome

activation; and plays an important role in both innate and acquired immunity. It has been describe that IL-18 stimulates T cells to produce IFN- γ , IL-13, and IL-5 [38], once IL-13 and IL-5 are potent Th2 cytokines, which play a critical pro-inflammatory role in atopic asthma, it is suggested that IL-18 ought to be a causative factor for asthma [21]. On the other hand, it has been shown that the ratio between IL and 18 and IL-18BP determines the severity of clinical pictures related to the activity of the inflammasome. When to ratios favor IL-18BP, the control of IL-18 activity increases, on the other side, when the ratios favor IL-18, it would increase signs and symptoms [39]. It was shown that inflammasome could be activated by fatty acids and high glucose, linking metabolic danger signals to the activation of chronic inflammation [40]. Our results showed high levels of IL-18 in obese patients, indicating activation of the inflammasome, a fact that would lead to the inflammatory cell death known as pyroptosis, that would induce tissue remodeling, a characteristic feature of degenerative processes such as asthma. On the other hand, our results show that the largest ratios were those in the phenotypic overlapping with the presence of allergy; however, we had smaller ratio in the phenotypic overlapping with non-allergic patients, which would indicate a greater persistence in the manifestation of asthma. The clinical significance of this finding will be explored in future studies.

Some other markers of local and systemic inflammation have been explored to evaluate the development of asthma. Gegin et al. developed a study in which they analyzed FENO and LBT4 in obese and non-obese asthmatic patients and compared these markers with healthy subjects; the authors concluded that FENO and LBT4 were increased in asthmatic patients compared to healthy subjects. However, there was no difference between asthmatic patients, regardless of whether they are obese [41].

This study adds the presence or absence of allergy to the equation, and we did not find differences in FENO in the different phenotypic overlapping. Nonetheless, when analyzing LTB4, we observed significant differences between allergic asthmatic patients compared those who are not. Leukotrienes (LTs) are lipid mediators that play pivotal roles in acute and chronic inflammation and allergic diseases. They exert their biological effects by binding to specific G-protein-coupled receptors. Specifically, LTB4 has been associated with allergic processes, including asthma [42]. The finding that we reported supports the efficient use of anti-leukotriene drugs to control moderate and severe asthma [43].

This study is not free of limitations, one of them is the number of patients enrolled; despite this, patients included had no prior pharmacological treatment, which allowed us a clearer analysis of the participating molecules and different phenotypic interactions without alterations. On the other hand, the measurements did not include components produced directly in respiratory way by obtaining of sputum or bronchiole alveolar lavage; however, results obtained in a systemic way open the possibility of future studies that analyze the phenotypes and molecules produced *in situ* separately.

Taken together, our results allow us to conclude that asthma does not develop as particular phenotypes but rather as an overlapping of multiple conditions that favor the development of this degenerative pathology. Although few molecules distinguish conditions, some can be used as markers and beyond this as possible therapeutic targets.

CRedit authorship contribution statement

Espiridión Ramos-Martínez: Conceptualization, Funding acquisition, Writing - review & editing. **Jorge Rojas-Serrano:** Visualization. **Warrison Andrade:** Visualization. **Araceli Rojas-Bernabé:** . **Ramcés Falfán-Valencia:** Visualization, Investigation, Writing - original draft. **Gloria Pérez-Rubio:** Visualization, Investigation, Writing - original draft. **Rosa Campos-Gutierrez:** Methodology, Supervision. **Rubén Cruz-Pantoja:** Methodology, Supervision. **Andrea Velasco-Medina:** Methodology, Supervision. **Guillermo Velázquez-Sámano:** Conceptualization, Funding acquisition, Writing - review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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