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Research article

Promoter methylation status of *RORC*, *IL17A*, and *TNFA* in peripheral blood leukocytes in adolescents with obesity-related asthma

José J. Leija-Martínez [a,b,1], Abraham Giacoman-Martínez [b,c,1], Blanca E. Del-Río-Navarro [a,d], Fausto Sanchéz-Muñoz [a,e], Adrián Hernández-Diazcouder [e], Onofre Muñoz-Hernández [a], Rodrigo Romero-Nava [b,f], Santiago Villafaña [f], Laurence A. Marchat [g], Enrique Hong [a,c], Fengyang Huang [a,b,*]

[a] *Universidad Nacional Autónoma de México, Programa de Maestría y Doctorado en Ciencias Médicas, Odontológicas y de la Salud, Mexico City, Mexico*
[b] *Hospital Infantil de Mexico Federico Gómez, Research Laboratory of Pharmacology, Mexico City, Mexico*
[c] *Department of Pharmacobiology, Centro de Investigacion de Estudio Avanzados del Instituto Politecnico Nacional, Calz. de Los Tenorios 235, Col. Granjas Coapa, Mexico City 14330, Mexico*
[d] *Hospital Infantil de México Federico Gómez, Department of Pediatric Allergy-Clinical Immunology, Mexico City, Mexico*
[e] *Departamento de Inmunología, Instituto Nacional de Cardiología "Ignacio Chávez", Mexico City, Mexico*
[f] *Laboratorio de Señalización Intracelular, Sección de Estudios de Posgrado e Investigación, Escuela Superior de Medicina, Instituto Politécnico Nacional, Mexico*
[g] *Laboratorio 2 de Biomedicina Molecular, ENMH, Instituto Politécnico Nacional, Mexico*

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ABSTRACT

A higher Th17-immune response characterises obesity and obesity-related asthma phenotype. Nevertheless, obesity-related asthma has a more significant Th17-immune response than obesity alone. Retinoid-related orphan receptor C (RORC) is the essential transcription factor for Th17 polarisation. Previous studies have found that adolescents with obesity-related asthma presented upregulation of *RORC*, *IL17A*, and *TNFA*. However, the mechanisms that cause these higher mRNA expression levels in this asthmatic phenotype are poorly understood. Methylation directly regulates gene expression by adding a methyl group to carbon 5 of dinucleotide CpG cytosine. Thus, we evaluated the relationship between *RORC*, *IL17A*, and *TNFA* methylation status and mRNA expression levels to investigate a possible epigenetic regulation. A total of 102 adolescents (11–18 years) were studied in the following four groups: 1) healthy participants (HP), 2) allergic asthmatic participants (AAP), 3) obese participants without asthma (OP), and 4) non-allergic obesity-related asthma participants (OAP). Real-time qPCR assessed the methylation status and gene expression levels in peripheral blood leukocytes. Remarkably, the OAP and AAP groups have lower promoter methylation patterns of *RORC*, *IL17A*, and *TNFA* than the HP group. Notably, the OAP group presents lower *RORC* promoter methylation status than the OP group. Interestingly, *RORC* promoter methylation status was moderately negatively associated with gene expression of *RORC* ($r_s = -0.39$, $p < 0.001$) and *IL17A* ($r_s = -0.37$, $p < 0.01$), respectively. Similarly, the promoter methylation pattern of *IL17A* was moderately negatively correlated with *IL17A* gene expression ($r_s = -0.3$, $p < 0.01$). There is also a moderate inverse relationship between *TNFA* promoter methylation status and *TNFA* gene expression ($r_s = -0.3$, $p < 0.01$). The present study suggests an association between lower *RORC*, *IL17A*, and *TNFA* gene promoter methylation status with obesity-related asthma and allergic asthma. *RORC*, *IL17A*, and *TNFA* gene promoter methylation patterns are moderately inversely correlated with their respective mRNA expression levels. Therefore, DNA methylation may regulate *RORC*, *IL17A*, and *TNF* gene expression in both asthmatic phenotypes.

* Corresponding author.
E-mail address: huangfengyang@gmail.com (F. Huang).
[1] Both authors contributed equally to this work.

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1. Introduction

Obesity has become a global epidemic, which has been related to a particular non-T2 asthma phenotype [1, 2]. In this asthmatic phenotype, the immunological mechanisms remain unclear; nevertheless, it has been reported that the immune profile is predominantly Th1/Th17 [3, 4, 5, 6, 7, 8]. Interleukin (IL)-17A is principally produced by Th17 lymphocytes, and retinoid-related orphan receptor C (RORC) is the essential transcription factor for Th17 polarisation [9, 10, 11]. Consequently, a RORγt insufficiency induced a severe impairment of Th17 cell differentiation with an important reduction of IL-17A levels [12, 13]. Tumour necrosis factor-alpha (TNF-α) and IL-17A play a critical role in obesity-related asthma, causing direct damage to the airway epithelium through the recruitment of neutrophils, wreaking serious histological damage, fibrosis, and bronchial remodelling [14, 15].

It is worth noting that Holguin et al. [16] described two phenotypes of asthma with obesity, the T2 asthma phenotype and the non-T2 asthma phenotype, respectively. The T2 asthma phenotype is characterised by the early onset, mainly in childhood before 12 years of age. Typically, these kinds of patients have an atopic march and atopic asthma and put on weight during their lives and get obese; the immunologic pathway of this asthmatic phenotype is elicited mostly through Th2 lymphocytes [3, 4, 5, 6, 7, 8, 16]. On the other hand, the non-T2 asthma phenotype is characterised by the late onset, often after 12 years of age and with a non-atopic phenotype. The immunological mechanisms in this non-allergic phenotype are triggered principally by Th17 cells, M1-type macrophages, and type 3 innate lymphoid cells (ILC3s) [3, 4, 5, 6, 7, 8, 16].

In addition, Choy et al. [17] showed in a transcriptional analysis of endobronchial tissue from adults with three different asthma phenotypes: Th2-high, Th17-high, and Th2–Th17 low, a significant inverse relationship between *IL13* and *IL17A* mRNA expression levels ($r_s = -0.35$, $p = 0.011$). Furthermore, Th2-high and Th17-high gene signatures in endobronchial biopsies were reciprocally exclusive: no asthmatic subjects had the Th2-high and Th17-high gene signatures at the same time. Recently, Chi et al. [18] documented the critical role of RORγt in preserving the Th17 cell differentiation by depleting Th2 polarisation. These reports indicate that both immune mechanisms are noticeably different and could be mutually exclusive. As we can notice, the best phenotype to assess the Th17-immune response and methylation status of *RORC*, *IL17A*, and *TNFA* is the non-T2 asthma phenotype; therefore, our research has focused on this phenotype.

Importantly, Wu et al. [19] found that adolescents with obesity-related asthma had higher *RORC* and *IL17A* mRNA expression levels. Similarly, we reported that adolescents with non-T2 asthma phenotype presented an upregulated gene expression of *RORC, IL17A*, and *TNFA* and higher serum IL-17A and TNF-α levels [20]. It is well-known that a Th17-immune response characterises obesity; however, the obesity-related asthma phenotype has a higher Th17 polarisation than obesity alone [20, 21, 22]. Nonetheless, the specific biological mechanisms which cause the upregulation of *RORC*, *IL17A*, and *TNFA* in this asthmatic phenotype remain poorly understood.

A potential explanation could be epigenetics mechanisms, especially the methylation pattern. Methylation directly regulates gene expression by adding a methyl group (CH3) to carbon 5 of dinucleotide CpG cytosine [23]. Consequently, the 5-methylcytosine constrains gene expression by impeding the binding of a transcription factor to DNA. In contrast, a gene promoter with unmethylated CpG dinucleotides in its DNA is better able to interact with transcription factors resulting in higher gene expression.

Therefore, we hypothesised that obesity-related asthma phenotype may have lower *RORC*, *IL17A*, and *TNFA* gene promoter methylation patterns; as a result, there could be an upregulated gene expression of *RORC, IL17A*, and *TNFA* [24]. The primary objective was to assess *RORC*, *IL17A*, and *TNFA* gene promoter methylation status in adolescents with non-allergic obesity-related asthma. We also explored the relationship between *RORC*, *IL17A*, and *TNFA* gene promoter methylation patterns with their respective mRNA expression levels to investigate a possible epigenetic regulation.

2. Methods

2.1. Study design

This was a cross-sectional study of 102 adolescents divided into four groups of participants according to their body mass index (BMI) and asthma phenotype (allergic and non-allergic). This study was conducted at the Children's Hospital of Mexico Federico Gómez, Mexico City. In addition, we carried out a convenience sample from outpatient adolescents.

2.2. Ethics approval

This study was carried out according to the Declaration of Helsinki, revised in 2013 [25]. This clinical research was approved by the local Ethics Committee (approval number: HIM/2013/015.SSa.1601 and HIM/2020/030.SSa.1667). After the study was approved, informed consent was obtained from all the participant's parents or guardians, and informed assent was obtained from all the adolescents.

2.3. Subjects

Adolescents of either gender aged 11–18 years were recruited and classified into the following four groups: 1) healthy participants (HP), 2) allergic asthmatic participants (AAP), 3) obese participants without asthma (OP), and 4) non-allergic obesity-related asthma participants (OAP). Obesity was diagnosed as a BMI ≥95th percentile [26]. BMI was measured by the classic formula of BMI = weight in kilograms/(height in metres)^2. A paediatric allergist/immunologist diagnosed asthma according to the Global Initiative for Asthma (GINA) criteria with a documented history of asthma at least 6 months prior to the enrolment visit [27]. Reversible airway obstruction was necessary to confirm asthma diagnosis; it was defined as an increase in forced expiratory volume in 1 s (FEV1) ≥12% and 200 ml relative to baseline after inhalation of a rapid-acting β2-agonist measured by spirometry. The asthma severity was graded as mild intermittent, mild persistent, and moderate persistent asthma [28]. The inclusion criteria for allergic asthma without obesity were a positive skin prick test (SPT) for at least one air allergen. Finally, we considered adolescents with obesity-related asthma with the following eligibility criteria: a BMI ≥95th percentile and obesity had to precede the onset of asthma. Importantly, these asthmatic adolescents had to have a non-allergic asthma phenotype defined as a negative SPT. We precluded all participants with chronic diseases and treatment with systemic corticosteroids.

2.4. Skin prick test

A paediatric allergist carried out SPTs using a standard inhalant allergen panel (dust mites, cockroaches, dog epithelium, standardised cat hair, and pollen of weeds, trees, and grasses) (ALK-Abelló®, Copenhagen, Denmark). We used histamine (10 mg/mL) and glycerine as the positive and negative control, respectively. Additionally, a wheal diameter of ≥3 mm was considered positive, as well as allergic asthma.

2.5. DNA extraction and sodium bisulfite modification

According to the manufacturer's instructions, DNA from peripheral blood leukocytes was extracted using the Master Pure™ Kit (Epicentre®, San Diego, CA, USA). First, Nanodrop assessed DNA purity (ratio 260/280: ~1.8 y 2.0), and DNA integrity was checked by 1% agarose gel electrophoresis by showing a defined band without degradation. Next, DNA was subjected to bisulfite modification to convert all unmethylated cytosines into uracils leaving methylated cytosines unaltered, allowing specific detection of methylated or unmethylated DNA. Finally, following the manufacturer's protocol, bisulfite modification of 4 μg genomic DNA was conducted with the EpiTect® Fast DNA Bisulfite kit (QIAGEN, Venlo, Netherlands).

Cycling conditions of bisulfite conversion were at 95 °C (denaturation) for 5 min, followed by at 60 °C (incubation) for 20 min, at 95 °C (denaturation) for 5 min, at 60 °C (incubation) for 20 min, and finally at 20 °C. Subsequently, modified DNA's purification and recovery process continued according to the manufacturer's protocol and stored at -30°c for further experiments.

2.6. Primer design

We searched *RORC* and *IL17A* gene promoter sequences at National Center for Biotechnology Information (NCBI) platform [29], analysing at least 2.5 kb upstream for each gene. Primers for methylation-specific PCR were designed using MethPrimer (Li LC, Dahiya R, 2002), searching regions or CpG islands with at least 50% of CpG's (Table 1). The selected regions for methylated and unmethylated primer design were more proximal to the gene (1 kb upstream), considered the most crucial region to expression gene, considering that the primers were complementary regions for CpG's susceptible to methylation events. For the promoter region of the *TNFA* gene, we used the primers reported by Cordero et al. [30] (Table 1).

2.7. Methylation-specific PCR

DNA methylation analysis was performed by real-time qPCR. The modified DNA was used as a template to assess the methylation pattern of a gene promoter sequence using FastStart SYBR™Green Master technology in a thermal cycler Prime Pro Real-time qPCR (TECHNE, Staffordshire, UK). Cycling conditions were at 94 °C for 5 s, followed by 57 °C–58 °C (depending on each set of primers, see Table 1) for 30 s and 72 °C for 30 s. The melting curves were performed to ensure that non-specific products were not amplified. The following formula determined the percentage of methylation.

$$Methylation\ \ status = \left(\frac{Methylated\ \ DNA}{Methylated\ \ DNA + Unmethylated\ \ DNA}\right) * 100$$

2.8. Statistical analysis

Numerical data were presented as the median and interquartile range (IQR). We performed the Mann-Whitney *U* and Kruskal-Wallis tests to compare the medians between two and more than two groups, respectively. Pearson's chi-squared test was carried out for analysing categorical variables. Spearman's correlation coefficients were applied to all correlations for analysis. Finally, we conducted a multivariate linear regression model to adjust confounding variables. To meet the assumptions for a multivariate regression model, we transformed all quantitative variables into the natural logarithm to obtain more robust data with a normal distribution. All data were analysed using SPSS version 22 (SPSS, Inc., IBM, Chicago, IL, USA), and box-plot graphs were performed by GraphPad Prism software version 8 (GraphPad Software, La Jolla, Calif). A p-value of less than 0.05 was considered significant, and all p-values are two-sided.

Table 1. Gene sequences for forward (F) and reverse (R) primers used for methylation-specific RT-qPCR.

Gene promotor	Primer sequence (5′-3′)	Annealing temperature (°C)
TNFA (M)	F: TTAGAAGATTTTTTTCGGAATC	57 °C (40)
	R: TATCTCGATTTCTTCTCCATCG	57 °C (40)
TNFA (UM)	F: GGTTTAGAAGATTTTTTTTGGAATT	57 °C (40)
	R: TCTATCTCAATTTCTTCTCCATCAC	57 °C (40)
RORC (M)	F: TTTAATAAGGGGATTGGGTACG	58 °C (40)
	R: GTCCTTTCAAAACAAAACGATA	58 °C (40)
RORC (UM)	F: TTAATAAGGGGATTGGGTATGTG	58 °C (40)
	R: CATCCTTTCAAAACAAAACAATA	58 °C (40)
IL17A (M)	F: TGGGAGGTAAAGTGTCGTTA	58 °C (40)
	R: AAACAATATAAAAACTCCCTACGCA	58 °C (40)
IL17A (UM)	F: GTGATTTGGGAGGTAAAGTGTT	58 °C (40)
	R: ACAATATAAAAACTCCCTACACAA	58 °C (40)

M, methylated; UM, unmethylated.

3. Results

3.1. Demographic and clinical characteristics

As we can see, Table 2 summarises the demographic and clinical characteristics of all participants. The adolescents with obesity (OAP and OP groups) had more significant anthropometric measures compared to the normal-weight participants (HP and AAP groups). In addition, the obesity-related asthma group presented a more significant history of obesity in years compared to the obese adolescents without asthma (Table 2).

The majority of the asthma severity of all asthmatic participants was awarded as mild persistent asthma. Obesity-related asthma and allergic asthma groups had the same treatment based on inhaled corticosteroids and antileukotrienes (Table 2). Additionally, there is no statistically significant difference in the history of asthma in years between both asthmatic phenotypes.

3.2. RORC, IL17A, and TNFA gene promoter methylation status

RORC gene promoter methylation status was lower in the OAP group (43.9% (interquartile range (IQR)):40.9–48.2) compared to the HP group (54.8% (IQR: 52.4–57.5), $p < 0.001$) or the OP group (50.6% (IQR: 47.2–53.1), $p < 0.01$). However, there is no statistically significant difference between the OAP and AAP groups (43.9% (IQR: 40.9–48.2) vs 45.6% (IQR: 41.8–52.4), $p = 0.5$) (Figure 1A, Supplementary Table S1, and Supplementary Table S2). Furthermore, the *RORC* gene promoter methylation pattern was lower in Tanner stage III adolescents compared to Tanner stage II ($\beta = -5.4$, 95% CI (−10.4, −0.5), $p = 0.03$) (Supplementary Table S2). Interestingly, the more significant the asthma history in years is, the lower the *RORC* gene promoter methylation levels are ($\beta = -4.8$ 95% CI (−8.9, −0.7), $p = 0.02$) (Supplementary Table S3).

As illustrated in Figure 1B, *IL17A* gene promoter methylation status was lower in the OAP group (47.3% (IQR: 45.2–49.2)) than in the HP group (53.4% (IQR: 52.0–55.5), $p < 0.001$). On the contrary, there are no significant differences between the OAP group versus the AAP group (47.3% (IQR: 45.2–49.2) vs 44.45% (IQR: 42.6–49.2), $p = 0.1$) and between the OAP group versus the OP group (47.3% (IQR: 45.2–49.2) vs 45.4% (IQR: 44.3–47.0), $p = 0.05$) (Figure 1B and Supplementary Table S1). In addition, *IL17A* gene promoter methylation status was greater in mild intermittent asthmatics adolescents than in those in mild persistent ($\beta = -6.7$, 95% CI (−11.7, −1.6), $p = 0.01$) and moderated persistent ($\beta = -9.7$ 95% CI (−15.6, −3.8), $p = 0.002$) (Supplementary Table S3).

TNFA gene promoter methylation status was lower in the OAP group (46.6% (IQR: 44.1–49.2)) compared to the HP group (55.2% (IQR: 53.9–56.0), $p < 0.001$). In contrast, no statistically significant differences were found between the OAP group versus the AAP group (46.6% (IQR: 44.1–49.2) vs 47.6% (IQR: 46.7–49.4), $p = 0.09$) and between the OAP versus OP groups (46.6% (IQR: 44.1–49.2) vs 46.6% (IQR: 44.7–48.5), $p = 1.0$) (Figure 1C and Supplementary Table S1).

As reported in Supplementary Table S1 and Supplementary Table S2, *TNFA* gene promoter methylation levels were lower in Tanner stage IV adolescents compared to in Tanner stage II ($\beta = -3.4$, 95% CI (−6.7, −0.1), $p = 0.04$) and ($\beta = -5.5$, 95% CI (−10.5, −0.5), $p = 0.03$), respectively.

It is worth highlighting that both the AAP and OP groups have lower promoter methylation patterns of *RORC*, *IL17A*, and *TNFA* compared to the HP group (Figure 1A, Figure 1B, and Figure 1C).

Table 2. Demographic and clinical characteristics.

	HP (n = 23)	AAP (n = 29)	OP (n = 28)	OAP (n = 22)	P value
Age, years	16.0 (13.0–18.0)	13.0 (12.0–17.0)	14.0 (13.0–17.7)	14.0 (11.0–17.0)	0.2$
Female, n (%)	12 (52.2%)	17 (58.6%)	17 (60.7%)	11 (50.0%)	0.9*
Tanner, n II/III/IV/V	1/6/6/10	3/11/7/8	4/6/10/8	4/7/5/6	0.8*
Weight, kg	54.0 (45.0–58.5)	48.0 (41.6–57.3)	74.8 (70.3–84.9) &#	71.4 (64.0–81.1) &#	<0.001$
Height, cm	160.0 (154.0–165.0)	155.0 (147.5–164.5)	159.1 (155.0–162.0)	159.1 (152.7–165.5)	0.5$
BMI, kg/m^2	21.0 (17.8–23.4)	20.1 (18.5–21.1)	30.5 (28.0–32.3) &#	27.9 (26.7–31.2) &#	<0.001$
BMI percentile, %	52.0 (43.0–65.0)	70.0 (41.0–76.5)	97.0 (96.0–98.8) &#	97.0 (96.0–98.1) &#	<0.001$
BMI z-score	0.2 (0.0–0.3)	0.4 (−0.2–0.8)	1.8 (1.7–2.0) &#	1.8 (1.7–2.1) &#	<0.001$
C. abdomen, cm	76.0 (70.4–83.0)	74.0 (69.4–80.0)	98.0 (93.3–102.7) &#	96.5 (90.4–99.9) &#	<0.001$
C. waist, cm	74.2 (67.0–81.8)	72.0 (65.5–79.0)	92.4 (89.6–98.8) &#	90.5 (84.5–96.8) &#	<0.001$
C. hip, cm	85.5 (78.3–90.8)	82.0 (79.3–89.8)	103.0 (99.9–109.1) &#	101.0 (94.7–108.6) &#	<0.001$
Time with obesity, (years)			5.0 (5.0–5.0)	9.0 (7.0–10.3)	<0.001¶
Time with asthma, (years)		6.0 (5.0–8.0)		5.0 (5.0–6.0)	0.1¶
Severity of asthma					0.08*
Mild intermittent (n, %)		6 (20.7%)		1 (4.5%)	
Mild persistent (n, %)		18 (62.1%)		12 (54.5%)	
Moderate persistent (n, %)		5 (17.2%)		9 (40.9%)	
Inhaled steroid (n, %) ++		15 (52%)		12 (54.5%)	0.8*
Antileukotriene (n, %)		5 (17.2%)		7 (31.8%)	0.2*

$ Kruskal-Wallis, * Pearson's chi-square, and ¶ Mann-Whitney U tests. Values are expressed as the medians and interquartile ranges. Abbreviations: BMI, body mass index; C, circumference; HP, healthy participants; AAP, allergic asthmatic participants; OP, obese participants without asthma; OAP, non-allergic obesity-related asthma participants.

++ Dose equivalent to 200–400 μg of budesonide.

& $p < 0.001$ vs the healthy group; #$p < 0.001$ vs the allergic asthma group.

Reproduced and modified with permission from Elsevier, reference Leija-Martínez et al. [20].

3.3. Correlations between gene promoter methylation status and mRNA expression levels for RORC, IL17A, and TNFA

In this same clinical sample of adolescents, we previously found that *TNFA* mRNA expression levels were more significant in the OAP group compared to the HP group or the OP group (Figure 2A) [20]. Similarly, TNF-α levels were greater in the OAP group compared to the OP group (Figure 2B). Additionally, *IL17A* mRNA expression was more significant in the OAP group than in the HP group, the AAP group, or the OP group (Figure 2C) [20]. Our previous report also showed that serum IL-17A levels were greater in the OAP group compared to the HP group or the AAP group (Figure 2D). Importantly, *RORC* mRNA expression levels were more significant in the OAP group compared to the HP group, the AAP group, or the OP group (Figure 2E) [20].

As a result, we evaluated the relationship of methylation levels with mRNA expression to investigate a potential epigenetic regulation. Interestingly, there is a moderate inverse association between *RORC* gene promoter methylation status and *RORC* mRNA expression levels ($r_s = -0.39$, $p < 0.001$) (Figure 3A). A similar correlation was also observed between *IL17A* gene promoter methylation status and *IL17A* mRNA expression levels ($r_s = -0.3$, $p < 0.01$) (Figure 3B). Notably, a moderate negative relationship was also found between *RORC* gene promoter methylation status and *IL17A* mRNA expression levels ($r_s = -0.37$, $p < 0.01$) (Figure 3C). Likewise, a moderate negative association was observed between *TNFA* gene promoter methylation status and *TNFA* mRNA expression levels ($r_s = -0.3$, $p < 0.01$) (Figure 3D).

4. Discussion

Our findings reveal that *RORC*, *IL17A*, and *TNFA* gene promoter methylation status is lower in obesity-related asthma phenotype than in healthy adolescents. Similarly, Jeong et al. [31] showed that the methylation status of the NLRP3-IL1B-IL17 axis, including *RORC* and *IL17A* gene promoters, was different between healthy adults and non-atopic obesity-related asthma.

This study also shows that allergic asthmatic adolescents have lower promoter methylation patterns of *RORC*, *IL17A*, and *TNFA* than healthy adolescents. However, although we previously documented that the obesity-related asthma participants had higher *RORC* and *IL17A* mRNA expression levels and more significant serum IL-17A levels than the allergic asthmatic participants, this research was unable to find statistically significant differences between these groups in promoter methylation levels [20].

Possibly, the Th17 pathway may also play an essential role in the pathophysiology of allergic asthma; our results also showed that allergic asthmatic adolescents presented more significant *RORC*, *IL17A*, and *TNFA* mRNA expression levels compared to healthy participants [20]. Apparently, IL-17A and TNF-α may also be causing lung damage in this allergic phenotype, as obesity-related asthma does [32, 33, 34].

It is well-known that the immune response of allergic asthma is orchestrated mainly by Th2 lymphocytes, which release IL-4, IL-5, and IL-13; consequently, there is a specific IgE synthesis by B cells and eosinophils recruitment in the respiratory epithelium [32, 33, 34]. In contrast, the immune response in obesity-related asthma is mediated primarily by Th17 cells, ILC3s, and M1-type macrophages; as a result, there is an increase in TNF-α, IL1-β, IL-6, IL-17A, and IL-23 [3, 4, 5, 6, 7, 8, 32, 33, 34]. It is worth emphasising that the immunological mechanisms noticeably differ between these asthmatic phenotypes, as asthma development has different pathways. Therefore, due to different immunological pathways and our findings, it is complicated to claim that the immunologic differences between these phenotypes are associated with *RORC*, *IL17A*, and *TNFA* promoter methylation status.

Even though our previous research found that obesity-related asthma had more significant *IL17A* and *TNFA* mRNA expression levels than in the obesity alone group, this study cannot demonstrate that the obesity related-asthma adolescents had lower promoter methylation patterns of *IL17A* and *TNFA* than obesity alone [20]. Similarly, our previous findings showed no statistically significant differences between these groups of adolescents in serum IL-17A [20]. Besides, the obesity alone group shows lower *RORC*, *IL17A*, and *TNFA* gene promoter methylation status than

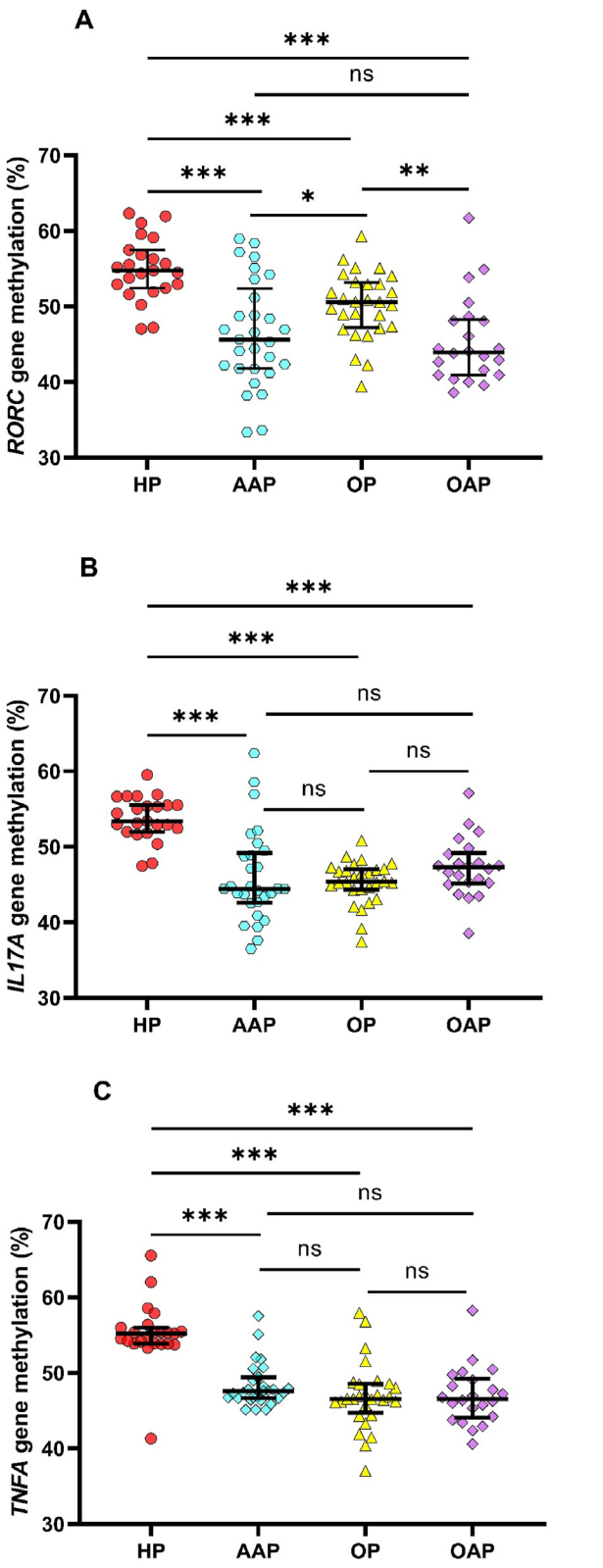

Figure 1. *RORC*, *IL17A*, and *TNFA* gene promoter methylation status in the four groups of adolescents. (A) *RORC* promoter methylation levels, (B) *IL17A* promoter methylation levels, and (C) *TNFA* promoter methylation levels. The data are presented as the median and interquartile range. The Kruskal-Wallis test with the Mann-Whitney *U* test for post hoc analysis was performed to analyse significant differences in the medians. Abbreviations: ns, not significant; HP, healthy participants; AAP, allergic asthmatic participants; OP, obese participants without asthma; OAP, obesity-related asthma participants. *p < 0.05, **p < 0.01, ***p < 0.001.

the healthy participants. Presumably, the methylation status of these gene promoters may be as crucial in the physiopathology of obesity as in the physiopathology of non-T2 asthma phenotype; both have a Th17-immune response [20, 21, 22].

RORC is entirely essential to differentiate naïve CD4 + T cells into Th17 lymphocytes; obesity-related asthma had higher *RORC* mRNA expression levels compared with obesity alone [10, 11, 20]. Thus, we believe that the most critical point in this research is that the obesity-related asthma adolescents have a lower *RORC* gene promoter methylation status compared to the obesity alone group; as a result, the obesity-related asthma phenotype may have more significant *RORC* and *IL17A* mRNA expression levels than obesity alone. We also showed a moderate negative correlation between *RORC* gene promoter methylation status with *RORC* and *IL17A* mRNA expression levels. Hence, the big question is why some adolescents with obesity become non-allergic asthmatics while other obese adolescents do not? The answer might be associated with the promoter methylation status of critical cytokines linked to the chronic low-grade inflammation of obesity, for instance, the *RORC* gene promoter [24].

Furthermore, it is worth highlighting that *RORC* gene promoter methylation plays a fundamental function in Th17 polarisation. Importantly, Mazzoni et al. [35] demonstrated that promotor methylation levels of *RORC* and *IL17A* in Th17 cells were nearly unmethylated (~0.5% and ~1.5%, respectively); conversely, the *RORC* and *IL17A* methylation status in the naïve CD4 + T lymphocytes was far more significant (~62% and ~78%, respectively). In addition, we showed a moderate negative association between *RORC* gene promoter methylation status with *RORC* and *IL17* mRNA expression levels. Arguably, the *RORC* gene promoter methylation pattern may manage the Th17 cells for producing *RORC* and *IL17A* mRNA expression levels.

Taken together, these findings indicate that DNA methylation may regulate *RORC*, *IL17A*, and *TNFA* gene expression in obesity-related asthma and allergic asthma. To evaluate our hypothesis, we performed correlations of promoter methylation patterns with their respective gene expression in peripheral blood leukocytes. As a result, we confirmed inverse correlations between the promoter methylation status of *RORC*, *IL17A*, and *TNFA* with their respective gene expression levels.

Finally, it is also worth highlighting that methylation patterns could be continually changing with ageing, Tanner stage, asthma severity, and the evolution time of asthma [36, 37]. For example, ageing is characterised by global hypomethylation and regions of CpG island hypermethylation [36, 37]. These modifications in epigenetics might generate lower gene promoter methylation status in older adolescents.

Evidently, our research cannot show that the promoter methylation status of *IL17A* and *TNFA* was inferior in the obesity-related asthma group compared to the allergic asthma participants or the obesity alone group. Therefore, our results suggest that the promoter methylation patterns of *IL17A* and *TNFA* are not explicitly related to obesity-related asthma but to both asthmatic phenotypes.

Nonetheless, we believe that only *RORC* gene promoter methylation status is specifically associated with obesity-related asthma due to a lower *RORC* gene promoter methylation status than the obesity alone group, a moderate negative relationship between *RORC* gene promoter methylation pattern with *RORC* and *IL17* mRNA expression levels, and the immunological similarities between obesity-related asthma and obesity alone. Moreover, indisputably, the pathophysiological process of non-T2 asthma phenotype firstly involves obesity and, afterwards, the development of obesity-related asthma.

It is worth highlighting that the above is a hypothesis in the testing process. Therefore, we acknowledge that our findings reported are too premature to substantiate our hypothesis; more work is needed to study this topic in more detail.

This study has a few critical limitations: 1) As this is an observational study, we cannot infer causality. In addition, this cross-sectional design is susceptible to possible recall bias. 2) This research has a small sample size. We should have tested our hypothesis in a large sample of

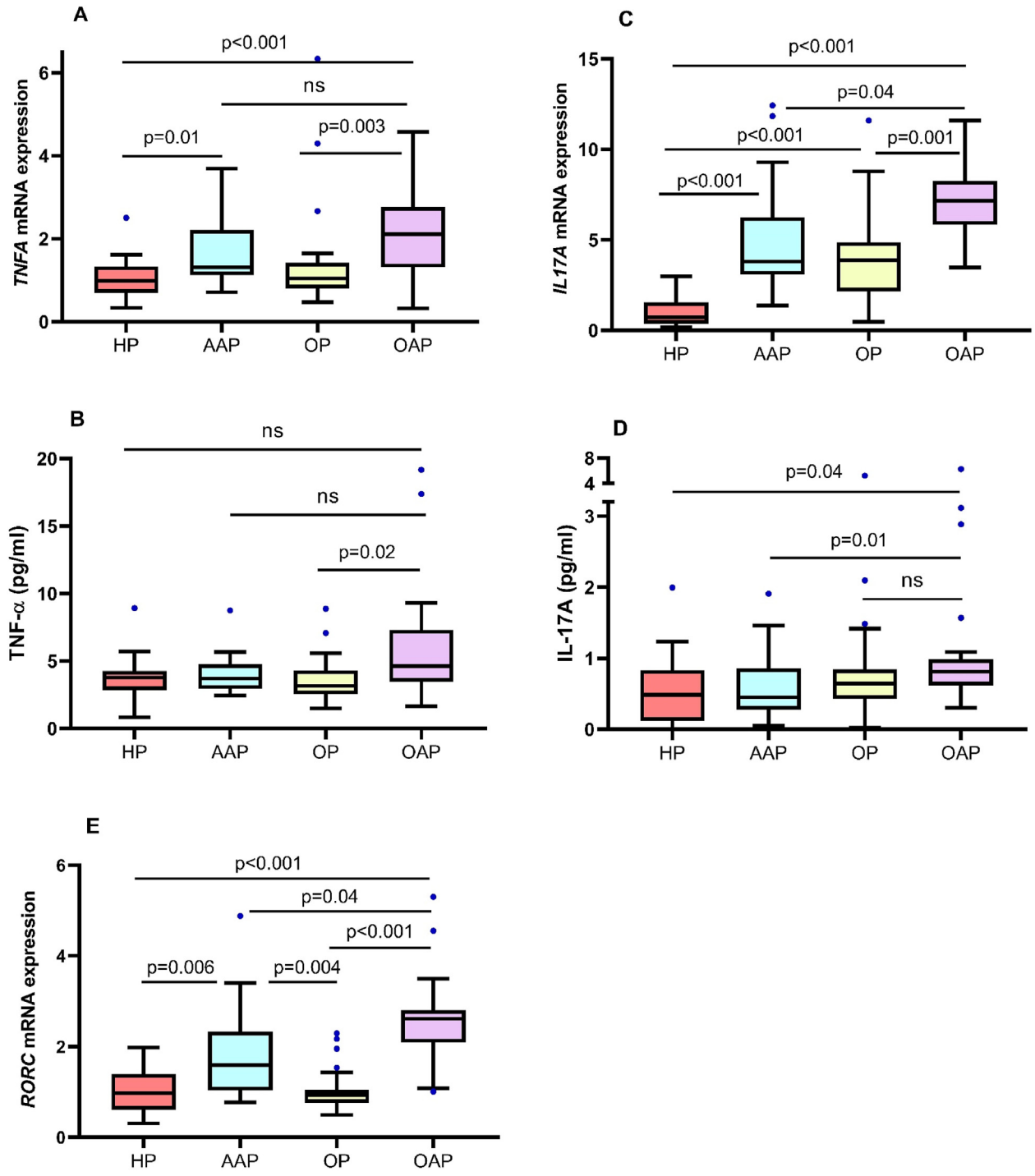

Figure 2. *TNFA*, *IL17A*, and *RORC* mRNA expression levels and TNF-α and IL-17A levels in the four groups of adolescents. (A) *TNFA* mRNA expression levels, (B) serum TNF-α levels, (C) *IL17A* mRNA expression levels, (D) serum IL-17A levels, and (E) *RORC* mRNA expression levels. The data are presented as the median and interquartile range. The Kruskal-Wallis test with Dunn's test for post hoc analysis was performed to analyse significant differences in the medians. Relative mRNA expression was calculated with the $2^{\Delta\Delta Ct}$ method and normalised to that of the housekeeping gene *GAPDH*. Abbreviations: ns, not significant; HP, healthy participants; AAP, allergic asthmatic participants; OP, obese participants without asthma; OAP, obesity-related asthma participants. Reproduced and modified with permission from Elsevier, reference Leija-Martínez et al. [20].

adolescents with obesity in a longitudinal study. 3) The methylation pattern was measured in peripheral blood leukocytes. To better answer our research question, the best kinds of cells to carry out the methylation process are IL-17-producing cells, especially Th17 lymphocytes and ILC3s; thus, our results should be cautiously interpreted.

However, our study has focused on the RORC transcription factor since it is fundamental to the polarisation of Th17 lymphocytes and, consequently, in synthesising IL-17A [10, 11]. Interestingly, like Th17 lymphocytes, IL-17A is also released by other cells of adaptive immunity, such as CD8+ (Tc17) cells, as well as several types of innate lymphocytes, including γδT17 cells, invariant natural killer T (iNKT) cells, natural killer T (NKT) cells, ILC3s, and "natural'' Th17 cells [9, 38]. Previous studies have also found that myeloid-lineage cells, such as neutrophils and microglia, produce IL-17A [39, 40, 41, 42]. Notably, they all express

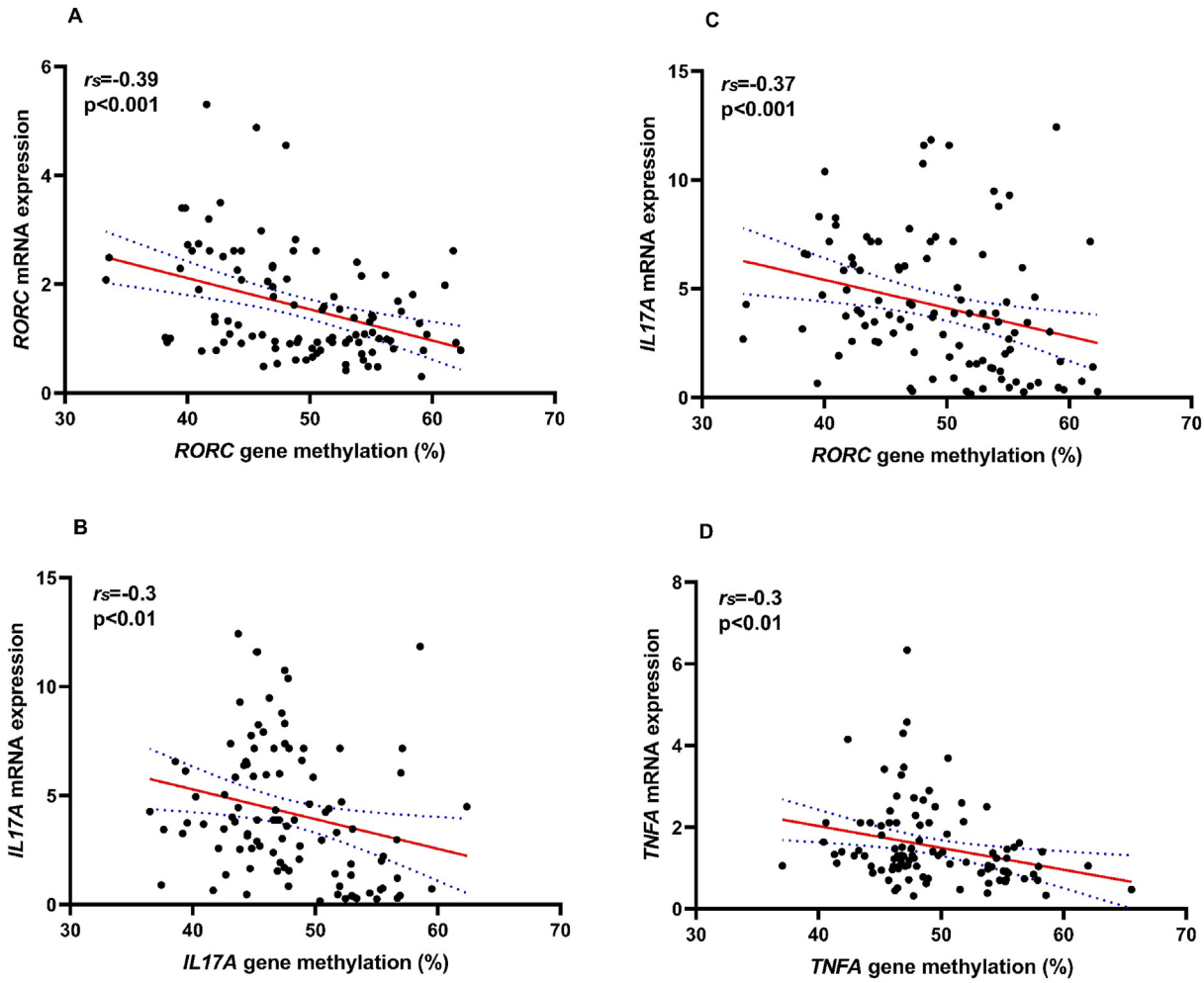

Figure 3. Correlations between gene promoter methylation status and mRNA expression levels for *RORC*, *IL17A*, and *TNFA* in the four groups of adolescents. (A) *RORC* promoter methylation status and *RORC* mRNA expression levels, (B) *IL17A* promoter methylation status and *IL17A* mRNA expression levels, (C) *RORC* promoter methylation status and *IL17A* mRNA expression levels, and (D) *TNFA* promoter methylation status and *TNFA* mRNA expression levels. Spearman's correlation coefficients were conducted for analysis; p values are shown in the figure.

the RORC transcription factor for producing IL-17A; moreover, most of them are located in peripheral blood [9, 38, 43]. Thus, our experiment was conducted in peripheral blood leukocytes to include all these types of cells with their respective RORC transcription factor.

We also acknowledge that using mixed peripheral blood leukocytes, composed of monocytes, B cells, and T cells, could reduce our capacity to identify T cell-specific modifications. Nonetheless, these recent findings contribute to a base of the possible role of *RORC* methylation in obesity-related asthma phenotype and add pioneer data to substantiate our results in forthcoming studies. Consequently, additional studies of *RORC* methylation patterns in each type of IL-17-producing cell should be warranted to understand better the role of *RORC* promoter methylation in the process of IL-17-producing cell differentiation, especially in Th17 lymphocytes. One strength of this study is that these results indicate that the *RORC* methylation pattern in peripheral blood leukocytes in obesity-related asthma is different and suggests activation of innate and adaptive immunity, including T cell differentiation. Future investigations are required to explore the potential role of each immune cell in the methylation process of *RORC* individually and to corroborate our observations, particularly in the paediatric population.

Additionally, other epigenetics mechanisms such as ubiquitination, acetylation, SUMOylation, phosphorylation and, Swi/Snf chromatin remodelling complex also regulate *RORC* gene expression [44, 45]. Thus, these epigenetics mechanisms should also be studied in detail in obesity-related asthma.

In conclusion, the present study suggests an association between lower *RORC*, *IL17A*, and *TNFA* gene promoter methylation status in peripheral blood leukocytes with obesity-related asthma and allergic asthma. Furthermore, *RORC, IL17A*, and *TNFA* gene promoter methylation patterns are moderately inversely correlated with their respective mRNA expression levels. Therefore, DNA methylation may regulate *RORC*, *IL17A*, and *TNF* gene expression in both asthmatic phenotypes. However, because of the critical limitations of this study, the DNA methylation status should study in more detail in individual IL-17-producing cells in a large cohort of adolescents with obesity.

Declarations

Author contribution statement

José J. Leija-Martínez: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Wrote the paper.

Abraham Giacoman-Martínez: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Wrote the paper.

Blanca E. Del-Río-Navarro: Conceived and designed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Fausto Sanchéz-Muñoz: Conceived and designed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Adrián Hernández-Diazcouder: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data.

Onofre Muñoz-Hernández: Conceived and designed the experiments; Analysed and interpreted the data; Wrote the paper.

Rodrigo Romero-Nava: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data.

Santiago Villafaña: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data.

Laurence A. Marchat: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Wrote the paper.

Enrique Hong: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data.

Fengyang Huang: Conceived and designed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

The data that has been used is confidential.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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