

***RUNX1* gene expression changes in the placentas of women smokers**

LITZY GISELLA BERMUDEZ¹, ITHZAYANA MADARIAGA¹, MARIA ISABEL ZUÑIGA², MERCEDES OLAYA²,
ALEJANDRA CAÑAS³, LUZ-STELLA RODRIGUEZ¹, OLGA MARIA MORENO¹ and ADRIANA ROJAS¹

¹Institute of Human Genetics, School of Medicine, Pontificia Universidad Javeriana;
Departments of ²Pathology and ³Internal Medicine, School of Medicine, Pontificia Universidad Javeriana,
Hospital Universitario San Ignacio, Bogotá 110231, Colombia

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Abstract. The placenta can be affected by environmental factors, such as exposure to cigarette smoke. This exposure in the fetal context is considered a risk factor for the development of short-term postnatal diseases, such as asthma. Asthma is an inflammatory disease characterized by predominant acquisition of CD4 T lymphocytes (TLs) of the Th2 type. Transcription factors such as GATA binding protein 3 (GATA3) and STAT6 actively participate in the differentiation of virgin TLs towards the Th2 profile, while transcription factors such as STAT1, T-Box transcription factor 21 (T-BET), RUNX1 and RUNX3 participate in their differentiation towards the Th1 profile. The objective of the current study was to evaluate the impact of exposure to cigarette smoke on the gene expression of *STAT1*, *T-BET*, *GATA3*, *IL-4*, *RUNX1* and *RUNX3* during the gestation period, and to determine whether the expression levels of these genes are associated with changes in global methylation. *STAT1*, *GATA3*, *RUNX1* and *RUNX3* protein and mRNA expression levels in the placental tissue of women smokers and non-smoking women were determined via immunohistochemistry and quantitative PCR (qPCR) respectively. Additionally, *T-BET* and *IL-4* mRNA expression levels were determined by qPCR. On the other hand, global methylation was determined via ELISA. In the present study, significant increases were observed in *RUNX1* transcription factor expression in placentas from women smokers when compared with placentas of non-smoking women. Similarly, significant increases in the expression of *GATA3*, *IL-4* and *RUNX3* mRNA were observed. The changes in gene expression were not associated with changes in the global methylation levels. Finally, a higher frequency of low-birth-weight infants were

identified in cases of exposure to cigarette smoke during pregnancy when compared with infants not exposed to cigarette smoke during pregnancy. Thus, the data of the present study contributed to the understanding of the genetic and clinical impacts of exposure to cigarette smoke during pregnancy and its importance in maternal and fetal health.

Introduction

The placenta is a specialized organ that is considered to be the vascular interface between the maternal and fetal circulatory systems (1). Genetic, environmental, transcriptional and epigenetic factors are actively involved in the formation of this interface (1). Numerous studies in mice and humans have revealed that factors such as malnutrition, smoking, alcoholism, drug use and pollution can induce alterations in gene expression; these alterations can lead to morphological and physiological alterations that can produce disturbances in placental and fetal growth, which can affect the health of the individual in the long term (2-8).

Specifically, exposure to cigarette smoke has been associated with alterations in placental development, such as inhibition of trophoblast invasion, which generates placental hypoxia (9), thickens the trophoblastic basement membrane (10) and increases umbilical cord blood flow resistance (11). This is because compounds such as nicotine, polycyclic aromatic hydrocarbons and nitrosamines, all of which are present in tobacco, manage to cross the transplacental barrier and accumulate in the fetal environment (12). Further to the aforementioned alterations, exposure to cigarette smoke during pregnancy is also considered to be a risk factor for poor pregnancy outcomes, such as: i) Intrauterine growth restriction; ii) premature birth (13); iii) low birth weight (14); and iv) diseases in early childhood, such as allergies and asthma (15).

Asthma is a phenotypically heterogeneous inflammatory disease of the airways and is associated with intermittent respiratory signs and symptoms, bronchial hyperreactivity and reversible airflow obstruction (16). At the immune response level, asthmatic individuals characteristically exhibit more Th2 type CD4 T lymphocytes (TLs), which produce a spectrum of cytokines, such as IL-4, IL-5 and IL-13 (17).

Correspondence to: Professor Adriana Rojas, Institute of Human Genetics, School of Medicine, Pontificia Universidad Javeriana, 40-62 Carrera 7, Bogotá 110231, Colombia
E-mail: rojas-adriana@javeriana.edu.co

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The process of cell differentiation from virgin CD4 T_H1s to effectors requires interactions between antigen-presenting cells, specifically dendritic cells and CD4 T_H1s (18). During this interaction, signaling pathways are activated that allow the expression of genes needed to establish the T_H1 profile according to the initial antigenic stimulus (18). Therefore, for the acquisition of the T_H2 phenotype, activation of STAT6 is required, which, along with other factors, activates the expression of GATA binding protein 3 (GATA3) (18,19). However, for the process of cell differentiation from virgin T_H1s towards the T_H1 profile, the initial expression of signal transducer and activator of STAT1 is required. The transcription factor STAT1 activates the expression of T-Box transcription factor 21 (T-BET), which is required for the differentiation of T_H1s towards the T_H1 phenotype (Fig. S1) (20-24).

RUNX family proteins are transcription factors that participate in processes associated with embryonic development, such as: i) Cartilage, bone and nervous system formation; ii) angiogenesis; iii) hematopoiesis; and iv) the immune system response (20). RUNX1 and RUNX3 mediate the normal maturation of various components of the immune system; specifically, it has been revealed that RUNX1 attenuates the differentiation of T_H2 T_H1s concomitant with the repression of GATA3 (20). By contrast, RUNX3 is relevant for the differentiation of T_H1s to the T_H1 lineage, which occurs through its interaction with T-BET (21).

Data describing the effects of cigarette smoke exposure on *RUNX1* and *RUNX3* gene expression are conflicting. In a murine model, it has been reported that exposure to nicotine and tobacco smoke during gestation significantly decreases *RUNX1* and *RUNX3* expression levels in lung tissue of 3- and 5-day-old neonatal mice (22). However, *RUNX1* expression levels in humans are increased in neonates from pregnant women smokers, as reported in a meta-analysis study in which aberrant expression of *RUNX1* was associated with the onset and progression of acute lymphoblastic leukemia (23). Additionally, it has been demonstrated that the expression of RUNX transcription factors in the placenta play fundamental roles in the formation of placental hematopoietic stem and progenitor cells (HSPCs). Pregnancy complications that result in preterm births differentially affect placental HSPC localization and *RUNX1* expression (24,25).

In the present study, the biological impact of exposure to cigarette smoke during the gestation period in the gene expression of *STAT1*, *T-BET*, *GATA3*, *IL-4*, *RUNX1* and *RUNX3* between placentas from women smokers and non-smoking women was evaluated using quantitative PCR and western blot assays. To determine whether these changes in gene expression were associated with epigenetic mechanisms such as DNA methylation, the global methylation levels were evaluated by ELISA.

Materials and methods

Study population. A total of 34 paraffin-embedded placentas were obtained from 14 women smokers (median age, 28 years; age range, 19-38 years) and 20 non-smoking women (median age, 28.5 years; age range, 18-37 years). The samples were collected between January 2013 and November 2019 at the Pathology Department of the Hospital Universitario San

Ignacio (Bogotá, Colombia) with the corresponding clinical information. The placentas were part of a previous study, and the subjects signed informed consent authorizing their use in future studies.

The present study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and all procedures were approved by the Ethics Committee of the Pontificia Universidad Javeriana and the Hospital Universitario San Ignacio (approval no. FM-CIE.0224-16).

For sample selection, the corresponding medical records were reviewed and the information routinely reported by the patients on cigarette smoking was analyzed. Placentas from neonates born at 29 weeks or later whose mothers reported smoking during pregnancy or up to 1 year before pregnancy were selected according to the criteria of the World Health Organization (26). For the controls, placentas from neonates older than 29 weeks were selected whose mothers were: i) Healthy before pregnancy; ii) did not develop complications during pregnancy (except for 2 patients who presented hypertensive disorders of pregnancy); iii) reported no history of smoking; and iv) had healthy neonates without peripartum complications. Chromosomal diseases and congenital malformations were used as exclusion criteria. To describe the results, experimental cases are placentas from mothers with a history of smoking, referred to as 'women smokers', and controls are placentas from non-smoking mothers, referred to as 'non-smoking women'.

Cell culture. Human Caucasian gastric adenocarcinoma cell line AGS (cat. no. 89090402; Sigma-Aldrich; Merck KGaA) was cultured in Ham's F12 Medium supplemented with 10% fetal bovine serum and 5% antibiotics (ampicillin and streptomycin) at 37°C in a humidified 5% CO₂ atmosphere. Cells were cultured to 80% confluence to perform the extraction of genomic DNA.

Histopathological findings. From the macroscopic findings of the 34 placentas, the following anatomical characteristics of the umbilical cord were considered abnormal: i) The presence of true knots; ii) abnormal insertions into the placenta; iii) furcata (early loss of Wharton's jelly leaving funicular vessels exposed); iv) marginal (entry of the cord at the very edge of the placenta); and v) velamentous (cord reaching the membranes). The following were also considered: i) Excessive or decreased coiling (with the definition of a normal coiling index being between 0.07 and 0.3 coils/cm); ii) the number of umbilical vessels (defined as the abnormal presence of a single umbilical artery); iii) the presence of retroplacental hematoma; iv) circular weight; and v) placental weight (according to gestational age) and its percentile.

Immunohistochemistry. Placental samples were fixed in 10% buffered formaldehyde for 48 h, embedded in paraffin, sliced into 3- μ m sections and mounted on microscope slides. Analysis of the immunohistochemical markers was carried out using the antibodies listed in Table I (all Santa Cruz Biotechnology, Inc.) and processed by the pathology department of the Hospital Universitario San Ignacio. The paraffin-embedded sections were rehydrated and incubated for

Table I. Characteristics of the monoclonal antibodies used to detect the markers of interest, and paraffin tissues selected as a positive control for each marker.

Marker of interest	Concentration of primary antibody	Incubation, min	Cat. no.	Control
GATA3	1:100	50	sc-268	Breast
STAT1	1:100	60	sc-417	Thymus
RUNX1	1:200	60	sc-101146	Epiglottis
RUNX3	1:200	60	sc-101553	Lymph node

All antibodies supplied by Santa Cruz Biotechnology, Inc. GATA3, GATA binding protein 3.

55 min at 20°C in methanol containing 10% hydrogen peroxide to block endogenous peroxidase activity (EnVision™ FLEX kit; Dako; Agilent Technologies, Inc.). The pretreatment of the samples was performed using the FLEX Peroxidase-Blocking reagent (5 min at room temperature) to facilitate the recovery of the antigen and increase the permeability of the membrane to the antibodies (27). Immunohistochemistry of the samples and the positive and negative controls of selected tissues was performed at room temperature using an Autostainer Link 48 from Dako (Agilent Technologies, Inc.). The positive reaction was observed following incubation with the HRP-conjugated secondary antibodies (20 min) with 3,3-diaminobenzidine according to the manufacturer's instructions. The sections were counterstained with Harris hematoxylin for 1 min at room temperature, dehydrated and covered with a slide for later observation under a light microscope. The entire slides were examined under x4 and x40 magnification. Due to the availability of tissue and reagents, immunohistochemical analyses were performed on 28 samples, including 14 non-smoking women and 14 women smokers.

Anonymization was performed on the two placental groups. Each antibody was validated by verifying the positive control as suggested by the manufacturer's instructions. The positive controls were tissues (breast, thymus, epiglottis and lymph node) used routinely for diagnosis based on immunohistochemistry in the Pathology Department of Hospital Universitario San Ignacio, which obtained informed consent from patients or relatives for the use of tissues from autopsies for research. The analysis was performed discriminating between extension and intensity, classifying each one into four categories and assigning the corresponding grade. In the case of extension: i) 0% (grade 0); ii) <30% (grade 1); iii) 30-60% (grade 2); and iv) >60% (grade 3). In the case of intensity: i) not expressed (grade 0); ii) weak (grade 1); iii) moderate (grade 2); and iv) strong (grade 3) according to Olaya-C *et al* (27). Each placental cell was tested, and immunohistochemical scores are presented as the median + interquartile range and were analyzed using the non-parametric Mann-Whitney U test.

RNA isolation and quantitative (q)PCR. Total RNA was extracted from paraffin-embedded placentas with a Quick-DNA/RNA™ FFPE kit (cat. no. R1009; Zymo Research Corp.) according to the manufacturer's protocol and RNA quality was assessed by the optical density (OD) 260/280 nm and OD 260/230 nm ratios. For each sample, an equal amount of RNA (2 µg) was reverse transcribed into cDNA using

a ProtoScript® First Strand cDNA Synthesis kit according to the manufacturer's instructions (New England BioLabs, Inc.). qPCR was performed using an SYBR-Green I Master real-time PCR kit (Roche Diagnostics) on a LightCycler® Nano instrument (Roche Diagnostics). The reaction conditions were as follows: Initial denaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 10 sec at 95°C, annealing for 15 sec at 59°C for RUNX1, 64°C for RUNX3, 59°C for GATA3, 61°C for STAT1, 62°C for T-Bet and 60°C for 18S, and elongation for 20 sec at 72°C. Data are presented as the relative mRNA levels of the gene of interest normalized to the 18S mRNA level, and the $2^{-\Delta\Delta Cq}$ method was used to analyze the mRNA expression of the studied genes (28). The sequences of the primers used to amplify the genes of interest are described in Table II. Due to the RNA quality and quality criteria for the expression analyses at the mRNA level, 23 samples, 11 from non-smoking women and 12 from women smokers, were selected.

Genomic DNA isolation. Genomic DNA was extracted and purified from paraffin-embedded placentas with a Quick-DNA/RNA FFPE kit (cat. no. R1009; Zymo Research Corp.) according to the manufacturer's instructions. Isolation of genomic DNA consisted of three steps: i) Deparaffinization; ii) tissue digestion; and iii) purification. Briefly, pretreatment of paraffin-embedded tissue representative sections was performed with the deparaffinization solution (1 min at 55°C), proteinase k (overnight at 55°C) and its corresponding buffer, followed by loading of the deparaffinized tissue sample onto the Zymo-Spin™ IC Column provided in the kit (Zymo Research Corp.) for consecutive washes and purification. Genomic DNA quality was assessed by the OD 260/280 nm and OD 260/230 nm ratios. Genomic DNA of the AGS cell line was extracted and purified with a Quick-DNA Miniprep Plus Kit (cat. no. D4069; Zymo Research Corp.).

Global methylation analysis. The levels of 5-methylcytosine (5-mC) were assayed by ELISA using a 5-mC DNA ELISA kit (cat. no. D5325; Zymo Research Corp) according to the manufacturer's protocol. Briefly, 96-well plates were coated with 100 ng denatured DNA extracted from the paraffin-embedded samples and were incubated at 37°C for 1 h. A mixture of anti-5-methylcytosine and secondary antibody conjugated to HRP suspended in ELISA buffer was added to each well and incubated for 1 h at 37°C. Subsequently, the wells were washed with the washing solution, and ELISA was developed

Table II. Primer sequences used for RT-qPCR and methylation-specific PCR.

A, RT-qPCR	
Gene	Primer sequence
GATA3	FW: 5'-TGGGCTCTACTACAGCTTCACAATAT-3' RV: 5'-TTGCTAGACATTTTTTCGGTTTCTG-3'
STAT1	FW: 5'-ATGGCAGTCTGGCGGCTGAATT-3' RV: 5'-CCAAACCAGGCTGGCACAATTG-3'
RUNX1	FW: 5'-TGCATGATAAAAGTGGCCTTGT-3' RV: 5'-CGAAGAGTAAAACGATCAGCAAAC-3'
RUNX3	FW: 5'-GAGTTTCACCCTGACCATCACTGTG-3' RV: 5'-GCCCATCACTGGTCTTGAAGGTTGT-3'
STAT6	FW: 5'-TGGGCCGTGGCTTCAC-3' RV: 5'-CCGGAGACAGCGTTTGGT-3'
T-BET	FW: 5'-GATGTTTGTGGACGTGGTCTTG-3' RV: 5'-CTTTCCACACTGCACCCACTT-3'
18S	FW: 5'-ACGGACCAGAGCGAAAGCAT-3' RV: 5'-GCGGGTCATGGGATAACG-3'
B, Methylation-specific PCR	
Gene	Primer sequence
β -actin	FW: 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3' RV: 5'-AACCAATAAAACCACTCCTCCCTTAA-3'
T-BET U-met	FW: 5'-GGTTTTGTAGTATTTGTTAAGAGTGT-3' RV: 5'-CAACAAACCACTATCACTAAAATCAC-3'
T-BET Met	FW: 5'-GTTTTGTAGTATTCGTTAAGAGCGT-3' RV: 5'-GAACCGCTATCACTAAAATCG-3'

RT-qPCR, reverse transcription-quantitative PCR; FW, forward; RV, reverse; GATA3, GATA binding protein 3; T-BET, T-Box transcription factor 21; Un-met, unmethylated; Met, methylated.

using 3,3',5,5'-tetramethylbenzidine plus hydrogen peroxide. The absorbance was read at 405 nm on an ELISA plate reader (Multiskan EK; Thermolab Scientific Equipments). Determination of the 5-mC percentage in the genomic DNA sample was performed by interpolating the results from a standard curve of 7 methylated DNA controls with 0, 5, 10, 25, 50, 75 and 100% methylation provided by the manufacturer.

Methylation-specific PCR. Samples from non-smokers (sample nos. 4, 13, 14, 18 and 23) and smokers (sample nos. 2, 6, 19, 25 and 27) were evaluated by methylation-specific PCR. DNA bisulfite conversion was carried out using an EZ DNA Methylation kit (cat. no. D5001; Zymo Research Corp.) following the manufacturer's instructions. Briefly, 0.5-1.0 μ g of genomic DNA was mixed with 130 μ l of CT Conversion Reagent prepared following the manufacturer's instructions. The mixture was incubated in a thermocycler with 19 thermal cycles at 98°C for 30 sec and 64°C for 15 min. The bisulfite-converted DNA samples were loaded onto the Zymo-Spin IC Column provided in the kit for desulfonation and purification. The bisulfite-converted DNA quality was analyzed by β -actin amplification and PCR was performed

using IMMOLASE™ DNA Polymerase (Bioline) on a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.). The reaction conditions were as follows: Activation at 95°C for 10 min, followed by 37 cycles of denaturation at 95°C for 10 sec, annealing at 57°C for U-Met T-BET and 58°C for Met T-BET for 15 sec, and extension at 72°C for 20 sec. The amplification product was visualized by electrophoresis on a 2% agarose gel with Gel Red (cat. No. 41003; Biotium). Additionally, for experimental validation, the positive control (methylated DNA) and negative control (unmethylated DNA) provided by the same manufacturer were used (cat. no. D5014; Zymo Research Corp.) The sequences of the primers used to amplify the genes of interest are described in Table II.

Statistical analysis. For data analysis, the samples were categorized into two groups: Samples of placentas from women smokers and samples of placentas from non-smoking women. Statistically significant differences in the expression levels of *RUNX1*, *RUNX3*, *STAT1*, *T-BET*, *IL-4* and *GATA3* at the protein and mRNA levels and methylation results were determined using the Mann-Whitney U test as non-parametric variables of independent samples. In all the figures, horizontal

Table III. Clinical characteristics of patients involved in the study.

Clinical characteristic	Women smokers (n=14)	Non-smoking women (n=20)	P-value	Odds ratio	Estimated 95% CI
Maternal age, years (%)			0.38	0.21	0.02-2.19
18	0 (0)	1 (5)			
19-35	13 (92.8)	15 (7)			
>35	1 (7.2)	4 (20)			
Maternal background, n (%)			0.66	2.455	0.35-17.08
Gastrointestinal diseases	2 (14.5)	0 (0)			
Hematological diseases	1 (7.2)	0 (0)			
Malignant diseases	0 (0)	1 (5)			
Obstretical complications, n (%)			0.83	1.3	0.20-7.75
Hypertensive disorders of pregnancy	5 (35.7)	2 (10)			
Gestational diabetes	3 (21.4)	0 (0)			
Sexual transmission infection	1 (7.2)	0 (0)			
Premature rupture of membranes	0 (0)	0 (0)			
Newborn sex, n (%)			0.10	0.223	0.047-1.052
Female	3 (21.4)	11 (55)			
Male	11 (78.6)	9 (45)			
Gestational age, weeks (%)			0.60	0.54	0.13-2.17
≤37	7 (50)	7 (35)			
>37	7 (50)	13 (65)			
Birth weight, g (%)			0.29	2.7	0.66-11.09
<2,500	9 (64)	8 (40)			
≥2,500	5 (35.7)	12 (60)			
Perinatal complications, n (%)			0.84	4.24	0.16-111.65
Intrauterine growth restriction (below the 10th percentile for gestational age)	1 (7.2)	0 (0)			

bars represent the median. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, Inc.).

Association between histological findings and interest groups was evaluated by calculating raw odds ratio (OR) with 2-sided Fisher's exact test with 95% confidence intervals (CIs); the means and percentages were determined to summarize the data. Qualitative variables with absolute frequencies and extreme or aberrant data were analyzed in detail in relation to the different variables. The hypothesis test to determine the P-value was null hypothesis (H_0), $OR=1$ and two-tailed alternative hypothesis (H_a), different from $OR=1$. Only variables with $P < 0.05$ were retained in the final statistical models. Statistical analysis was performed with Stata 14.2 (StataCorp, LLC).

Results

Clinical characterization. Within the sample group, 92.8% of women smokers were in the age range of 18-35 years, with a median age of 26 years. In the control group, 75% of the women classified as non-smokers were within the same age range, with a median age of 28 years. Of the women smokers, 50% had term births, compared with 65% of the control

group who had term births. A total of 78.6% of neonates in the sample group were male, compared with 55% of female neonates in the control group of non-smoking women.

In 7.2% of women smokers, intrauterine growth restriction was recorded as a perinatal complication. An association between low birth weight and exposure to cigarette smoke during pregnancy was observed. Specifically, 64% of neonates born to women smokers displayed low birth weight (Table III).

Analysis of obstetric complications revealed that 35.7% of the women smokers had hypertensive disorders of pregnancy compared with only 10% of non-smoking women who had hypertensive disorders of pregnancy. In the group of women smokers, 21.4% (3 cases) developed gestational diabetes and 7.2% (1 case) had a sexually transmitted disease. Analysis of chronic maternal pathologies revealed 14.5% (2 cases) of women smokers had a gastrointestinal disease (gastritis), 7.2% (1 case) of women smokers had a history of a hematological disease (von Willebrand disease) and 5% (1 case) of non-smoking women had a history of cancer (treated thyroid carcinoma). There were no cases of premature rupture of membranes in the two groups. The histopathological findings are summarized in Table SI. Due to the data being collected from patients in a level 4 hospital, a number of individuals (15 cases) included in this study presented comorbidities (Table III). Due to the low

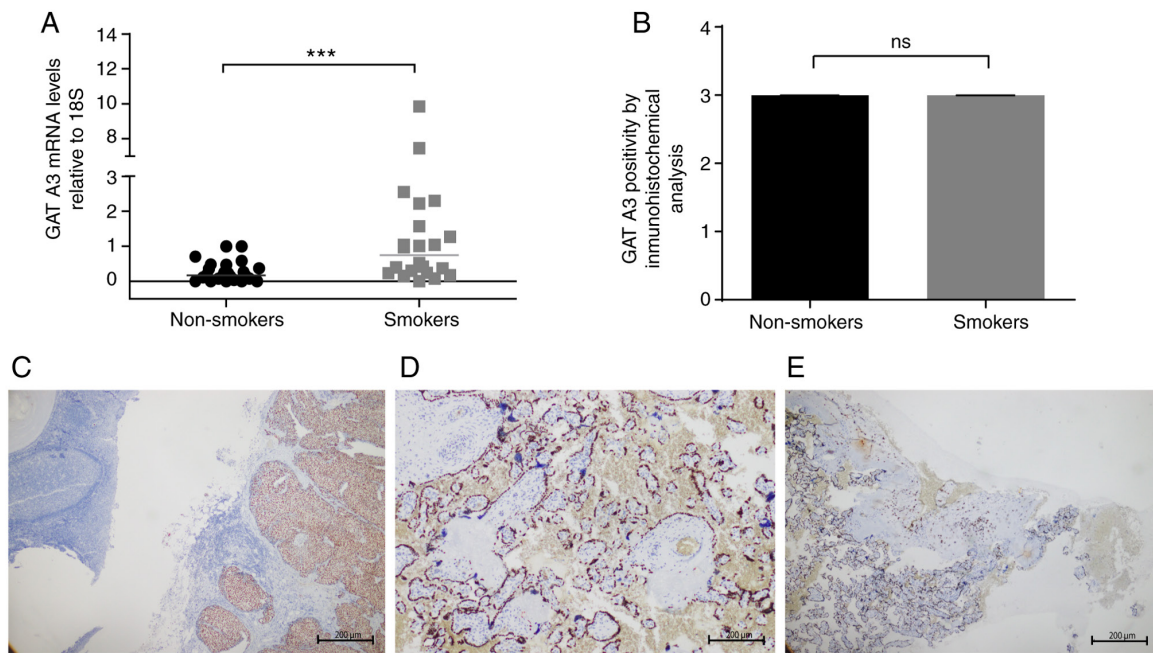


Figure 1. Analysis of the relative expression of GATA3 at the mRNA and protein levels in the placenta. (A) Expression levels of *GATA3* relative to *18S* in placentas from non-smoking women compared with placentas from women smokers. The horizontal bar represents the median of relative expression. (B) Intensity of positivity of GATA3 in placental samples from non-smoking women and women smokers. (C) Positive control biopsy slice of breast cancer and the nuclear staining pattern, with a strong intensity (magnification, x4). (D) Placental slice of a non-smoking woman displaying positive nuclear staining patterns in syncytiotrophoblasts, strong intensity, proportion between 30 and 60% of the total observed (magnification, x10). (E) Placental slice of a woman smoker, positive nuclear staining pattern in syncytiotrophoblasts, strong intensity, proportion <30% of total observed (magnification, x4). A Mann-Whitney U test was applied. *** $P < 0.001$. GATA3, GATA binding protein 3; NS, not significant.

number of cases for each entity, it was not possible to make groupings and analyze their impact on the results.

Changes in the gene expression of transcription factors are associated with the differentiation of virgin TLs towards the Th1 and Th2 profiles. To evaluate the changes in the gene expression of transcription factors associated with the differentiation of virgin TLs towards the Th1 and Th2 TL profiles as a result of exposure to cigarette smoke during pregnancy, the mRNA levels of *STAT1* and *GATA3* relative to *18S* were determined via qPCR. Immunohistochemical assays were performed to detect expression at the protein level in all the samples selected for the study. The results indicated a statistically significant increase in the *GATA3* mRNA level in placental samples from women smokers compared with non-smoking women (Fig. 1A). In the immunohistochemical analysis, GATA3 demonstrated a strong nuclear staining pattern, mainly in syncytiotrophoblasts and extravillous trophoblasts of the analyzed samples, with negativity in the middle section and positivity towards the external sections (maternal and fetal side) of the slice (Fig. 1D and E). Fig. 1C demonstrates the nuclear staining pattern with a high intensity in a positive control biopsy section of breast cancer tissue. Analysis of these pooled data numerically represented the intensity and extent of positivity, as described in the methodology, and did not show significant differences between the groups analyzed (Fig. 1B). Additionally, qPCR was performed to detect RNA expression of *IL-4* (Fig. S2) and the results demonstrated a statistically significant increase in placental samples from women smokers compared with non-smoking women.

Contrary to these findings, no changes were detected in the analysis of *STAT1* mRNA levels when comparing placental samples from women smokers and non-smoking women (median, 186 vs. 234; Fig. 2A). Immunohistochemical analysis demonstrated a positive pattern in extravillous trophoblasts (Fig. 2D) and areas of strong positivity in intravillous Hofbauer cells (Fig. 2E) in samples from women smokers and non-smoking women. Fig. 2C demonstrates the nuclear staining pattern and intensity in a positive control thymus tissue section. This labeling was associated with inflammation and was not observed when H&E staining was performed (data not shown). Analysis of these pooled data numerically represented the intensity and extent of positivity and did not exhibit significant differences between the analyzed groups (Fig. 2B).

Additionally, qPCR assays were performed for *T-BET*, a gene that is translated into a master transcription factor in the differentiation of virgin TLs towards the Th1 TL profile. These results revealed an increase in the relative expression of *T-BET* mRNA in women smokers compared with non-smoking women (Fig. S3A). However, it is important to highlight that in five samples of women smokers, decreases in the mRNA expression of *T-BET* were observed (Fig. S3B). To determine whether the expression decrease was due to changes in DNA methylation patterns, end-point methylation-specific PCR was performed. It was revealed that 100% of the samples exhibited hypomethylation of the promoter of the *T-BET* gene (Fig. S3C and D).

*Changes in *RUNX1* and *RUNX3* gene expression are associated with exposure to cigarette smoke during pregnancy.* Based on the roles of *RUNX1* and *RUNX3* in the process of differentiation from virgin TLs towards Th1 and

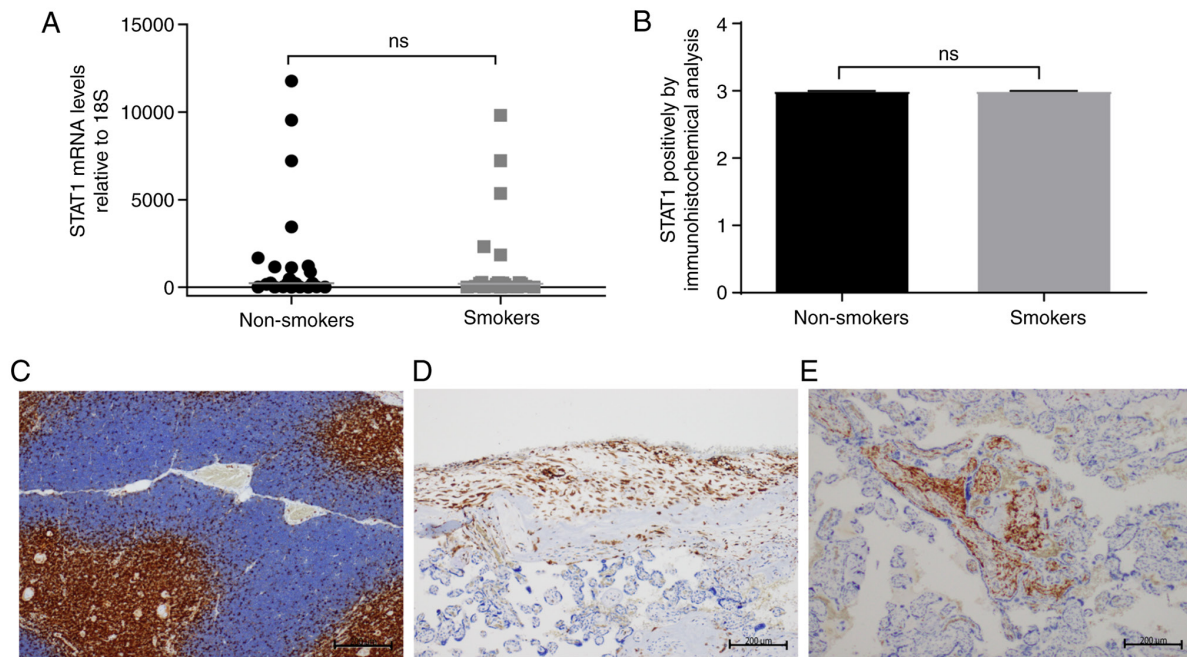


Figure 2. Analysis of the relative expression of STAT1 at the mRNA and protein levels in the placenta. (A) Expression levels of *STAT1* relative to *18S* in grouped samples of placentas from non-smoking women compared with placentas from women smokers. The horizontal bar represents the median of relative expression. (B) Level of intensity of STAT1 labeling via immunohistochemistry in samples from women smokers and non-smoking women. (C) Positive control thymus tissue slice, nuclear staining pattern, strong intensity (magnification, x10). (D) Placental slice of a non-smoking woman displaying positive nuclear staining pattern in extravillous trophoblasts and the decidua, strong intensity, proportion >60% of the total observed (magnification, x10). (E) Placental slice of a woman smoker, positive nuclear staining pattern in intravillous histiocytes, strong intensity (magnification, x10). A Mann-Whitney U statistical test was applied. NS, not significant.

Th2 TLs and in the outcome of postnatal diseases in early and late life in humans (18), the changes in gene expression resulting from exposure to cigarette smoke were evaluated. To this end, the mRNA levels of *RUNX1* and *RUNX3* relative to *18S* were evaluated via qPCR, and immunohistochemical assays were performed to detect protein expression in all the samples selected for study. According to the relative expression levels of mRNA, *RUNX1* increased significantly in placental samples from women smokers compared with non-smoking women (median, 0.5 vs. 0.145; Fig. 3A). In addition, in the immunohistochemical analysis of *RUNX1*, a strong nuclear staining pattern was observed in the decidua and in chorion stromal cells (Fig. 3D and E). Differences were observed in chorion stromal cells, with a greater labeling intensity in placental samples from women smokers compared with those from non-smoking women (Figs. 3B and S4). Fig. 3C demonstrates the nuclear staining pattern with medium intensity of the positive control epiglottis tissue section.

The relative expression levels of *RUNX3* mRNA revealed significant differences between groups and were higher in placental samples from women smokers than in those from non-smoking women (median, 0.7 vs. 0.1; Fig. 4A). However, immunohistochemical determination of the levels of expression of *RUNX3* at the protein level demonstrated a negative pattern in all placental cells (Fig. 4C and D). Fig. 4B demonstrates the positive control lymphoid tissue slice with a nuclear staining pattern and strong intensity.

Changes in global placental methylation in women smokers. To assess whether exposure to cigarette smoke induced changes

in the overall DNA methylation level, the methylation profiles of the extracted genomic DNA from the paraffin-embedded tissue were analyzed by detecting the 5-mC marker using ELISA. The results demonstrated that there were no differences in the methylation levels detected between the placental samples from women smokers and non-smoking women. It is important to note that the methylation levels detected were <15%. Although the screening kit included both positive and negative methylation controls, a sample of genomic DNA from a human Caucasian gastric adenocarcinoma cell line for which high levels of overall methylation have been reported was included. In this case, the results demonstrated a 60% methylation level, which proved the efficiency of the kit (Fig. 5).

Discussion

In the present study, a first approximation was made in regard to the biological impact of exposure to cigarette smoke during gestation. Comparisons were made between the expression levels of genes encoding the transcription factors STAT1, GATA3, *RUNX1* and *RUNX3*, which are involved in the process of differentiation from virgin TLs towards Th1 and Th2 TLs. In the current study, placentas from women who smoked during pregnancy and placentas from women non-smokers were analyzed.

Significant increases were observed in *GATA3*, *IL-4*, *RUNX1* and *RUNX3* mRNA expression levels in placentas from women smokers compared with those from non-smoking women. In parallel, a decrease in the expression of *T-BET* was observed in 5 women smokers compared with non-smoking

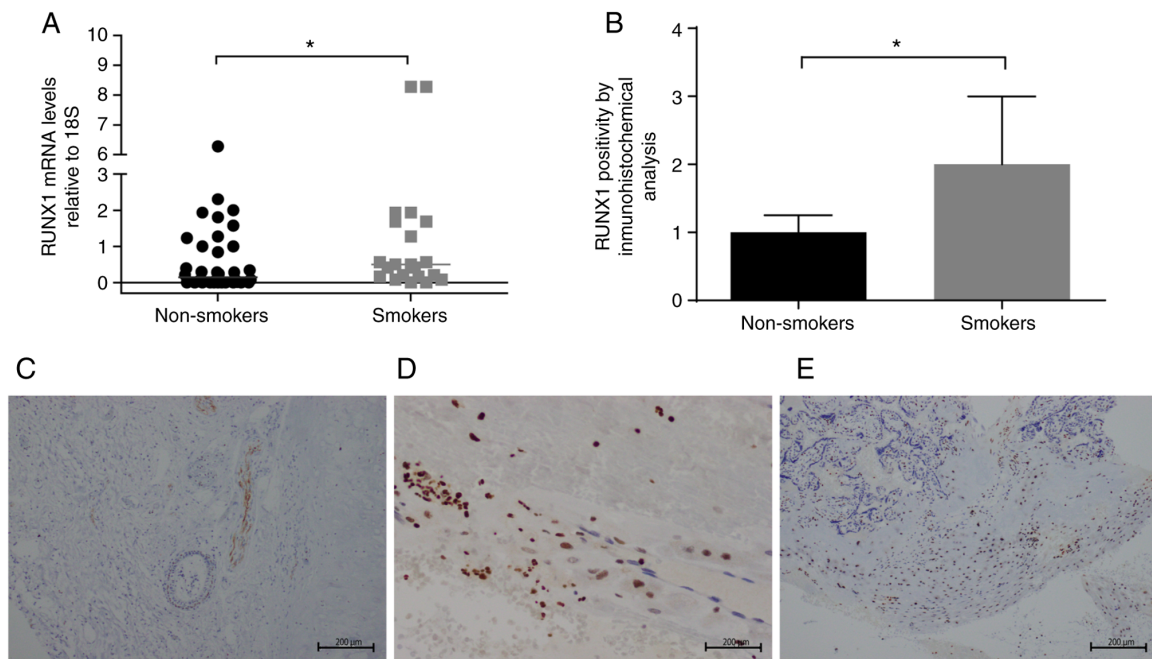


Figure 3. Analysis of the relative expression of *RUNX1* at the mRNA and protein levels in the placenta. (A) Expression levels of *RUNX1* relative to *18S* in placentas from non-smoking women compared with placentas of women smokers. The horizontal bar represents the median relative expression. (B) Level of intensity of *RUNX1* labeling via immunohistochemistry in samples grouped by placentas from women smokers and non-smoking women. There was increased detection of *RUNX1* protein in stromal cells of the chorion of placentas from women smokers compared with placentas from non-smoking women. (C) Positive control epiglottis tissue slice, nuclear staining pattern, medium intensity (magnification, x10). (D) Placental slice of a non-smoking woman, nuclear staining pattern positive in polymorphonuclear cells and negative in endothelial cells (magnification, x40). (E) Placental slice of a woman smoker, positive nuclear staining pattern for the decidua, strong intensity, proportion >60% of the total observed (magnification, x10). A Mann-Whitney U statistical test was applied. * $P < 0.05$.

women. With respect to the expression of *STAT1*, the results were not statistically significant.

Early dysregulation of the newborn's immune response is associated with the development of allergies and asthma in childhood. In this regard, changes have been reported in the generation and differentiation processes of virgin CD4 T cells towards the Th1 and Th2 type profiles (29,30). These variations are associated with genetic predispositions and prenatal environmental exposures, such as exposure to cigarette smoke (31). Most of the research carried out to date evaluates the association between smoking during pregnancy and the outcome of newborns with allergic or asthmatic phenotypes experimentally, by determining the IgE levels in umbilical cord blood, exacerbating the neonatal immune response to specific antigens, and quantifying Th2 type cytokines and changes in specific methylation levels of placental tissue as a result of exposure to cigarette smoke (32-35). *GATA3* is the master transcription factor of Th2 lymphocyte differentiation. This process is initiated by the binding of *IL-4* to the receptor, which leads to phosphorylation and dimerization of *STAT6*; *STAT6* dimers are translocated into the nucleus, where they promote *GATA3* expression, favoring transcription and translation of *IL-4*, a characteristic Th2 cytokine, which also contributes to the continuous stimulation of differentiation towards this phenotype (Fig. S1) (36).

The increase in *GATA3* expression in placentas from women smokers compared with non-smoking women supports the hypothesis that transcription of genes involved in the Th2 lymphocyte differentiation pathway is associated with the predisposition to an allergic phenotype, caused by exposure to cigarette smoke during pregnancy. This finding is consistent

with the observed increase in *IL-4* RNA expression and decrease in *T-BET* expression, and reflects a possible imbalance in the response of CD4 T cells. Transcriptional activation of *GATA3* may be associated with the presence of histone covalent modifications associated with activation, which are associated with an epigenetic mechanism underlying the transcriptional modulation of the allergic response (37,38). For the protein expression of *GATA3* detected by immunohistochemistry, the results did not show significant changes between the groups analyzed. However, it is important to highlight the existence of a differential and tissue-specific expression pattern that has not been previously reported, with positivity towards both maternal and fetal extreme regions.

Placental samples from 5 women smokers revealed decreases in *T-BET* mRNA expression compared with those of non-smoking women. As described above, in the differentiation pathway towards a Th1 T cell profile, *T-BET* expression is induced by T cell receptor signaling (TCR) and is strongly elevated by activation of the transcription factor *STAT1*, which occurs in a positive feedback loop in response to autocrine *IFN γ* (39). Likewise, *T-BET* plays an antagonistic role in the differentiation of Th2 lymphocytes by inhibiting *GATA3* (40). The significantly decreased levels of *T-BET* and the decreasing trend of *STAT1* detected in placentas from women smokers are in line with the elevated transcriptional levels of *GATA3* and *IL-4* detected in the present study. It is important to highlight that one of the limitations of the current study is the lack of information on the protein expression of *IL-4* and *IFN γ* .

It has been identified that dysregulation of *T-BET* expression plays a role in the immunopathogenesis of type 1 diabetes by generating an imbalance in Th1/Th2 differentiation in

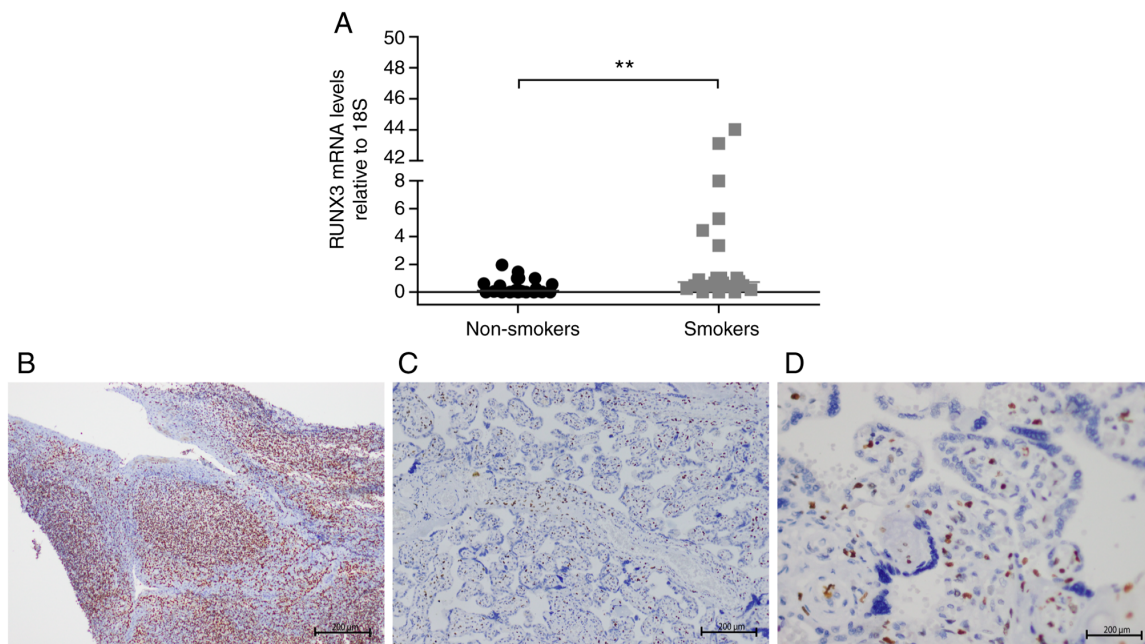


Figure 4. Analysis of the relative expression of RUNX3 at the mRNA and protein levels in the placenta. (A) Expression levels of *RUNX3* relative to *18S* in grouped samples of placentas from non-smoking women compared with placentas from women smokers. The horizontal bar represents the median of relative expression. (B) Positive control lymphoid tissue slice, nuclear staining pattern, strong intensity (magnification, x10). (C) Placental slice of a non-smoking woman, positive nuclear staining pattern in polymorphonuclear cells and negative nuclear staining pattern in placental cells (magnification, x40). (D) Placental slice of a woman smoker, negative pattern for all placental cells (magnification, x10). A Mann-Whitney U statistical test was applied. ** $P < 0.01$.

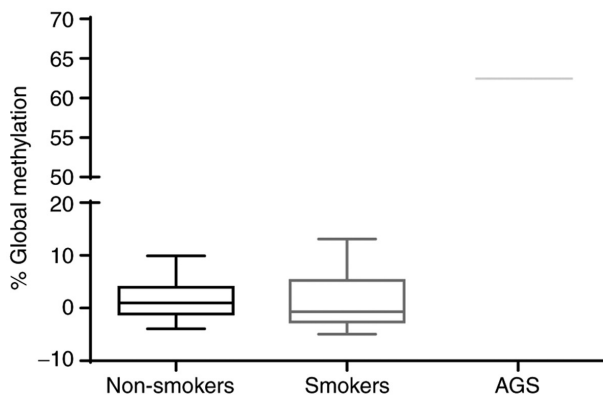


Figure 5. Analysis of the overall percentage of methylation in the placenta. Placental samples with methylation percentages $< 15\%$ were analyzed regardless of exposure to cigarette smoke during pregnancy. The graph shows the percentage of the overall methylation of genomic DNA from the placentas of non-smoking women and women smokers, as well as the AGS human gastric adenocarcinoma cell line to allow the sensitivity of the test for identifying overall methylation markers to be validated.

peripheral blood mononuclear cells (41). The expression of *T-BET* has been described as being regulated by epigenetic mechanisms, such as DNA methylation (42). For this reason, specific methylation PCR tests were carried out on a CpG island of the promoter in the current study. In this way, samples from non-smoking and smoking women were evaluated. Notably, the results demonstrated that for all the samples analyzed, the presence of the unmethylated condition was evident in the analyzed region for both women smokers and non-smoking women. These results indicated that the changes in the expression in *T-BET* are independent of the DNA methylation of the analyzed region and it would therefore be interesting to

consider the existence of additional epigenetic mechanisms for DNA methylation, such as covalent histone modification (43). In this regard, it has been reported that this type of mechanism is mostly affected by exposure to solvents and toxic agents, such as tobacco and particulate matter, among others (44,45).

RUNX1 and *RUNX3* are known to be involved in T-cell immunity. T lymphocytes differentiate into subsets with distinct functions. In some T-cell subsets, *RUNX1* and *RUNX3* are equivalently expressed and exhibit redundant activities (46). However, in other T-cell subsets, *RUNX1* and *RUNX3* exert distinct functions. These differences depend on the unique expression patterns of these proteins in a particular T-cell subset (47). *RUNX1* but not *RUNX3* is found in naive T lymphocytes, whose TCR stimulation immediately down-regulates *RUNX1* protein expression; on the other hand, Th1-committed cells express only *RUNX3*, whereas Th2 cells express both *RUNX1* and *RUNX3* (47).

RUNX1 promotes the development of Th1 lymphocytes from virgin CD4 T lymphocytes by activating the IL-4 repressor, leading to their transcriptional inactivation and increased expression of $\text{IFN}\gamma$, a cytokine characteristic of the Th1 response (48). However, it is important to emphasize that for this process to happen, there must be a parallel repression of *GATA3* (47). In the placental samples analyzed in the current study, exposure to cigarette smoke generated significant increases in the protein and mRNA expression levels of *RUNX1*. These results are consistent with a study in a murine model by Haley *et al* (22), in which changes in the expression patterns of *RUNX1* due to exposure to cigarette smoke during pregnancy are described. Haley *et al* (22) report the existence of single nucleotide polymorphisms in *RUNX1* that are associated with airway responsiveness in asthmatic children. These associations are reported to be modified by

exposure to cigarette smoke during pregnancy, which tends to increase the expression levels of *RUNX1* (22). Other exposures during pregnancy, such as alcohol consumption, have been associated with the differential expression of ~304 genes identified by microarray experiments in the placental tissue of rats (49). It has been suggested that *RUNX1* demonstrates decreased expression in placentas from rats that consumed ethanol compared with controls (46).

In relation to *RUNX3* expression, the results of the present study revealed very low levels of gene expression in placental samples from both women smokers and non-smoking women. However, a significant increase in *RUNX3* mRNA expression levels was reported in women smokers compared with non-smoking women. Despite the fact that *RUNX3* has been established to be fundamental for promoting the Th1 phenotype through the *IL-4* repression induced by its interaction with T-BET (50), the significance of *RUNX3* expression in Th2 cells remains unclear (47). It remains to be determined whether the significant increase detected in the expression of *RUNX1* and *RUNX3* is generated in response to the transcriptional increase detected for *GATA3*.

Finally, to assess whether exposure to cigarette smoke had an effect on the overall DNA methylation patterns, a screening test was performed using a 5-mC DNA ELISA kit. For this particular evaluation, results of the present study demonstrated no change between the experimental groups. It is important to highlight that during early embryonic development, the placenta presents generalized hypomethylation compared with normal somatic tissue, which contributes to the processes of trophoblast differentiation and later to correct placentation (51-53). Thus, changes in this global stage have been associated with environmental exposure, such as exposure to pollution and alcohol consumption during the third trimester of pregnancy (49,51,54). It has been reported that even slight changes in methylation levels (~3%) can be considered important (52). However, there is little evidence available in the literature from clinical and preclinical experimental studies to support the association between exposure to cigarette smoke and increases in overall placental methylation.

Analysis of the characteristics and clinical outcomes of the study population allowed an association to be established between exposure to cigarette smoke during pregnancy and low birth weight (<2,500 g) (53). With a representative percentage of 64% in women smokers, the findings of the present study are consistent with reports that have previously described maternal smoking during pregnancy as a risk factor for low birth weight (55). This association is direct and dose-dependent according to studies demonstrating that birth weight decreases as the number of cigarettes consumed per day increases, with a reduction in weight from 6-10 cigarettes daily (56). In addition, placental pathologies such as chronic ischemia and secondary changes associated with inadequate perfusion (57) have been identified as being associated with both low birth weight and exposure to cigarette smoke, highlighting the importance of an optimal environment for fetal development and the impact of maternal smoking on development.

In conclusion, it was found maternal smoking is associated with low birth weight and changes in the expression of genes that encode important transcription factors for

hematopoiesis and T lymphocyte differentiation. In the present study, 5 placental samples exposed to cigarette smoke during pregnancy favored the presence of transcription factors and cytokines involved in the differentiation of T_H2 cells, such as *GATA3* and *IL-4*. These transcription factors predispose cells to differential dysregulation and cause an imbalance in the Th2/Th1 ratio, characteristic of the asthmatic phenotype with an exacerbated Th2 TL profile. In the present study, significant increases in *RUNX1* expression were also detected as a consequence of exposure to cigarette smoke. These results were consistent with previous scientific reports that associate this altered expression in *RUNX1* with the occurrence of hematological malignancies in early life (23). In addition, an analysis of the overall methylation of placental samples demonstrated changes in methylation that were not directly associated with exposure to cigarette smoke, but provided additional evidence for overall placental methylation levels, for which there are limited reports in the literature. Results of the present study indicated the impact of exposure to cigarette smoke on gene expression and the possible impact on health of the individuals in the short, medium and long term. It is important to highlight that one of the limitations of the present study is the low number of samples. However, the results obtained open possibilities for novel research in the area to be conducted to demonstrate the impact of environmental exposure on maternal-fetal health. Finally, further studies are necessary to evaluate the asthma status of newborns from women smokers analyzed in the present study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LGB performed the experiments, analyzed and interpreted the data and wrote the paper. IM and MIZ performed some experiments and analyzed and interpreted the data. AC, LSR and OMM contributed reagents, materials, analysis tools or data, and analyzed and interpreted the data. MO and AR conceived and designed the experiments, analyzed and interpreted the data, contributed reagents, materials, analysis tools or data, and wrote the paper. MO and AR confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All research protocols were approved by the Ethics Committee of the Pontificia Universidad Javeriana and the Hospital

Universitario San Ignacio (approval no. FM-CIE.0224-16). Informed consent was obtained from patients or relatives for the use of placental tissue and positive control tissues for research.

Patient consent for publication

The data and results presented in the present study were anonymized and patients provided consent for publication.

Competing interests

The authors declare that they have no competing interests.

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