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Association between TNF-α, cortisol levels, and exposure to PM10 and PM2.5: a pilot study

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Abstract

Purpose The most harmful atmospheric pollutant for human health is particulate matter (PM). We analyzed the correlation between short-term lag exposure to PM10 and PM2.5, salivary cortisol and TNF-α level, and methylation levels of the TNF-α promoter.

Methods A pilot study including 20 subjects. Eight salivary samples for each subject at various times of the day were collected for comparing cortisol levels and TNFα detection. TNFα promoter methylation levels on salivary DNA were analyzed. Regression analyses were performed using generalized linear mixed models between the different outcomes and 4, 3, 2 and 1 day's lag values of PM10/PM2.5.Generalized additive mixed model (GAMM) was used to evaluate any potential deviation from linearity.

Results Area under the curve with respect to the ground (AUCg) showed a statistically positive association with 4-, 3-, 2-, and 1-day lag of exposure to PM10. Area under the curve with respect to the increase (AUCi) showed a statistically negative association with 4-, 3- and 1-day lag of exposure to PM10. TNFα showed statistically significant association with both exposures, PM10 and PM2.5, at 4-, 3-, 2-, and 1-day lag.

Conclusions Regarding cortisol levels there is an increase of overall hormone levels but a less dynamism of the system to answer to external stressors. Increase of TNF-α may reflect increased levels of oxidative stress and inflammation due to pollution exposure.

Keywords Salivary testing, Tumor necrosis factor-alpha, Environmental pollution, Epigenetic process, Methylation

Introduction

The World Health Organization (WHO) has identified air pollution as the main environmental risk for human health in the European Union (EU). In fact, according to a recent report, up to 2016, around 4.2 million premature deaths were attributable to it, as well as substantial health-related diseconomies amounting to hundreds of billions of euros [1, 2]. In Italy, in 2016, 723 DALYs (Disability-Adjusted Life Years) per 100,000 inhabitants were lost due to air pollution, and there were 49 deaths per 100,000 inhabitants associated with it [3]. People living in cities and urban areas are particularly exposed to this risk, as the higher population density compared to rural areas results in greater release of atmospheric pollutants and makes their dispersion more difficult. According to the WHO, one of the most harmful atmospheric pollutants for human health is particulate matter (PM).

Source of pollution and route of exposure

Main source of this pollution is represented by several factors: Industries such as power plants, manufacturing

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facilities, and refineries emit large quantities of PM through the combustion of fossil fuels and other processes. These activities release a variety of particles, including metals, sulfates, nitrates, and organic compounds [4].

The transportation sector is another major contributor to PM pollution, particularly in urban areas. Exhaust from cars, trucks, and buses, especially those using diesel engines, releases a significant amount of fine particles into the atmosphere. Brake and tire wear also contribute to PM levels [5].

Moreover, activities such as construction, demolition, and road works generate dust and other particulate matter, contributing to elevated PM levels in surrounding areas [6, 7].

The route of exposure to PM is primarily through inhalation. When individuals breathe in air containing PM, the particles can deposit in different parts of the respiratory system depending on their size.

- PM10: These larger particles tend to deposit in the upper respiratory tract, including the nose and throat. They can cause irritation and exacerbate conditions like asthma and bronchitis [8].
- PM2.5: Due to their smaller size, PM2.5 particles can penetrate deeper into the lungs, reaching the alveoli. Once in the alveoli, these particles can enter the bloodstream, leading to systemic effects such as cardiovascular and neurological impacts [9].

Adverse effects on human health

The health effects of PM exposure can vary based on several factors, including the duration and concentration of exposure, as well as individual susceptibility. Chronic exposure to high levels of PM can lead to serious health outcomes, including respiratory and cardiovascular diseases, and has been linked to increased mortality rates [10].

Moreover, recent studies have shown that exposure to air pollution may be a complex scenario influenced by several elements, such as other pollutants or meteorological conditions, indicating that assessing this relationship requires a multidimensional approach to be better quantified [11–13]. Despite this complexity, exposure to environmental pollutants has been clearly linked to multiple negative health outcomes, including short-term symptoms, chronic effects on morbidity, and premature mortality [14]. Environmental pollution is associated with a high risk of developing certain diseases such as cardiovascular diseases [15], diabetes [16], asthma [17, 18], chronic obstructive pulmonary disease (COPD) [19], and emergency room visits for respiratory diseases [20]. Furthermore, recently, the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) have classified air pollution as a Group 1 carcinogen [21].

In this context, prevention remains a challenge of primary importance. The main obstacle in recognizing and managing the potential risks of chemicals is the lack of information regarding citizens' exposure, including workers, to chemicals and their interaction with other substances related to the environment or lifestyle. Many studies have shown the important role of green and blue spaces, identified, respectively, as natural or artificial areas characterized by the presence of vegetation, such as parks, gardens, forests, and meadows, and natural or artificial environments characterized by the presence of water, such as seas, rivers, lakes, ponds, canals, and even swimming pools, in bringing positive effects on human health [22]. Meta-analyses have shown how green spaces may positively affect diastolic pressure, cortisol production, and heart rate, also reducing all-cause mortality [23]. The reason for this could be a pleiotropic effect, also obtained through a reduction of air pollution thanks to the presence of vegetation [24].

Biological pathways and mechanisms behind the association of Tnf-α, cortisol levels, and exposure to PM10 and PM2.5

One of the main studied hypotheses is the pathogenic role of PM as a trigger of the stress response. Exposure to particulate matter (PM), especially PM10 and PM2.5, has been shown to activate the hypothalamic–pituitary–adrenal (HPA) axis, a central stress response system [25, 26]. The inhalation of fine particles leads to oxidative stress and inflammation in the respiratory tract, which can trigger the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) [27–30]. TNF-α is a key cytokine involved in systemic inflammation. It is produced by various cells, including macrophages, and its elevated levels lead to chronic inflammation, which is associated with a range of adverse health outcomes, including respiratory and cardiovascular diseases [31]. This cytokine can stimulate the HPA axis, leading to increased production and release of cortisol from the adrenal glands [32–35]. Regarding stress and inflammation, several animal studies have shown changes in cortisol and related hormones following short-term exposure to fine particulate matter like PM2.5, implying activation of the hypothalamic–pituitary–adrenal axis [36, 37].

Some epidemiological studies have investigated the relationship between cortisol levels and exposure to air pollution but with different, heterogeneous, and sometimes null results, for example for exposure to PM2.5

while observing some effects for other exposures such as NO2 [38, 39]. Very few epidemiological studies have examined short-term exposure to urban air pollution and response to salivary cortisol, and only one [40, 41], to our knowledge, specifically studied the relationship between salivary cortisol and both exposure to PM2.5 and PM10. Most studies have concentrated on the long-term effects of PM exposure, while the short-term lag effects on biomarkers like cortisol and TNF-α are less explored. This gap is crucial because short-term exposure can also trigger significant physiological responses, which are important for understanding the immediate impact of air pollution on human health [42, 43].

In addition, TNF-α is involved in systemic inflammation [44], and it has been linked to human body reactions to exposure to air pollution in murine models. Its blood circulating levels showed a possible role of this cytokine in modifying and affecting human physiology and pathology [45–47]. An interesting in vitro study showed how seasonal variability of PM composition may influence cytokine cell production like TNF-α or IL-6 [48]. Nevertheless, the relationship between salivary levels of TNF-α and short-term exposure to both PM10 and PM2.5 has not yet been deeply investigated, since only one study, to the best of our knowledge, reported an association of TNF-α salivary levels and very short-term exposure, within hours, to PM2.5 [49]. Moreover, the TNF-α pathway related to air pollution has been studied from an epigenetic point of view, since methylation levels in this relationship between air pollution exposure and health outcomes involve not only association but also mediation [50–52]. Methylation is a key factor in terms of epigenetic regulation, as its presence changes and modulates gene expression [53, 54], and air pollution could exert its harmful effects also through this pathway by modifying the quantity levels of the TNF-α cytokine in the human body. However, its salivary promoter methylation levels and their relationship with salivary cortisol and PM are not fully elucidated.

Our study addresses these gaps by examining the association between short-term lag exposure to PM10 and PM2.5 and salivary cortisol and TNF-α methylation levels, thereby providing new insights into the immediate biological responses to air pollution. In addition, we provide comparative data on PM exposure effects in urban and rural populations, which can inform region-specific public health strategies. Furthermore, the inclusion of TNF-α promoter methylation analysis helps elucidate the epigenetic mechanisms that may mediate the health effects of PM exposure, bridging a crucial gap in the existing literature. In conclusion, the aim of this study is to analyze the association between short-term lag exposure to PM10 and PM2.5, salivary cortisol and TNF-α levels, and methylation levels of the TNF-α promoter.

Methods

Study population

To define the study population, we reviewed air pollution data recorded by the local Environmental protection Agency (Agenzia Regionale Protezione Ambientale Marche—ARPAM), recorder through fixed registration units. We used records coming from two fixed registration units located in the areas, one rural and one urban, where our subjects have their residency. Validation of these data are guaranteed by ARPAM agency that is a government regional agency which activities and registrations are regulated by Italian law. ARPAM considers registrations coming from these units as a good approximation of the exposure of people that are residents in the municipal areas where the units are located. These data are publicly available. According to these data, two areas with different level of exposure to air pollution have been selected, one with the lowest and another one with the highest exposure. Selected 4 days lag values before saliva specimens were collected to look at possible effect of short-term air pollution. We recruited 20 subjects, 10 from the above-mentioned urban area with an historical major exposure to air pollution and 10 from the rural area with an historical lower exposure to air pollution [55–57]. First set of saliva samples have been collected among July 2018 while second set has been collected during February 2019. Thirty-nine observations have been used in the present analysis, since one saliva sample collected has been undetermined both on TNF-alfa and Cortisol quantification. All subjects have been requested to answer some question regarding their health conditions, possible presence of chronic diseases like hypertension or diabetes, dietary habits, lifestyles, social interactions. Inclusion criteria were being resident in the two areas analyzed and being aged more than 18 years old. Recruitment has been carried out on voluntary basis and subjects have been contacted through their general practitioners.

Exclusion criteria were being affected by chronic diseases like chronic liver diseases (viral, NASH, autoimmune cirrhosis, drug), previous myocardial infarction, angina presence, previous stroke, BMI > 30, cortisone drugs therapies, sexual hormones therapies.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical approval for the study was obtained from by the local ethics committee (Comitato Etico Regionale Marche—CERM, Prot. N. 2017-0317OR).

Saliva collection and extraction

Procedures were conducted as previously described [58]. At least 1 mL of saliva was collected in a Salivette® (Sarsted Aktiengesellschaft & Co., Nümbrecht, Germany). Subjects were instructed not to consume water or food (including candies or chewing gum) or brush their teeth within 30 min prior to sample collection. Saliva samples were centrifuged at 1000 rpm for 2 min, and the supernatant was collected and stored at − 20 °C [59]. Sampling was carried out using the same standard procedure in both July and February: the first saliva sample was collected rigorously 30 min after awakening to ensure that the morning peak of cortisol secretion was not missed (7:00 am). The other samples were collected at 7:15 AM, 7:45 AM, 11:30 AM, 3:00 PM, 6:00 PM, 8:00 PM, and 10:00 PM.

Cortisol and TNF-alfa measuring on saliva

A commercial enzyme immunoassay kit to determine salivary cortisol (®DRG International, Inc) was used according to manufacturer's instructions. This is an enzyme immunoassay for the quantitative determination of cortisol, based on the principle of competitive binding. The microtiter wells are coated with a monoclonal antibody directed towards an antigenic site on the Cortisol molecule. Endogenous Cortisol of a sample competes with a Cortisol-horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is inversely proportional to the concentration of Cortisol in the sample. After addition of the substrate solution, the intensity of color developed is inversely proportional to the concentration of Cortisol in the sample. The level of TNF-α, was established in the saliva by using the commercially available kit Diaclone SAS, France. This is an enzyme immunoassay for the quantitative determination of TNF-α, based indirect method. Regarding equations performed to calculate cortisol area under the curve with respect to the ground (AUCg) and cortisol area under the curve with respect to the increase (AUCi) we used following equation as indicated in previous works [60]:

$$AUC_g = \sum_{i=1}^{n-1} \frac{\left(m_{(i+1)} + m_i\right) * t_i}{2}$$

$$AUC_i = \left(\sum_{i=1}^{n-1} \frac{\left(m_{(i+1)} + m_i\right) * t_i}{2}\right) - \left(m_I \sum_{i=1}^{n-1} t_i\right)$$

DNA extraction from saliva

Genomic DNA extraction from the specimens was performed using a PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions with some modifications. DNA concentrations were determined spectrophotometrically using Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California, USA).

Methylation assay

To study methylation levels of promoter region of TNFα gene on DNA extracted from saliva, we used methylation DNA immune precipitation purification technique (MeDIP) which is used in molecular biology to enrich for methylated DNA sequences. It consists of isolating methylated DNA fragments via an antibody raised against 5-methylcytosine (5mC). This technique was described previously [61]. To perform the technique, we used the MagMeDIP qPCR/Auto MagMeDIP qPCR Kit from Diagenode following manufacturer protocol. Then the immunoprecipitated samples have been amplified trough real time PCR using following primers:

TNF-alpha promoter

Forward primer: TTGATGCTTGTGTGTCCCCAA.
Reverse primer: CTCCCTCTTAGCTGGTCCTCT.

We designed our primers based on the same region of the TNFα promoter analyzed in previous studies [62, 63].

Methylation it has been than calculated as percentage of methylation after adjusting each sample with its corresponding input, a fixed amount of DNA that has been processed in the same way of corresponding sample except for immunoprecipitation step.

Statistical analysis

Univariate analysis has been performed to evaluate baseline characteristic of the subjects followed by bivariate analyses to study possible association of variables for being exposed to air pollution or not. Exposure has been considered as present if subject was resident in the urban area and not present if resident in rural area.

Data distribution of outcomes variables AUCg, AUCi, TNFα and TNFα methylation levels have been checked trough histogram graphical representation, showing a not normally distribution of the values for all outcomes considered.

Regression analyses were performed using generalized linear mixed models [64–66] with a linear link between the different outcomes (Cortisol mean, TNFα mean, AUCi, AUCg, TNFα methylation) as the dependent variables and 4,3,2 and 1 days lag values of PM10 and PM2.5 as the independent variable, adjusting for potential confounders age (continuous), gender (male/female), alcohol consumption(yes/no), marital status(single, married,

divorced/separated, and widowed), if rural or urban area (1 = urban, 0 = rural), smoking(yes/no), Body Mass Index (continuous), Beta blockers assumption (yes/no), Diuretics assumption (yes/no), Psychotropic drugs assumption (yes/no), Hypertension (yes/no), Arthritis/AI diseases (yes/no), Chronic Pain (yes/no), Severe depression (yes/no), effective sleep time m(continuous). Lag days values refer to the delay between the day we performed biological sampling on subjects and their exposure to PM. To clarify, we evaluated the possible association between cortisol and methylation levels and values of PM10 and PM2.5 from 1, 2, 3, and 4 days before the sampling event. We used random intercepts to account for repeated measures and within-subject clustering. To assess any potential deviation from linearity, we also fit a generalized additive mixed model (GAMM) with a penalized spline for air pollution values and used generalized cross-validation to select the optimal number of degrees of freedom for this association. Generalized cross-validation estimated that the best-fitting number of degrees of freedom was 1, suggesting that a linear association was the best fit. This result was valid for all our models except for TNFα vs PM2.5 3 days lag, AUCg vs PM10 4 days lag, AUCg vs PM2.5 4 days lag, AUCi vs PM10 and vs 2.5 4 days lag and AUCi vs PM10 3 days lag that showed slopes with degree of freedom > 1. All our models have been adjusted for age, sex, alcohol consumption, marital status, if rural or urban area, smoking, Body Mass Index, Beta blockers assumption, Diuretics assumption, Psychotropic drugs consumption, Hypertension, Arthritis/AI diseases, Chronic Pain, Severe depression, effective sleep time. The cutoff for statistical significance was set at the 95% confidence level (p value ≤ 0.05). All analyses have been performed with STATA® 15/SE. Graphical representations of GAMM models have been obtained using R (v 4.1.1) (R Core Team 2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

Results

The population studied is composed of 20 subjects; 39 observations have been used in the present analysis, since one saliva sample collected was undetermined for both TNF-α and cortisol quantification. The main characteristics of the subjects at baseline are shown in Table 1. We performed a bivariate analysis with baseline characteristics of our subjects to evaluate possible statistically significant differences between the two populations based on different residency areas, rural or urban (Table 2). Only hypertension and smoking reached statistical significance. Regarding levels of exposure, Table 3 shows

Table 1 Univariate analyses for baseline characteristics of participants

Variables	*N*	%
Gender		
Male	10	50
Female	10	50
Age		
Mean	60,6 (C.I. 54,38/66,82)	
Hypertension		
Yes	6	30
No	14	70
Type2 diabetes		
Yes	1	5
No	19	95
Chronic pain		
Yes	8	40
No	12	60
Smoke		
Yes	4	20
No	16	80
BMI		
Mean	25.41 (C.I. 23.87–26.96)	
Severe depression		
Yes	1	5
No	19	95
Psychosis		
Yes	1	5
No	19	95
Arthritis or other autoimmune diseases		
Yes	2	10
No	18	90
Presence of disease or infirmity in the family		
Yes	7	35
No	13	65
Marital status		
Single	2	10
Married	16	80
Widowed	2	10
Alcohol units/week		
0	11	55
1–5	1	5
6–10	2	10
11–15	5	25
β-blockers		
Yes	2	10
No	18	90
Diuretics		
Yes	18	90
No		
Psychotropic drugs		
Yes	2	10
No	18	90

the mean exposure for all time periods considered for both PM10 and PM2.5. Means have been stratified by season (winter/summer) since, as already described, samples were collected in February and July, corresponding, respectively, to winter and summer seasons at our latitude. Next, we examined the relationship between TNF-α and exposure to PM10 and PM2.5 at different lag days. We found a positive association for both PM2.5, which was statistically significant, and PM10, which was statistically significant in all exposure periods analyzed from 4 days lag to 1 day lag, except for the relationship between PM2.5 at 3 days lag and TNF-α, which was not statistically significant (Table 4). Looking at the relationship between AUCg and air pollution, we found a positive statistically significant association with PM10 at 4-day, 3-day, 2-day, and 1-day lag exposure, and a positive but not statistically significant association with PM2.5 at 4-day, 3-day, 2-day, and 1-day lag exposure (Table 5). The association between AUCi and PM10 at 4 days lag and PM2.5 at 4 days lag was evaluated; we found a statistically significant negative association with PM10 at 4 days lag, 3 days lag and 1 day lag (Table 6). This association maintained the same trend but did not reach statistical significance for 2 days lag. Regarding the association with PM2.5, all lag periods considered showed a negative but not statistically significant association (Table 6). The general trend of our relationships considering all time periods from 1 to 4 days lag showed a linear association except for a few specific cases as shown in figures (Figs. 1, 2, and 3). Figure 1 shows GAMM modeling for TNF-α, displaying a general trend of a linear relationship from 1 to 4 days lag, with the only exception being exposure to PM2.5 at 3 days lag. Figure 2 shows GAMM modeling for AUCg, indicating a general trend of a linear relationship from 1 to 4 days lag, with the only exception being exposure to 4 days lag for both PM10 and PM2.5. Figure 3 shows GAMM modeling for AUCi, illustrating a general trend of a linear relationship from 1 to 4 days lag, with the only exception being exposure to 4 days lag for both PM10 and PM2.5. We then performed methylation analyses through MagMeDIP to analyze the methylation levels of the TNF-α promoter. GAMM models (Fig. 4) showed a general linear association except for exposure to PM10 at 3- and 1-day lag. Interestingly, the association with increasing air pollution was negative except for exposure at 4 days lag, which was positive for both PM10 and PM2.5 exposure. In Table 7, we report β values of the association of TNF-α promoter methylation levels with different exposures to air pollution, both in terms of lag exposure and PM type. The β values confirmed the directionality of the association found through GAMM models. None of them reached statistical significance.

Table 2 Bivariate analyses for association with Exposure

Variables	Exposure		*p* value
	No (rural)	Yes (urban)	
Gender			0.37
Female	6(30%)	4(20%)	
Male	4(20%)	6(30%)	
Age			0.09
18–34	1(5%)	0(0%)	
35–54	2(10%)	2(10%)	
55–64	5(25%)	1(5%)	
65 and over	2(10%)	7(35%)	
Hypertension			**0.05**
No	9(45%)	5(25%)	
Yes	1(5%)	5(25%)	
Diabetes			0.30
No	10(50%)	9(45%)	
Yes	0(0%)	1(5%)	
Chronic pain			0.65
No	4(20%)	5(25%)	
Yes	6(30%)	5(25%)	
Smoking			**0.02**
No	10 (50%)	6 (30%)	
Yes	0 (0%)	4 (20%)	
BMI			0.07
Normal weight	3(15%)	7(35%9	
Overweight	7(35%)	2(10%)	
Obese	0(0%)	1(5%)	
Severe depression			0.30
No	10(50%)	9(45%)	
Yes	0(0%)	1(5%)	
Arthritis or other autoimmune diseases			0.14
No	10(50%)	8(40%)	
Yes	0(0%)	2(10%)	
Marital status			0.14
Single	2(10%)	0(0%)	
Married	8(40%)	8(40%)	
Widow	0(0%)	2(10%)	
Alcohol units/week			0.34
0	5(25%)	7(35%)	
1–5	2(10%)	0(0%)	
6–10	2(10)	1(5%)	
11–15	1(5%)	2(10%)	
β-blockers			0.14
No	10(50%)	8(40%)	
Yes	0	2(10%)	
Diuretics			0.14
No	10(50%)	8(40%)	
Yes	0(0%)	2(10%)	
Psychotropic drugs			1
No	9(45%)	9(45%)	
Yes	1(5%)	1(5%)	

Highlighted in bold results with statistical significance p value ≤ 0.05

Discussion

Association between PM exposure and salivary cortisol

Our results show an interesting statistically significant association between exposure to PM10 and PM2.5 and salivary cortisol. It is very important to underline that comparing cortisol levels is extremely complex, especially when there are repeated measures with many samples, as our study design provided. It is crucial to take into consideration changes over time of this hormone [58, 60]. Therefore, we also analyzed two additional measures that are usually used in studies with repeated cortisol sampling: AUCi and AUCg. They showed different characteristics of association: AUCg showed a statistically positive association with 4-, 3-, 2-, and 1-day lag of exposure to PM10. On the other hand, AUCi showed a statistically negative association with 4-, 3-, and 1-day lag of exposure to PM10. Considering that AUCg is more related to "total hormonal output" and AUCi is more related to the sensitivity of the system, it could be possible to speculate that there is an increase in overall hormone production but, at the same time, a reduced dynamism of the system to respond to external stressors [58, 60]. Since cortisol is a fundamental part of the human body's hormonal axis to respond to modifications of normal homeostasis induced by internal or external stimuli, a significant increase in the overall quantities of this hormone reflects an increase in a subject's stress and a reduced capability to respond to stressors, when necessary, since the basal production is already high. This point is illustrated by the negative association between exposure to PM10 and AUCi. These findings are in accordance with previous studies looking at the relationship between cortisol levels and PM exposures [67–69]. Regarding the association of AUCg and AUCi with continuous PM2.5, the magnitude and directionality of β coefficients reflect the same kind of association found for PM10 without statistical significance. So, the absence of statistical significance in AUCi and AUCg analyses may be due to the different nature of the particles that constitute PM10 and PM2.5: the smaller diameter of the particles in the PM2.5 mixture allows them to penetrate deeper into the lower airways, resulting in less permanence in the mouth and consequently a lower effect on salivary cortisol.

Linear and non-linear associations in GAMM analysis

Using generalized additive mixed models (GAMM) in the R environment, we decided to check for any deviation from linearity regarding the different relationships investigated. Even though some specific associations showed a nonlinear pattern, taken together, all GAMM performed showed a general trend of linear association for the outcomes AUCg and AUCi with PM10 and PM2.5. Moreover, the directionality of our GAMMs confirmed estimates from regression analyses.

TNF-α levels and air pollution exposure

Regarding regression models involving TNF-α and PM10 and PM2.5, continuous models showed a statistically significant association with both exposures at 4-, 3-, 2-, and 1-day lag, and we found positive trends that have been confirmed by GAMM models for both PM10 and PM2.5, showing a general trend of linear association. From a biological point of view, exposure to air pollution may cause oxidative stress and inflammation, as evidenced by increased salivary TNF-α levels. Our result, even with different exposure times, is in accordance with findings by Zhu X et al. [41] and is also supported by other studies linking air pollution with inflammatory responses and TNF-α [70–72].

TNF-α promoter methylation and PM exposure

We then decided to analyze the association of promoter methylation levels of TNF-α with exposure to PM10 and

Table 3 Mean exposure levels for all time periods

PM10/PM2.5 by lag day	Exposure levels			
	Winter		Summer	
	Mean(μg/m^3)	C.I. 95%	Mean(μg/m^3)	C.I. 95%
PM10 4 days lag	29.02	22.38–35.66	16.58	15–18.17
PM10 3 days lag	23.93	20.46–27.40	16.10	15.20–17.01
PM10 2 days lag	27.51	23.39–31.62	19.28	18.13–20.43
PM10 1 days lag	27.61	24.32–30.89	19.78	18.70–20.86
PM2.5 4 days lag	17.33	11.66–23.02	8.25	7.43–9.06
PM2.5 3 days lag	12.88	9.02–16.73	9.07	7.98–10.16
PM2.5 2 days lag	15.44	11.93–18.96	9.59	8.65–10.53
PM2.5 1 days lag	16.33	11.83–20.83	11.09	9.97–12.22

Table 4 Association between TNF-α levels and PM levels during the previous four days of exposure

TNFα	Coef.	Std. Err.	*p* value	[95% Conf. Interval]	
PM10 4 days lag	**0.90**	**0.37**	**0.015**	**0.18**	**1.62**
PM10 3 days lag	**1.61**	**0.7**	**0.020**	**0.27**	**2.97**
PM10 2 days lag	**2.00**	**0.63**	**0.001**	**0.76**	**3.23**
PM10 1 day lag	**2.09**	**0.67**	**0.002**	**0.76**	**3.42**
PM2.5 4 days lag	**1,29**	**0.45**	**0.001**	**0.41**	**2.17**
PM2.5 3 days lag	**2.32**	**0.69**	**0.001**	**0.96**	**3.67**
PM2.5 2 days lag	**2.85**	**0.65**	**0.000**	**1.57**	**4.13**
PM2.5 1 day lag	**2.44**	**0.41**	**0.000**	**1.64**	**3.24**

Highlighted in bold results with statistical significance *p* value ≤ 0.05

Table 6 Association between AUCi and PM levels during the previous four days of exposure

AUC_i	Coef.	Std. Err.	*p* value	[95% Conf. Interval]	
PM10 4 days lag	**− 119.19**	**50.25**	**0.017**	**− 218.1**	**− 21.12**
PM10 3 days lag	**− 201.16**	**96.61**	**0.037**	**− 390.52**	**− 11.8**
PM10 2 days lag	− 147.8	93.46	0.114	− 330.97	35.4
PM10 1 day lag	**− 173.63**	**101.25**	**0.09**	**− 372.07**	**24.82**
PM2.5 4 days lag	− 89.11	67.73	0.188	− 221.88	43.64
PM2.5 3 days lag	− 83.2	122.68	0.498	− 323.64	157.24
PM2.5 2 days lag	− 116.34	110.85	0.294	− 333.60	100.92
PM2.5 1 day lag	− 54.24	93.48	0.562	− 237.46	128.98

Highlighted in bold results with statistical significance *p* value ≤ 0.05

PM2.5 at different lag days. GAMM models showed a general trend of linear association, which was positive for both PM10 and PM2.5 at 4 days lag and then negative at 3-, 2-, and 1-day lag. The directionality of these results was confirmed by β values estimates from regression models, but none of them reached statistical significance. The interpretation of these results is quite tricky: it could be possible to speculate that only very recent exposure, like 3-, 2-, and 1-day lag, could cause demethylation of the TNF-α promoter and subsequently an increase in the protein, as we found by analyzing the levels of this cytokine in salivary samples. In fact, it is well known that promoter demethylation allows an increase in gene transcription [53]. The difference in statistical significance levels between regression models involving the TNF-α protein and those involving the promoter of its gene may have multiple explanations: first, we were able to obtain methylation data from only 22 samples out of 39 because some samples did not have enough DNA required for a MagMeDip procedure, which could have caused a loss of information and statistical power. Moreover, there could also be biological reasons causing this difference since other epigenetic events together with methylation, like mRNA post-transcriptional modifications, may play an important role in TNF-α protein levels. So, the methylation levels we encountered are only a part of a more complex biological equation. In addition, we used site time of exposure in our analysis for both the protein and promoter methylation levels, but for epigenetic changes, much shorter time points might be needed, possibly just a few hours, since methylation and demethylation events could happen immediately after air pollution exposure.

Table 5 Association between AUCg and PM levels during the previous four days of exposure

AUC_g	Coef.	Std. Err.	*P* value	[95% Conf. Interval]	
PM10 4 days lag	**0.01**	**0.005**	**0.035**	**0.001**	**0.023**
PM2.5 4 days lag	0.01	0.007	0.192	− 0.005	0.02
PM10 3 days lag	**0.02**	**0.01**	**0.05**	**0.00**	**0.04**
PM2.5 3 days lag	0.01	0.013	0.515	− 0.02	0.03
PM10 2 days lag	**0.02**	**0.009**	**0.049**	**0.00**	**0.04**
PM2.5 2 days lag	0.02	0.01	0.173	− 0.01	0.04
PM10 1 day lag	**0.02**	**0.01**	**0.017**	**0.00**	**0.04**
PM2.5 1 day lag	0.01	0.01	0.301	− 0.00	0.03

Highlighted in bold results with statistical significance *p* value ≤ 0.05

Study strengths and limitations

To our knowledge, our study is the first to analyze together salivary cortisol levels, TNF-α salivary cytokine levels, and salivary promoter TNF-α methylation levels at short-term exposure expressed as 4-, 3-, 2-, and 1-day lag of exposure. We decided to look at this time period because too much lagged exposure could have lost its effect on very complex biological mechanisms such as cortisol and TNF-α levels, since these pathways can be influenced by many other factors and potential confounders may increase their effects when looking at longer periods of observation. Moreover, our study is longitudinal since we have studied our subjects with two different sampling periods and have been able to collect much information about their habits and lifestyles. In the end, our sample is composed of both men and women essentially in good health without any major diseases that could have influenced TNF-α and cortisol levels and production pathways. Regarding methylation analyses, our technique allowed us to focus better on only one gene by studying its promoter methylation with a method that is less expensive and much less time-consuming compared to next-generation sequencing techniques (NGS), which are more expensive and can generate BIG DATA whose interpretation may be quite tricky due to high levels of noise and biases, and data heterogeneity [61, 73].

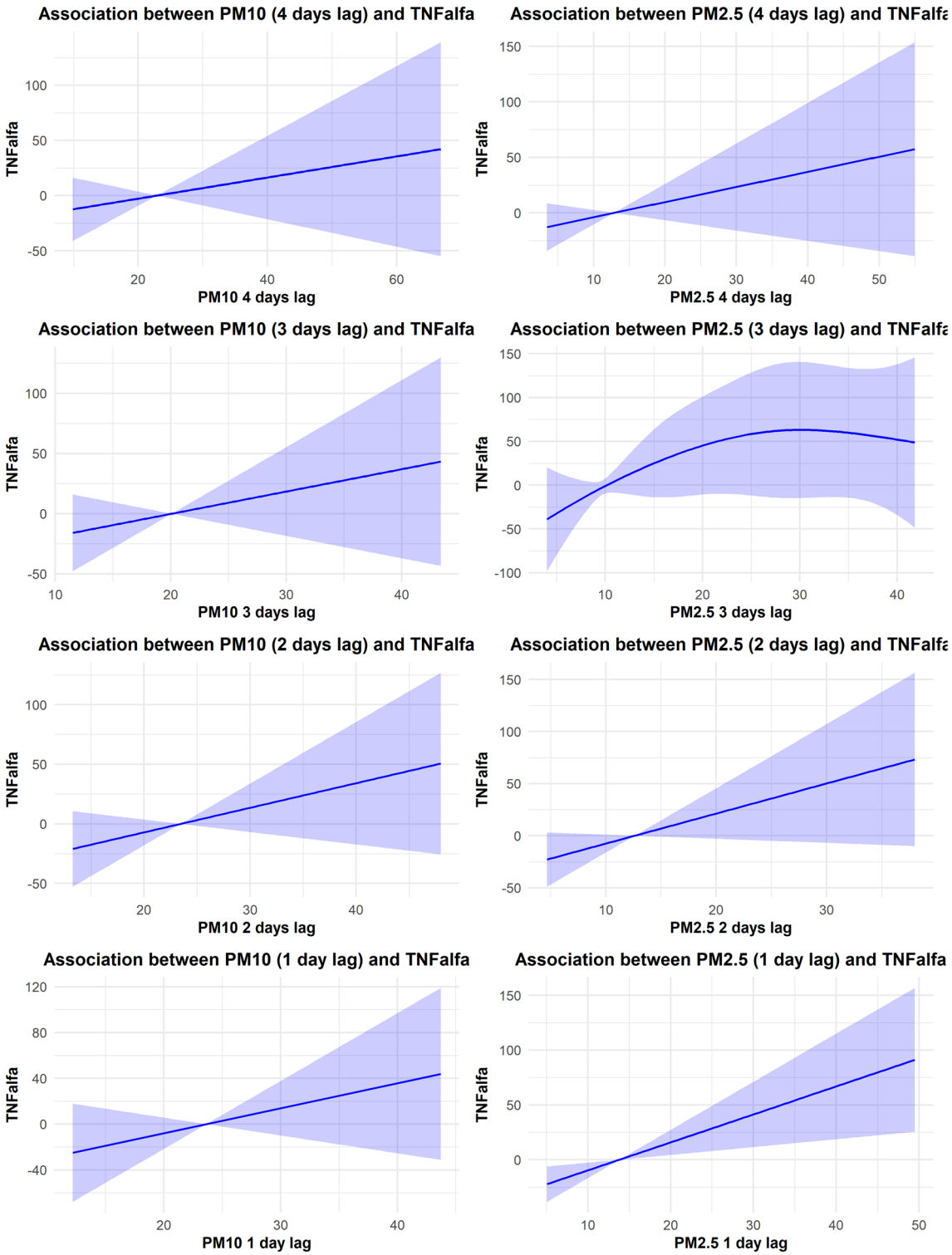

Fig. 1 GAMM models between lag days values of exposure levels PM10/PM2.5 and TNFα

There are some limitations to our study. The small sample size of our population, which resulted in a total of 39 observations, limited the statistical power of our findings. Although we collected 8 saliva samples from each subject throughout the day until 10:00 pm, we were unable to collect samples during nighttime due

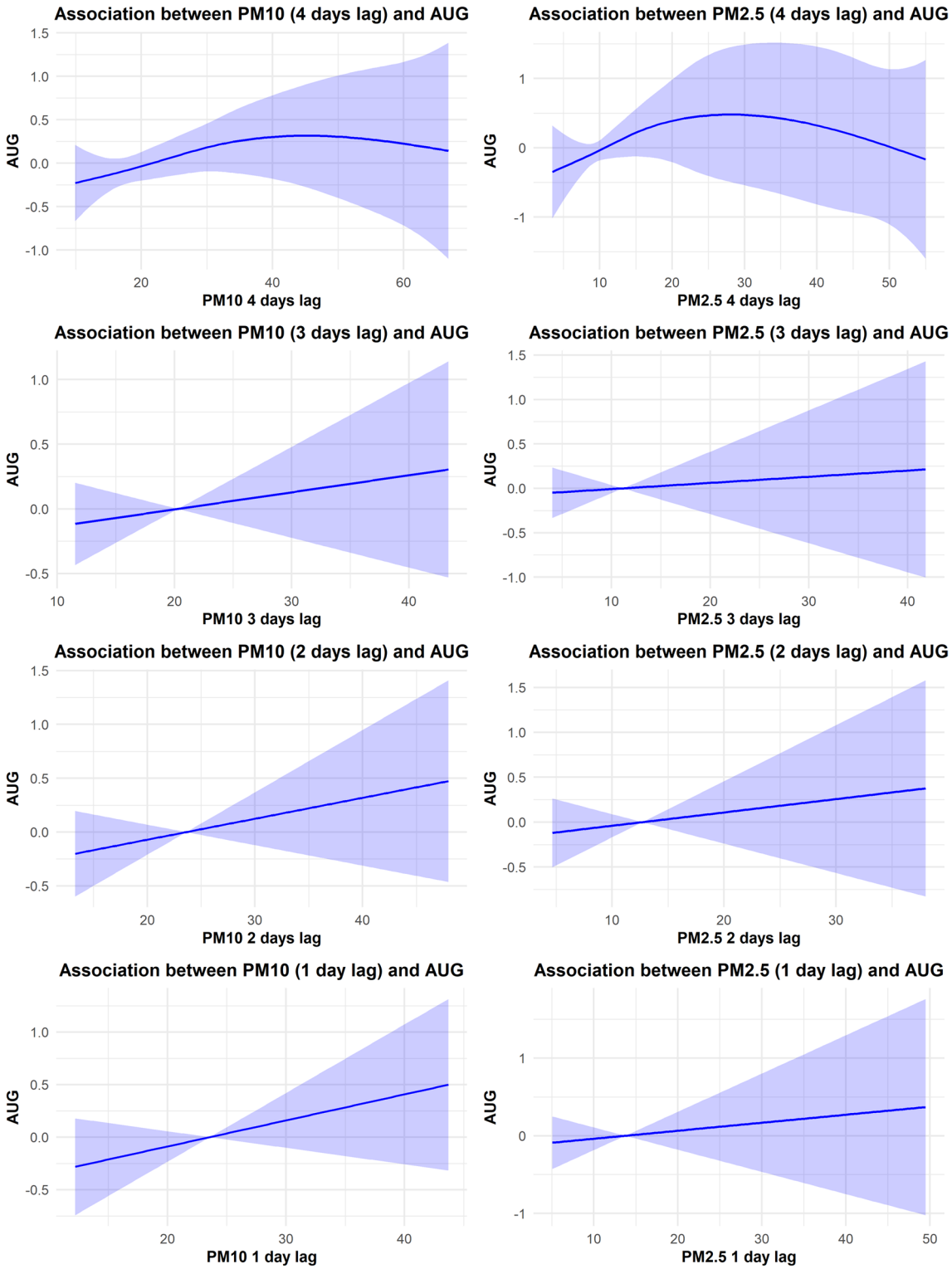

Fig. 2 GAMM models between lag days values of exposure levels PM10/PM2.5 and AUG

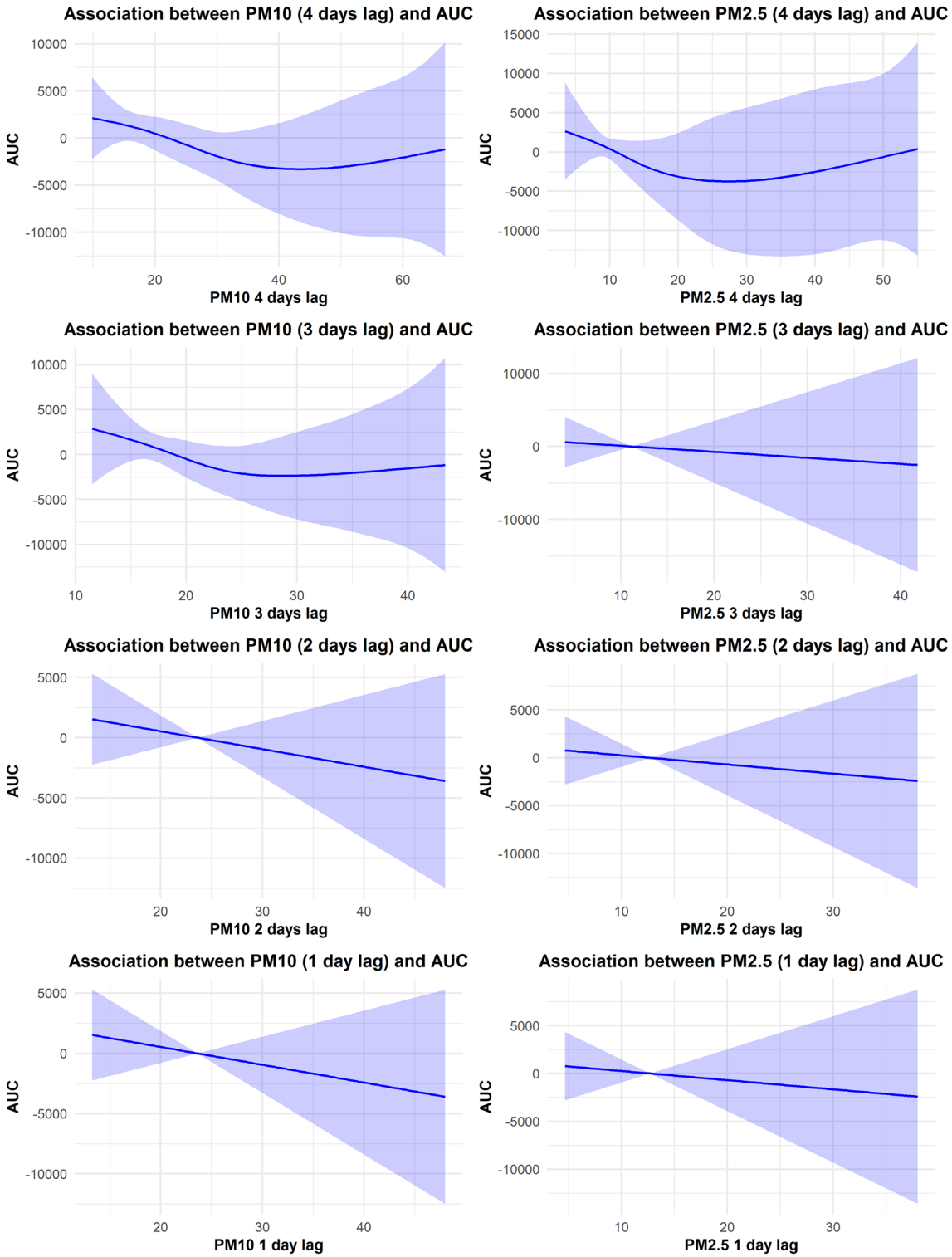

Fig. 3 GAMM models between lag days values of exposure levels PM10/PM 2.5 and AUC

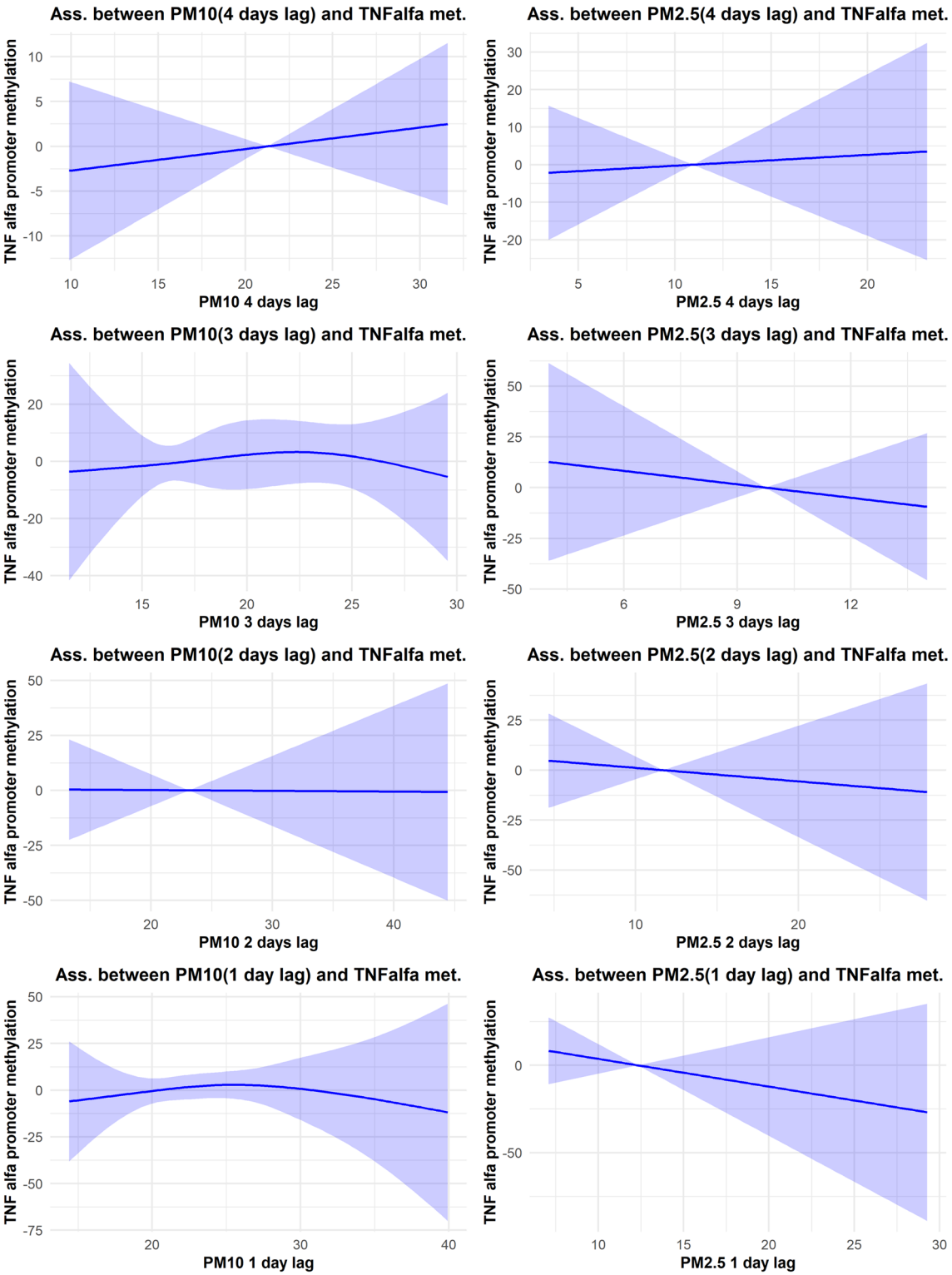

Fig. 4 GAMM models between lag days values of exposure levels PM10/PM 2.5 and TNF-alfa promoter methylation

Table 7 Association between TNF-α promoter methylation and PM levels during the previous four days of exposure

TNF-α promoter methylation	Coef.	Std. Err.	*P* value	[95% Conf. Interval]	
PM10 4 days lag	0.24	0.23	0.30	− 0.21	0.69
PM10 3 days lag	0.19	0.53	0.72	− 0.85	1.24
PM10 2 days lag	− 0.03	0.61	0.95	− 1.24	1.17
PM10 1 days lag	0.32	0.61	0.60	− 0.87	1.50
PM2.5 4 days lag	0.28	0.63	0.65	− 0.95	1.52
PM2.5 3 days lag	− 2.21	2.25	0.33	− 6.62	2.20
PM2.5 2 days lag	− 0.67	0.89	0.45	− 2.42	1.07
PM2.5 1 days lag	− 1.58	0.97	0.10	− 3.49	0.32

to logistical constraints. This limitation might have impacted the accurate measurement of biological parameters, especially cortisol levels. Consequently, this could have affected the robustness of some of our findings, particularly in terms of statistical significance. However, our results are in accordance with larger studies that found a statistically significant positive association between PM2.5 and salivary cortisol output [41, 74]. On the other hand, some studies considering multipollutant models and longer PM2.5 exposures have found a negative association with serum cortisol levels [75].

Another limitation is the reliance on fixed station data for PM exposure, as we did not have individual exposure information. This could have biased the exposure assessment. Moreover, even if we adjusted for many possible confounders, there may be some elements we did not consider that could influence the associations we analyzed. Regarding this aspect, we would like to point out that we are aware that we used many confounders for relatively few observations, but they have been selected due to biological and clinical reasons since each of them can influence our biological parameters, especially cortisol levels. We decided to take into consideration variables that may have a relevant biological magnitude even if not mirrored by statistical power due to the small size of our population. Other variables such as occupation, exercise history, or other socio-demographic characteristics were not included due to the limitations of our data collection process. These factors can significantly impact cortisol and TNF-α levels and should be considered in future studies.

Another limitation is the lack of meteorological variables like temperature in our study: in this kind of study, it has been shown how there could be an interaction between effects coming from temperature and air pollutants. However, some studies have shown interactions between temperature and air pollution effects, but these are often considered in the context of macroscopic events rather than single biological parameters like cortisol or TNF-α levels [76], and even at this large scale, studies are not always in accordance [77, 78]. Moreover, previous works looking at the relationship between cortisol levels and air pollution did not account for temperature [74, 75]. Certainly, more studies are needed to better address this point since some of them showed how PM may encounter seasonal variation in terms of pattern composition influencing inflammatory cytokine production in vitro like TNF-α and IL-6 [48]. This aspect could be related to seasonal differences that have been found in epidemiologic studies looking at air pollution and health outcomes, especially considering that some previous works encountered seasonal differences and variation in air pollution associated with various seasons and specificity of particular months [79].

Studying these temporal differences in terms of composition could clarify some of our results that lack statistical significance, such as air pollution exposure and the association with promoter TNF-α methylation levels, despite strong associations with the cytokine saliva levels. This may be the result of more complex interactions between PM components, size, and cytokine crosstalk that we cannot fully capture with the data at our disposal. Moreover, the relationship between promoter methylation levels and protein levels of TNF-α may be influenced by several factors such as post-transcriptional regulation: TNF-α protein levels can be regulated at multiple levels, including transcriptional, post-transcriptional, and post-translational modifications. Methylation of the promoter is just one regulatory mechanism, and other factors might play a more dominant role in regulating TNF-α levels in response to particulate matter exposure. A more detailed approach could also clarify mechanistic aspects rather than simple associations between air pollution and health outcomes. In addition, it might be interesting to follow our subjects for a longer period, taking more samples to better account for inter-person variability.

Regarding methylation analysis, unfortunately, some samples did not yield a significant amount of DNA to proceed with the immunoprecipitation technique. Moreover, our population may not be representative enough, being composed only of Caucasian males and females. More studies with larger and more ethnically diverse populations are needed to better investigate the relationships we described [80].

Conclusions

Cortisol levels, as well as protein levels of TNF-α, showed significant associations with different lag days of exposure to PM10 and PM2.5. Our findings reveal that there is

an increase in the overall cortisol hormone levels, accompanied by a reduced dynamism of the system to respond to external stressors. This suggests that prolonged exposure to air pollution could lead to a state of heightened stress with diminished adaptive capacity. The observed increase in TNF-α levels may indicate elevated levels of oxidative stress and inflammation as a result of air pollution exposure, highlighting the potential for air pollution to exacerbate inflammatory pathways.

In addition, our study contributes to the growing body of research exploring the relationship between air pollution and promoter methylation levels of TNF-α, although further investigation is needed to elucidate these epigenetic mechanisms. The novelty of our findings lies in the detailed temporal analysis of exposure effects, the integration of cortisol and TNF-α pathways, and the inclusion of epigenetic factors analyzed through the methylation DNA immunoprecipitation purification technique (MeDIP), which together provide a more comprehensive understanding of how air pollution impacts biological systems. These insights underscore the importance of addressing air pollution to mitigate its adverse health effects.

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Author contributions
JD, RL, and EP conceived the study and contributed to data acquisition. AL, LF and, SBS provided assistance with study design and EP provided assistance in preparing and editing the manuscript. Laboratory analyses were done by JD, RL, and LP. Statistical analyses were done by JD and PB. AG, MMD and, PB supervised the project. Funding acquisition, AG and MMD. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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