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STUDIO DELL'ASSOCIAZIONE TRA INQUINAMENTO ATMOSFERICO E DISTURBO DEPRESSIVO MAGGIORE: RUOLO DI MARCATORI BIOLOGICI ED EPIGENETICI

Tesi di Dottorato di Ricerca

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ABSTRACT

INTRODUCTION: Major Depressive Disorder (MDD) is the most common mental disorder worldwide, affecting about 13% of adults and representing the second leading cause of disability. Despite its important social impact, etiopathology of the disease is still unknown. It has been suggested that MDD is a multifactorial disease, where genetic, environmental and biological factors may play a role. Among biological changes, epigenetic, immunological, and hormonal abnormalities have been found in MDD patients. Furthermore, recent studies have investigated whether air pollution may be a potential contributor of the onset of the disease, finding positive associations.

AIMS: In a sample of MDD patients: evaluate the association between exposure to air pollution and MDD severity; evaluate the relationship between MDD severity and different biological (inflammatory, epigenetic, hormonal) markers; evaluate the association between air pollution and the biological variables of interest; quantify the specific contribution of the investigated biological variables in the chain of events linking air pollution exposure to MDD severity (in the present study, we will focus on epigenetic alterations only).

METHODS: Overall, 416 MDD patients accessing the psychiatry unit of the Policlinico Hospital in Milan (Italy) from September 2020 to December 2022 have been recruited. Enrolled patients answered two questionnaires to collect demographic and lifestyle information, and history and characteristics of depression; they also donated a blood sample to examine biomarkers of interest. Severity of MDD was evaluated through five severity-of-illness rating scales: Montgomery-Asberg Depression Rating Scale (MADRS); Hamilton Depression Rating Scale (HAMD); Clinical Global Impression (CGI); Global Assessment of Functioning (GAF) and Sheehan Disability Scale (SDS). Daily exposures to particulate matter with diameter less than or equal to 10 (PM_{10}) and 2.5 μm ($PM_{2.5}$), and nitrogen dioxide (NO_2) were estimated as daily means through the Flexible Air quality Regional (FARM) chemical transport model of the Lombardy regional Environmental Protection Agency (ARPA Lombardia), and assigned to each subject on the basis of his/her residential address. Daily average exposure to apparent temperature was also estimated, combining daily measurements of ambient temperature, humidity, and wind speed, retrieved from ARPA weather monitoring stations closest to patients' residential addresses. Daily estimates of both apparent temperature and air pollutants were averaged to obtain moving averages of exposure. Multivariate regression models were used to assess the associations between air pollutant concentrations and MDD severity scales, air pollutants and methylation of *CLOCK* (circadian locomotor output cycles protein kaput) and *CLOCK*-related genes, and methylation of *CLOCK* and *CLOCK*-related genes and MDD severity scales.

RESULTS: Two-thirds of included patients were females and about one-third had a family history of depression. Most women had depression with symptoms of anxiety, while men had predominantly

melancholic depression; they were also more likely to experience suicidal and addictive behaviors. Average exposure to NO_2 in the two weeks preceding recruitment (lag0-14) was associated with a worsening of MDD severity [HAMD: β=2.09, 95% CI (0.63; 3.56); CGI: β=0.27, 95% CI (0.02; 0.51); and GAF: β=-1.96, 95% CI (-3.60; -0.33)], while particulate matter exposure (PM_{10}, $PM_{2.5}$) was associated with MDD severity only when temperatures were low or among hypersusceptible subjects. Short-term exposure to PM_{10} was associated with hypomethylation of *CRY2* and hypermethylation of *OX1R*, *CRY1,* and *ARNTL* at different lags of exposure within the two weeks preceding recruitment. Long-term exposure to PM_{10} (average of the three- and six-months preceding recruitment) was positively associated with *CRY1* and negatively associated with *CRY2*. Results were similar for $PM_{2.5}$ exposure. When short-term exposure to NO_2 was considered, we observed an increase methylation of *CRY1* and a reduced methylation of *CRY2*. Long-term exposure to NO_2 was positively associated to the methylation of *CRY1* and *HERVW*. In the whole population, hypermethylation of *CLOCK* was associated with less severe scores of depression. This association was found to be stronger in the subtype of depression "with strong symptoms of anxiety". Hypermethylation of *OXTR* was associated with more severe "melancholic / psychotic / no prevalent" type depression.

CONCLUSIONS: Exposure to PM_{10} and $PM_{2.5}$ did not exert a direct effect on the severity of depressive symptoms, while their influence emerged more clearly among hypersusceptible subjects. In addition, PMs had a greater significant impact on MDD severity when temperatures were very low. NO_2 exposure was strongly associated with MDD severity in the whole population and showed higher effects among hypersusceptible subjects as well as with concomitant exposures to low temperatures.

Short- and long-term exposure to particulate matter resulted associated with altered methylation of CLOCK and CLOCK-related genes, which can be involved in circadian rhythms, often affected by depression.

The hypermethylation of *CLOCK* was associated with lower scores of MDD severity suggesting (conversely) that the hypomethylation of *CLOCK* could be associated with a worsening of depressive symptoms. A finding consistent with preliminary data from the literature (1) which have highlighted an increased expression of *CLOCK* in subjects affected by MDD.

Taken as a whole, these findings suggest a possible role of CLOCK and CLOCK-related genes in the pathway linking air pollution exposure to the worsening of MDD severity.

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1. INTRODUCTION

Major Depressive Disorder (MDD) is the most common mental disorder characterized by at least one discrete depressive episode lasting minimum 2 weeks and involving clear-cut changes in mood, interests and pleasure, and also changes in cognition and vegetative symptoms, such as disturbed sleep or appetite (2).

MDD is twice more common in females than males and affects about 13% of adults in their lifetime (3–5). Indeed, it is the second leading cause of disability, being also associated with an increased risk of developing conditions such as diabetes mellitus, heart disease and stroke, consequently increasing its burden of disease (6,7). Moreover, MDD patients have an increased risk of suicide. It has been demonstrated that about 50% of the total suicides per year occur within a depressive episode and patients with MDD have a 20-fold greater risk to die by suicide than the general population (8–10).

Despite the huge social impact of this condition, no established mechanism can explain all aspects of the disease. It has been demonstrated that MDD is a multifactorial disease, where heritability accounts for approximately 35% (11). In addition, research studies have reported that some environmental factors such as sexual, physical or emotional abuse during childhood, lack of a partner (e.g., owing to divorce or widowhood), recent negative life events, e.g., illness or loss of close relatives or friends, financial or social problems and unemployment are associated with a greater risk of MDD (12–14). On the other hand, biological mechanisms underlying MDD have been investigated and epigenetic, immunological and hormonal abnormalities have been found in subjects affected by this disorder when compared to healthy controls (15). Furthermore, severity of depressive symptoms seems to be directly associated with the degree of biological abnormalities (15).

As MDD is associated with other medical conditions (e.g., being overweight or sleep disturbances) characterized by alterations in circadian rhythms (16), recent studies have focused on the epigenetic mechanisms underpinning appetite and sleep problems in depressed patients (17). It has been observed that MDD is associated with higher core body temperature, higher cortisol levels and lower melatonin secretion, supporting an involvement of the circadian system, although results are contradictory (18–20).

Circadian rhythms are generated, in part, by clock genes that are under the control of a small pair of nuclei in the anterior hypothalamus, the suprachiasmatic nucleus (SCN). The SCN receives extensive input from many brain regions and serves as a primary modulator of virtually all cellular clocks in the body (21,22). The SCN maintains synchrony by resetting circadian rhythms via photic and non-photic signaling. The molecular basis of circadian rhythms involves a positive (+) and a negative (−) feedback. The positive feedback usually acts during the daytime and the negative one

during the night. In the positive one, the transcriptional activators BMAL1 and BMAL2 dimerize with *CLOCK* (circadian locomotor output cycles protein kaput), or possibly with neuronal NPAS2 protein in brain tissue, and this heterodimer binds to the promoter elements (CACGTG) present in clock and clock-controlled genes (CCGs) (23). The negative feedback includes the following clock genes: Period (*PER1*, *PER2*, *PER3*) and Cryptochrome (*CRY1* and *CRY2*) that are activated by the CLOCK– BMAL heterodimer. *PER1*, *PER2*, *PER3*, *CRY1*, and *CRY2* proteins form heterodimers that eventually enter the nucleus to inhibit transcription by binding to the CLOCK–BMAL complex (23). In addition to these transcriptional mechanisms, it is known that the regulation of these genes is mainly due to DNA methylation, a molecular mechanism of gene expression regulation, which is able to react and be reprogrammed by environmental stimuli (24).

On the other hand, the clock genes regulate the transcription of glucocorticoid receptors (25). This biological mechanism explains why patients affected by MDD show a paradoxical state of chronic systemic over-inflammation (26). The outcome, inflammation, is a biological host defense mechanism characterized by increased blood flow and recruitment of innate immune cells to the site of injury.

The link between increased inflammation and MDD has been largely studied since the 1990s (27,28), which has led to the formulation of the macrophage hypothesis of MDD (also known as the cytokine hypothesis of MDD (29,30). This model proposes that external and internal stressors trigger MDD by elevating the production of proinflammatory cytokines interleukin-1 (IL-1) and IL-6, as well as activating cell-mediated immunity. More recently, observational, experimental and clinical studies have observed that the activation of innate immune mechanisms, especially proinflammatory cytokines IL-1, IL-6 and tumor necrosis factor alpha (TNF-α), as well as C-reactive protein (CRP), may have a role in the initiation and progression of psychiatric diseases, including MDD (31–38). Several recent publications have focused on these associations (39–48) and while the majority of these involve pro-inflammatory cytokines and CRP, changes in the function and numbers of innate immune cells, namely natural killer (NK) cells, have also been investigated.

Recent research has also showed that MDD is characterized by hormonal abnormalities (26). In particular, abnormalities have been observed in the hypothalamic-pituitary-adrenal (HPA) axis (e.g. failed suppression in dexamethasone test) in MDD patients versus controls (49). In addition, inflammation might also alter the release and circadian rhythm of hormones and neuropeptides implicated in the regulation of human behavior such as oxytocin, vasopressin, kisspeptin, orexin and prolactin (50).

In the attempt to disentangle mechanisms outstanding MDD, air pollution has been hypothesized as a potential contributor to the onset of the disease, also in the light of the increasing

mental health social costs due to urbanization (51). Research studies have shown that air pollution may be involved in the onset of depressive symptoms (52–54). We conducted a meta-analysis of the studies published up to May 2021, and results have been published in Environmental Pollution (**Attached file 1**). The meta-analysis included 39 studies: 16 were cross-sectional, 5 cohort studies, 11 time-series, 6 case-crossover and one a nested case-control study. With regard to duration of exposure, 21 studies evaluated short-term effects of air pollution exposure, 16 studies long-term effects and two studies both short and long-term effects. Briefly, we estimated a meta-analytic increased risk of depression associated with long-term exposure (≥30 days) to $PM_{2.5}$ (relative risk: 1.074, 95% confidence interval: 1.021–1.129) and NO_2 (1.037, 1.011–1.064). Short-term exposure (<30 days) to PM_{10} (1.009, 1.006–1.012), $PM_{2.5}$ (1.009, 1.007–1.011), NO_2 (1.022, 1.012–1.033), SO_2 (1.024, 1.010–1.037), O_3 (1.011, 0.997–1.026), and CO (1.062, 1.020–1.105) were also positively associated with an increased risk of depression. Publication bias was present in half of the investigated associations and most of the meta-analytic estimates had high heterogeneity preventing us to draw very firm conclusions. On the other hand, when sensitivity analyses were conducted, all the estimates were coherent after excluding single studies, confirming the soundness of our results. Of note, none of included studies was on severity of depression. Moreover, air pollution may contribute in the progression of depressive symptoms through three different biological mechanisms: (i) producing systemic over-inflammation that in turn modifies neurotransmitter release and alters circadian rhythms (55), (ii) overcoming the blood–brain barrier and having a direct toxic effect on the CNS (56), and (iii) stimulating brain microglia by changes in bone marrow of the skull activated by chronic peripheral damage (e.g., in the respiratory system) (57).

1.1 Aims and Hypotheses

Given the above summarized available evidence, the relationships between air pollution exposure and inflammation, CLOCK and CLOKC-related gene methylation, and hormonal dysregulation appear a promising mechanism for explaining MDD development and worsening. The hypothesis is that air pollution exposure may exacerbate neuroinflammation with consequent epigenetic and hormonal dysregulation, resulting in the worsening of depressive symptoms (**Figure 1**).

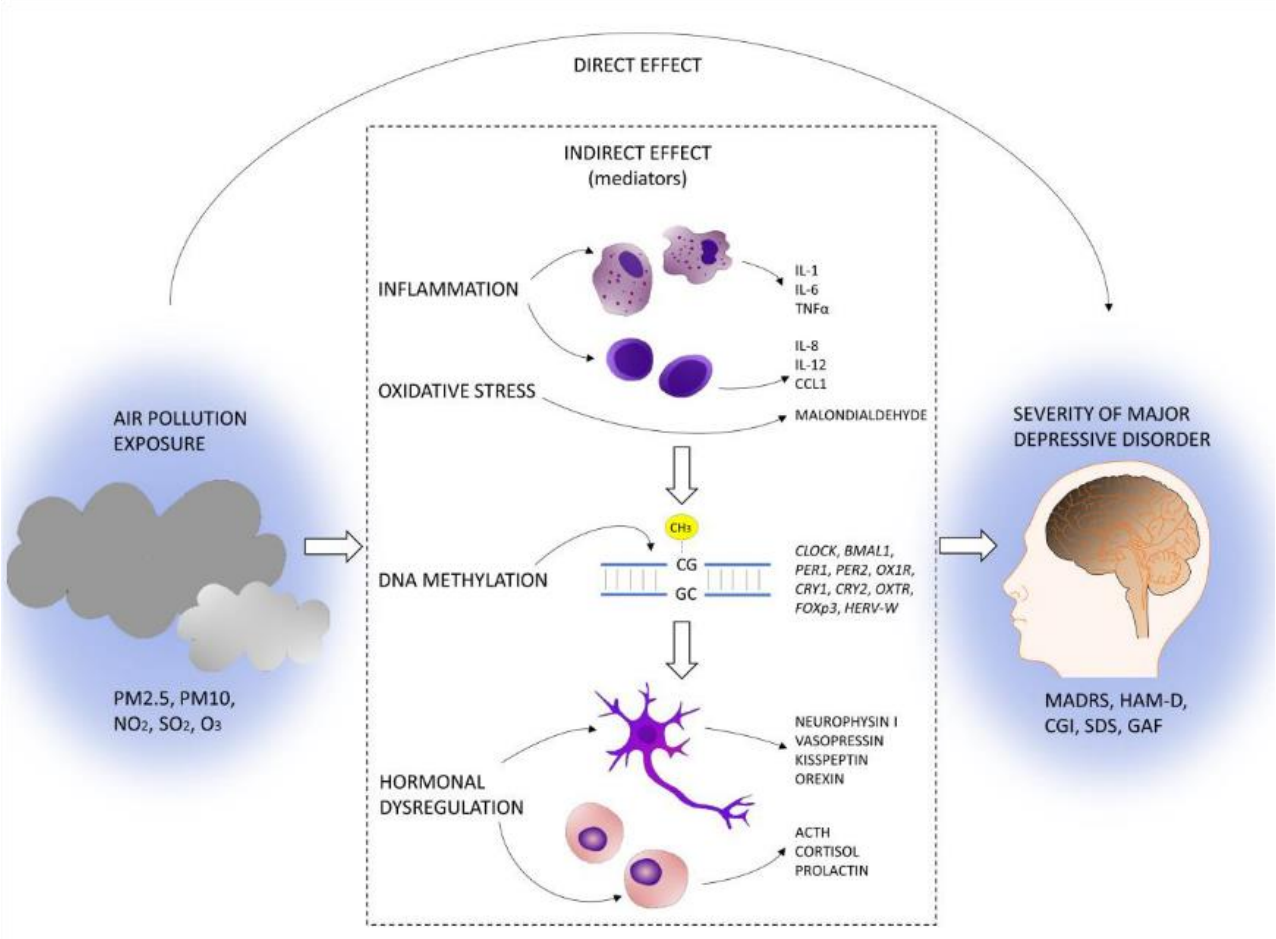


Figure 1: DeprAir conceptual framework. *Source: Borroni E, Pesatori AC, Nosari G, Monti P, Ceresa A, Fedrizzi L, et al. Understanding the Interplay between Air Pollution, Biological Variables, and Major Depressive Disorder: Rationale and Study Protocol of the DeprAir Study. Int J Environ Res Public Health. 2023 Mar 15;20(6):5196. https://doi.org/10.3390/ijerph20065196 (211).*

To verify the hypothesis, a multi-step approach has been adopted within a cross-sectional study with the aim to:

1. investigate the association between exposure to air pollution and MDD severity in a sample of patients with MDD, with an in-depth analysis of the role of apparent temperature;

2. assess the relationship between MDD severity and different biological (inflammatory, epigenetic, hormonal) markers, measured in blood samples collected from all the subjects recruited in step 1;

3. evaluate the association between air pollution and the biological variables of interest identified in step 2;

4. quantify the specific contribution of the investigated biological variables in the chain of events linking air pollution exposure to MDD severity.

2. METHODS

The DeprAir study is a cross-sectional study conducted in the Lombardy region, Italy, whose aim is to understand the interplay between air pollution, biological variables, and MDD.

2.1 Study Population

The study population includes 416 depressed patients who accessed the psychiatry unit of the Policlinico Hospital in Milan (Italy), from September 2020 to December 2022, and have been recruited by trained psychiatrists. Participants were recruited among hospitalized or day-hospital patients or outpatients, who accessed the hospital since 2003 for MDD. The physician contacted already known patients by phone or met them in person, if they were hospitalized or outpatients, described the study aims, and asked for participation in the study. In order to be eligible, patients had to fulfill the following criteria: being ≥18 years old at enrollment; having received a diagnosis of MDD and having signed the consent form. Patients were excluded when they: had a medical condition associated to behavioral disorders (e.g., unbalanced hypothyroidism or stroke); had abused of drugs in the last four weeks; had comorbidities related to other psychiatric disorders (except for personality disorders different from borderline personality disorder); had medical conditions which may alter inflammatory markers (e.g., autoimmune diseases); had known ongoing infections; were taking treatments which may influence biological markers of interest (e.g., corticosteroids or interferons); were pregnant; were < 18 years old.

2.2 Epidemiological and Clinical Data Collection

At recruitment, a consent form has been signed by each subject to: extract personal information from medical records (if already known); answer two questionnaires administered by the psychiatrist to collect demographic and lifestyle information, as well as depression history and characteristics; donate 30 mL of blood (five EDTA tubes of 6 mL each).

2.3 Questionnaire on Sociodemographic and Lifestyle Characteristics

Each patient was interviewed by the psychiatrist who filled in the questionnaire. The questionnaire included information on sociodemographic data (birth date, sex, height, weight, education, occupation status), recent residential history (current complete address, previous complete address if changed in the last year, traffic status in the residential area), smoking history, including passive smoking at home and at workplace (smoking status; duration of smoking; number of cigarettes smoked; age at starting; age at quitting if former smoker; number of smoking family members; number of smoking colleagues at work), current health status including information on

history of selected diseases (hypertension, hypercholesterolemia, diabetes, cancer, heart disease, renal failure) and medication, physical activity levels and sedentary behavior, type of diet (eating everything, vegetarian, vegan), and drinking habits (how much tea, coffee, wine, beer, and spirits).

2.4 Questionnaire on History and Characteristics of Depression

The anamnestic questionnaire collected information about depression history and characteristics, in details: family psychiatric history [including the type(s) of psychiatric disorder(s)], age at onset, duration of untreated illness in months, total duration of illness in years, duration of the latest episode in months, number of depressive episodes, hospitalizations (no vs. yes + total number of hospitalizations), suicide attempts (no vs. yes + total number of suicide attempts), psychotic symptoms (no vs. yes), seasonality of depression (no vs. yes), subtype of depression (melancholic, psychotic, with strong symptoms of anxiety, atypical), history of lifetime substances abuse (never, single-abuse or multiple-abuse and, if the subject ever suffered of substances abuse, type(s) of abuse(s) from alcohol, cocaine, cannabis, heroin, LSD, amphetamines, drugs, and Methylenedioxy methamphetamine (MDMA)), antidepressant treatment (no vs. yes + type of antidepressant assumed, active principle, dose, number of active principles ever assumed, suspension, and other treatments).

2.5 Diagnostic Criteria and Rating Scales

Diagnosis of MDD was confirmed by using Structural Clinical Interview for DSM-5 (SCID—Italian version) [33]. Depression severity of enrolled patients was evaluated by administering them the following rating scales, which are commonly used in clinical practice to assess the severity of affective symptoms:

- Montgomery-Asberg Depression Rating Scale (MADRS): this tool assesses core symptoms of MDD (e.g., anhedonia, sadness and agitation). It is composed of 10 items, as follows: apparent sadness, reported sadness, inner tension, reduced sleep, reduced appetite, concentration difficulties, lassitude, inability to feel, pessimistic thoughts, suicidal thoughts. Each item has a severity scale from 0 to 6, with higher scores reflecting more severe symptoms. Ratings can be summarized in an overall score (from 0 to 60), which allows to stratify severity of depression as: 0–6: no depression, 7–19: mild depression, 20–34: moderate depression, ≥35: severe depression (58);
- Hamilton Depression Rating Scale (HAM-D) 21-item: this tool assesses anxiety and somatization symptoms of MDD. It is composed of 21 questions on types of symptoms associated with depression such as anxiety, mood, insomnia, and somatic symptoms experienced within the past week. Each symptom is rated on a scale of 0–2, 0–3, or 0–4 with

0 being absent and 2, 3, or 4 being the most severe. To obtain the overall score of severity (from 0 to 67), ratings can be added, and the total score can be stratified as: 0–7: no depression, 8–16: mild depression, 17–23: moderate depression, ≥24: severe depression (59);

• Clinical Global Impression-severity of illness (CGI): this tool is used by the psychiatrist to evaluate the global severity of illness answering the following question: "Considering your total clinical experience with this particular population, how mentally ill is the patient at this moment?". The answer is given following this seven-point rating scale: 1 = normal, not at all ill; 2 = borderline mentally ill; 3 = mildly ill; 4 = moderately ill; 5 = markedly ill; 6 = severely ill; 7 = among the most extremely ill patients (60);

• Sheehan Disability Scale (SDS): this scale is used to evaluate the social dysfunction associated with MDD. It consists of a self-reported assessment of functional impairment composed of five items. The first three are global rating scales which assess impairment in work, home, and family responsibilities. There are two additional questions which measure perceived stress and social support. The items are scored individually on 10-point numerical rating scales, except for the "social support" one that can be scored 0–100 (61);

• Global Assessment of Functioning (GAF): this tool is used to evaluate the overall impairment associated with MDD. In particular, it measures how much a person's symptoms affect his/her day-to-day life on a scale of 0 to 100. This scale is broken into 10 sections, which are known as anchor points. The higher the score is, the better the patient is able to handle daily activities, suggesting that a lower score indicates a greater social disfunction associated with depression (62).

2.6 Blood Sample Collection

Specific laboratory standard operating procedures have been developed to ensure quality control of every step involved in biospecimen collection and storage. The psychiatrist performed directly the blood drawing. Each subject provided a 30 mL blood sample in five EDTA tubes, which were delivered to laboratory and processed within 4 h. One of the tubes is used for blood cell count, while the remaining ones were centrifuged and processed to obtain plasma and buffy coat fractions. Plasma and buffy coat samples were stored at −80 °C for subsequent quantification of inflammatory and hormonal markers and DNA methylation analysis, respectively.

2.7 Inflammatory Markers

As mentioned above, markers of both innate and adaptive immunity have been widely associated with the severity of MDD. Considering the innate immunity, the following markers have

been measured—IL-1, IL-6, and TNFα, while the following markers of adaptive immunity have been considered—IL-8, IL-12, and CCL1. In addition, the levels of malondialdehyde has been measured as a parameter of oxidative stress. All these markers have been evaluated on plasma by using ELISA (enzyme linked immunosorbent assay) kits.

2.8 DNA Methylation of Clock and Clock-Controlled Genes

We have selected 10 target genes (*CLOCK, BMAL1, PER1, PER2, OX1R, CRY1, CRY2, OXTR, FOXp3, HERV-W*), which included clock genes and genes directly stimulated by clock pathways, to measure DNA methylation by pyrosequencing. Following genomic DNA extraction from buffy coat, we performed this using a Promega kit (Madison, WI, USA), 3 μg DNA (concentration 25 ng/μL), which has been bisulfite-treated using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Bisulfite-treated DNA has been stored at −20 °C and used shortly after treatment. For each reaction, a 50 μL PCR has been carried out by adding 10 μL of bisulfitetreated genomic DNA to 25 μL of GoTaq Green Master mix (Promega, Madison, WI, USA) 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), and water. One of the primers is biotin-labelled and is used to purify the final PCR product by Sepharose beads. The PCR product has been bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden), and the Sepharose beads containing the immobilized PCR product have been purified, washed, denatured using a 0.2 M NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc., Westborough, MA, USA), as recommended by the manufacturer. Then, 0.3 μM Pyrosequencing primer has been annealed to the purified single-stranded PCR product, and Pyrosequencing has been performed using the PyroMark Q96 MD Pyrosequencing System (QIAGEN). Methylation quantification has been performed using the provided software (Pyro Q-CpG software, version 1.0.9—Biotage, Uppsala, Sweden)). The degree of methylation has been expressed as percentage of 5-methylated cytosines (%5mC) over the sum of methylated and unmethylated cytosines. Built-in controls have been used to verify bisulfite conversion efficiency.

2.9 Hormonal Markers

Hormonal changes, as well as inflammation, can be correlated with the severity of MDD. The following hormones (including neuropeptides) have been measured: adrenal corticotropic hormone (ACTH), cortisol, neurophysin I (a good marker of oxytocin levels in the CNS), vasopressin, kisspeptin, orexin, and prolactin. Plasma samples of the recruited subjects are collected at similar

time (around 11 a.m., as hormone levels change according to circadian rhythms) and measured using ELISA kits.

2.10 Exposure Assessment

Air pollution was defined as the exposure to the following pollutants: particulate matter with diameter less than or equal to 10 (PM_{10}) and 2.5 μm ($PM_{2.5}$), and nitrogen dioxide (NO_2). In order to assess the exposure of each pollutant, each patient's residential address was translated into spatial coordinates using the web tool GPS Visualizer (https://www.gpsvisualizer.com/, accessed on 17 January 2023) and geocoded using QGIS (QGIS Development Team, 2022. QGIS Geographic Information System. Open-Source Geospatial Foundation Project. http://qgis.osgeo.org, accessed on 17 January 2023). Air pollutants levels were assigned to each patient using daily mean estimates derived from the Flexible Air quality Regional (FARM) model (63,64). This type of Eulerian model considers the atmospheric chemistry, together with transport, dispersion, and deposition phenomena (65,66). By integrating data measured from the Regional Environmental Protection Agency (ARPA Lombardia) air quality and meteorological monitoring stations, emissions, concentrations at the beginning of the simulation period, and trend in adjacent areas, it estimates pollutants' concentrations as daily/hourly means covering the whole Lombardy territory with a grid of 1×1 km cell (providing 244x236 different grid cells). The daily average of pollutants' exposure estimated inside the grid cell where patients' residential address fell were assigned to each patient.

We chose to use the FARM model to estimate individual exposure instead of measurements derived from the monitoring stations since most of our study population is composed of subjects living in the city of Milan (341 (82%)). Although Milan is one the cities with the highest number of available air quality monitoring stations, the use of monitors to assign individual exposures would have resulted in more than 100 subjects sharing the same exposure measurements from a single station, thus strongly reducing inter-individual variability. The alternative tool (the FARM model), although based on estimates and not on measured data, has a finer spatial resolution leading to a better characterization of exposure and a higher inter-individual variability. For example, using FARM the maximum number of subjects attributed to the same grid cell in Milan was 16.

The use of personal monitors (i.e., wearable devices) was not feasible due to the high costs related to the relatively large study population as well as to the need of taking into account long-term exposures. In addition, although personal monitors can decrease measurement error, they can also bias exposure estimates due to confounding by personal characteristics and behaviors (67).

Daily estimates of each pollutants' exposure were obtained for each day starting from the day of recruitment (lag0) to 365 days before recruitment (lag365). Subsequently, daily estimates of

the day of recruitment were averaged with the levels of the day before (lag 0–1) and of each preceding day up to 365 days before (lag 0–365), thus obtaining moving averages of exposure. Different lags were considered in the analyses, representing short- (each lag from lag0-1 to lag0-7), middle- (lag0-14, lag0-21, lag0-30) and long-term (lag0-60, lag0-90, lag0-180, lag0-365) exposure.

Apparent temperature (AT) exposure was also estimated. In order to obtain AT estimates, meteorological data (e.g., ambient temperature, humidity, and wind speed) were retrieved from Regional Environmental Protection Agency (ARPA Lombardia) monitoring stations. Daily means of temperature, humidity, and wind speed measured at the station closest to the subject's residential address were assigned to each subject. Missing values for each meteorological variable on a specific day and monitor were imputed by computing the average of measurements of that variable for the previous and the following seven days. Daily AT estimates were obtained combining ambient temperature, humidity, and wind speed as follows:

$$AT = Ta + 0.33 * e - 0.70 * WS - 4.00$$

and

$$e = Rh/100 * 6.105 * exp(17.27 * Ta/(237.7 + Ta))$$

where Ta is the temperature (°C), Rh is the relative humidity (%), and WS is the average wind speed (m/s).

As for air pollutants' exposure, daily estimates of AT exposure were obtained for each day starting from the day of recruitment (lag0) up to 365 days before recruitment (lag365). Subsequently, daily estimates of the day of recruitment were averaged with the levels of the day before (lag 0–1) and of each preceding day up to 365 days before (lag 0–365), thus obtaining moving averages of AT estimates. Different lags were considered in the analyses, representing short- (each lag from lag0-1 to lag0-7), middle- (lag0-14, lag0-21, lag0-30) and long-term (lag0-60, lag0-90, lag0-180, lag0-365) exposure.

Extreme hot days and extreme cold days were also calculated: temperatures were considered extremely hot when daily maximum temperatures were above the 90th percentile of the daily distribution during summer months, i.e. June, July and August, while they were considered extremely cold when daily minimum temperatures were below the 10th percentile of the daily distribution during winter months, i.e. November, December, January and February.

Exposure to solar radiation (expressed in W/m^2) indicating the daily value of direct radiation and diffuse global radiation, in the unit of horizontal surface was also estimated. Daily mean estimates were retrieved from ARPA Lombardia monitoring stations closest to the subjects' residential addresses and assigned to each subject. Missing values on a specific day and monitor were imputed by computing the average of measurements of that variable for the previous and the following seven days. As for air pollutants and AT exposure, daily estimates of the day of recruitment were averaged

with the levels of the day before (lag 0–1) and of each preceding day up to 365 days before (lag 0–365). The same cumulative lags considered for air pollutants and AT were used in the analyses.

2.11 Statistical analyses

Descriptive analyses of study population characteristics, variables concerning the MDD characteristics, and related severity scales were performed. Descriptive analyses were conducted overall, and stratified by gender (males and females). When variables followed a continuous normal distribution, data was summarized reporting mean values and standard deviations; meanwhile, when variables were categorical, absolute and percentage frequencies were reported. Gender differences were tested using t-test for unmatched data for continuous normal variables, while chi-squared test was used for categorical variables.

To study the association between air pollutants exposure and severity of MDD, different models for each pollutant and for each severity scales were used. Multivariate linear regression models were used for all the severity scales except for CGI where a multivariate ordinal regression model was used, due to the nature of this severity scale. These models were adjusted for apparent temperature, gender, age, occupation, education, month and year of recruitment, and origin of recruitment. The role of hypersusceptibility (defined as the presence of at least one condition among the following: type II diabetes, current smoking, obesity (BMI>30), hypertension, hypercholesterolemia) and apparent temperature (stratified into ≤25th percentile vs >25th percentile) as possible effect modifiers was assessed with the same linear and ordinal regression models used previously, adding the interaction between pollutant and hypersusceptibility or apparent temperature, and extracting stratified estimates from these models. Graphs of the most relevant results were produced to show statistically significant interactions. Apparent temperature was used as adjusting covariate and as effect modifier covariate, as both the hypotheses have been tested in the literature.

Apparent temperature was also considered as a risk factor for developing more severe MDD: for this reason, the association between apparent temperature and severity of MDD was evaluated. Multivariate linear regression models were used for all the severity scales except for CGI where a multivariate ordinal regression model was used. Models were adjusted for gender, age, occupation, education, month and year of recruitment, origin of recruitment, precipitation, and solar radiation. The role of extreme cold days and extreme heat days were also investigated. The associations between extreme hot days and extreme cold days and severity of MDD were evaluated with the same models and adjusting variables used for the evaluation of the association between apparent temperature

exposure and MDD severity. Sensitivity analysis adjusting models for $PM_{2.5}$ and NO_2 exposure, with previously used covariates were conducted.

Among the different biological variables of interest, in the current study we focused on methylation of *CLOCK* and *CLOCK*-related genes. To study the association between air pollutants exposure and these biological markers, different models for each pollutant and for each gene were constructed. As *CLOCK*-related genes did not follow a normal distribution and also residuals from linear regression models did not follow a normal distribution, *CLOCK* and *CLOCK*-related genes were log-transformed. Multivariate linear regression models with log-transformation of the outcomes were used to assess the association between air pollution exposure and various methylation values of different genes. Models were adjusted for age, gender, occupation, month and year of recruitment, education, BMI, smoking, percentage of lymphocytes, antidepressant treatment (yes vs. no), and plate. Graphs representing results from different lag of exposure per each gene were produced.

In order to evaluate the third hypothesis about the association between the methylation of *CLOCK* and *CLOCK*-related genes and severity of MDD, different models for each severity scale of MDD and for each gene were constructed. Multivariate linear regression models were used for all the severity scales except for CGI where a multivariate ordinal regression model was used. Models were adjusted for age, gender, occupation, month and year of recruitment, education, BMI, smoking, percentage of lymphocytes, antidepressant treatment (yes vs. no), source of recruitment and plate. The role of subtype of depression as possible effect modifier was evaluated through the use of same linear and ordinal regression models as before, but the interaction between *CLOCK* and *CLOCK*-related genes and subtype of depression was added, and stratified estimates from these models were subsequently extracted.

All analyses were conducted with STATA statistical software, version 17.

3. RESULTS

3.1 Descriptive analyses

The main socio-demographic characteristics of the study subjects are summarized in **Table 1**. Two thirds of enrolled patients were females, which were older than males (52 vs. 48 years old, p-value=0.042) and more likely underweight. The largest majority of included patients was of normal weight, never smoker, employed and recruited during outpatient visits and had a high school degree, with no substantial differences between males and females.

In **Table 2**, the main characteristics of MDD are shown. On average, the disease onset was at 39 years old, with a duration of untreated MDD of 20 months. Mean number of MDD episodes was 3 and mean duration of last MDD episode was 9 months, with no differences across genders. Family history of psychiatric disorders accounted for 45% overall, with a higher prevalence among females (51% vs. 34% in males). A greater proportion among females was observed also with regard to family MDD history, while males were more likely hospitalized for MDD (35% vs. 24% in females). Overall, the prevalence of psychotic symptoms, suicide attempts and seasonality of MDD were 9%, 18%, and 27%, respectively, with no differences between males and females. The most frequent MDD subtypes were the melancholic one and the one characterized by strong symptoms of anxiety, with melancholic depression being more frequent in males and depression with strong symptoms of anxiety being more frequent in females. The largest majority of included patients was under antidepressant treatment, with Selective Serotonin Reuptake Inhibitors or Serotonin and Norepinephrine Reuptake Inhibitors.

Summary statistics of MDD severity rating scales are shown in **Table 3**. Based on the MADRS scale, about 70% of the subjects had moderate-to-severe depression (moderate: 44%, severe: 32%). Severe depression was also confirmed with the HAMD, classifying more than 45% of subjects in the highest category. On the other hand, the GAF scale returned an average score of about 59, identifying a category of patients with moderate symptoms or moderate difficulties in social functioning. When looking at CGI scores (based on subjective judgment of the clinician interviewing the patients), the enrolled population was spread across most of the scale categories, with the greatest proportion considered “moderately ill”. Finally, the subdomains of the SDS investigating the social, domestic and family impairment due to MDD returned average scores above 6, on a 10-point scale. Similar was the result on the perceived stress deriving from the disease. With regard to the final domain, it is worth noting that the question asked is “how much support (in percentage) have you needed from family/friends/colleagues in order to function properly?”: although, theoretically, the higher the score, the worst the disease, social support does also represent a powerful mean of contrasting MDD itself. As such, interpretation of the score regarding this subdomain might be seriously hampered. All severity scales showed similar scores in the two sexes.

Table 1: Demographic and lifestyle characteristics of the 416 included patients

Socio-demographic characteristics	**Mean (SD) / N (%)**			**p-value**
	Overall	**Males**	**Females**	
Age	50.8 (17.8)	48.4 (17.5)	52.1 (17.8)	**0.042**
Gender				
Females	266 (63.9%)	--	--	
Males	150 (36.1%)	--	--	--
BMI				
Underweight	28 (6.7%)	3 (2.0%)	25 (9.4%)	
Normal weight	228 (54.8%)	85 (56.7%)	143 (53.8%)	
Overweight	101 (24.3%)	39 (26.0%)	62 (23.3%)	
Obese	59 (14.2%)	23 (15.3%)	36 (13.5%)	**0.038**
Education level				
Primary school or less	23 (5.5%)	2 (1.3%)	21 (7.9%)	
Secondary school	78 (18.8%)	30 (20.0%)	48 (18.1%)	
High school	192 (46.2%)	72 (48.0%)	120 (45.1%)	
University	123 (29.6%)	46 (30.7%)	77 (29.0%)	**0.049**
Occupation				
Employed	170 (40.9%)	64 (42.7%)	106 (39.9%)	
Unemployed	102 (24.5%)	38 (25.3%)	64 (24.1%)	
Retired	98 (23.6%)	31 (20.7%)	67 (25.2%)	
Other	46 (11.1%)	17 (11.3%)	29 (10.9%)	0.778
Smoking status				
Never smoker	225 (54.1%)	70 (46.7%)	155 (58.3%)	
Former smoker	49 (11.8%)	21 (14.0%)	28 (10.5%)	
Current smoker	142 (34.1%)	59 (39.3%)	83 (31.2%)	0.073
Second-hand smoking exposure				
Yes	134 (32.2%)	47 (31.3%)	87 (32.7%)	
No	282 (67.8%)	103 (68.7%)	179 (67.3%)	0.773
Residence traffic exposure				
Mild	101 (24.3%)	40 (26.7%)	61 (22.9%)	
Moderate	152 (36.5%)	50 (33.3%)	102 (38.4%)	
Heavy	163 (39.2%)	60 (40.0%)	103 (38.7%)	0.535
Source of recruitment				
Outpatients	158 (38.0%)	55 (36.7%)	103 (38.7%)	
Day-hospital	77 (18.5%)	25 (16.7%)	52 (19.6%)	
Hospitalizations	82 (19.7%)	40 (26.7%)	42 (15.8%)	
Already known outpatients reconducted for the study	99 (23.8%)	30 (20.6%)	69 (25.9%)	0.052

Table 2: Major Depressive Disorder (MDD) characteristics of the 416 included patients.

Major Depressive Disorder (MDD) characteristics	**Mean (SD) / N (%)**			**p-value**
	Overall	**Males**	**Females**	
Age at onset of MDD	39.3 (17.7)	38.6 (17.3)	39.8 (18.0)	0.527
Number of MDD episodes	2.9 (2.8)	2.8 (2.5)	3.0 (3.0)	0.515
Family history of psychiatric disorders				
Yes	187 (45.0%)	51 (34.0%)	136 (51.1%)	
No	229 (55.1%)	99 (66.0%)	130 (48.9%)	**0.001**
Family history of MDD				
Yes	134 (32.2%)	39 (26.0%)	95 (35.7%)	
No	282 (67.8%)	111 (74.0%)	171 (64.3%)	**0.042**
Total duration of untreated MDD in months	20.3 (49.7)	20.4 (46.8)	20.2 (51.3)	0.973
Total duration of MDD in years	10.8 (12.7)	9.5 (10.7)	11.5 (13.7)	0.130
Duration of last MDD episode in months	9.1 (12.9)	9.0 (15.2)	9.2 (11.5)	0.907
Hospitalizations for MDD				
Yes	115 (27.6%)	52 (34.7%)	63 (23.7%)	
No	301 (72.4%)	98 (65.3%)	203 (76.3%)	**0.016**
Among those hospitalized for MDD (115), N of hospitalizations	1.8 (1.5)	1.7 (1.2)	1.9 (1.8)	0.541
Psychotic symptoms				
Yes	36 (8.7%)	14 (9.3%)	22 (8.3%)	
No	380 (91.4%)	136 (90.7%)	244 (91.7%)	0.711
Suicide attempts				
Yes	75 (18.0%)	31 (20.7%)	44 (16.5%)	
No	341 (82.0%)	119 (79.3%)	222 (83.5%)	0.293
Seasonality of MDD				
Yes	113 (27.2%)	36 (24.0%)	77 (29.0%)	
No	303 (72.8%)	114 (76.0%)	189 (71.1%)	0.276
MDD subtype				
Melancholic	159 (38.2%)	67 (44.7%)	92 (34.6%)	
Atypical	60 (14.4%)	20 (13.3%)	40 (15.0%)	
Psychotic	19 (4.6%)	10 (6.7%)	9 (3.4%)	
Strong symptoms of anxiety	146 (35.1%)	35 (23.3%)	111 (41.7%)	
No prevalent type	32 (7.7%)	18 (12.0%)	14 (5.3%)	**0.001**
Lifetime substances abuse				
Single abuse	61 (14.7%)	28 (18.7%)	33 (12.4%)	
Multiple abuse	25 (6.0%)	16 (10.7%)	9 (3.4%)	
No	330 (79.3%)	106 (70.7%)	224 (84.2%)	**0.001**
Type(s) of abuse (among ever abusers (86))				
Alcohol	45 (52.3%)	25 (56.8%)	20 (47.6%)	0.393
Cannabis	37 (43.0%)	21 (47.7%)	16 (38.1%)	0.367

Heroine	8 (9.3%)	6 (13.6%)	2 (4.8%)	0.157
Cocaine	14 (16.3%)	9 (20.5%)	5 (11.9%)	0.283
LSD	5 (5.8%)	3 (6.8%)	2 (4.8%)	0.684
Amphetamine	4 (4.7%)	3 (6.8%)	1 (2.4%)	0.329
Methylenedioxy methamphetamine (MDMA)	1 (1.2%)	0 (0.0%)	1 (2.4%)	0.303
Drugs	18 (20.9%)	5 (11.4%)	13 (31.0%)	**0.026**
Any antidepressant treatment				
Yes	365 (87.7%)	133 (88.7%)	232 (87.2%)	
No	51 (12.3%)	17 (11.3%)	34 (12.8%)	0.665
Treatment type (among 280 subjects taking treatment)				
Selective Serotonin Reuptake Inhibitors (SSRI)	228 (62.5%)	86 (64.7%)	142 (61.2%)	
Serotonin and Norepinephrine Reuptake Inhibitors (SNRI)	59 (16.2%)	19 (14.3%)	40 (17.2%)	
Tricyclics	32 (8.8%)	9 (6.8%)	23 (9.9%)	
Bupropion	2 (0.6%)	1 (0.8%)	1 (0.4%)	
Mirtazapine	14 (3.8%)	7 (5.3%)	7 (3.0%)	
Vortioxetine	11 (3.0%)	5 (3.8%)	6 (2.6%)	
Trazodone	12 (3.3%)	1 (0.8%)	11 (4.7%)	
Others	7 (1.9%)	5 (3.8%)	2 (0.9%)	0.138

Table 3: Scales of Major Depressive Disorder (MDD) severity in the 416 included patients.

Major Depressive Disorder (MDD) severity	**Mean (SD) / N (%)**			**p-value**
	Overall	**Males**	**Females**	
Montgomery-Asberg Depression Rating Scale (MADRS; 0-60)	28.3 (12.6)	28.7 (12.7)	28.1 (12.5)	0.632
0-6 (no depression)	17 (4.1%)	6 (4.0%)	11 (4.1%)	
7-19 (mild depression)	84 (20.2%)	29 (19.3%)	55 (20.7%)	
20-34 (moderate depression)	182 (43.8%)	62 (41.3%)	120 (45.1%)	
≥35 (severe depression)	133 (32.0%)	53 (35.3%)	80 (30.1%)	0.745
Hamilton Depression Rating Scale (HAMD; 0-67)	23.9 (12.3)	23.8 (12.3)	23.9 (12.4)	0.929
0-7 (no depression)	27 (6.5%)	11 (7.3%)	16 (6.0%)	
8-16 (mild depression)	93 (22.4%)	32 (21.3%)	61 (22.9%)	
17-23 (moderate depression)	108 (26.0%)	43 (28.7%)	65 (24.4%)	
≥24 (severe depression)	188 (45.2%)	64 (42.7%)	124 (46.6%)	0.715
Global Assessment of Functioning (GAF; 100-0)	58.6 (15.1)	57.2 (16.0)	59.3 (14.6)	0.164
Clinical Global Impression (CGI; 0-7)				
Normal, not at all ill	18 (4.3%)	5 (3.3%)	13 (4.9%)	
Borderline mentally ill	38 (9.1%)	9 (6.0%)	29 (10.9%)	
Mildly ill	92 (22.1%)	32 (21.3%)	60 (22.6%)	
Moderately ill	129 (31.0%)	48 (32.0%)	81 (30.5%)	
Markedly ill	75 (18.0%)	27 (18.0%)	48 (18.1%)	
Severely ill	52 (12.5%)	22 (14.7%)	30 (11.3%)	
Among the most extremely ill patients	12 (2.9%)	7 (4.7%)	5 (1.9%)	0.353
Sheehan Disability Scale (SDS)				
Impairment at work (0-10)	7.1 (2.8)	7.2 (2.7)	7.0 (2.7)	0.513
Impairment in home relationships (0-10)	6.8 (2.6)	7.0 (2.7)	6.7 (2.5)	0.195
Impairment in family responsibilities (0-10)	6.6 (2.8)	6.7 (2.8)	6.5 (2.8)	0.570
Perceived stress (0-10)	6.3 (2.8)	6.5 (3.0)	6.2 (2.7)	0.453
Perceived social support (100-0)	57.5 (27.2)	53.6 (28.6)	59.7 (26.1)	0.028

3.2 Association between air pollution and temperature exposure and severity of MDD

The association between exposure to air pollutants and severity of MDD assessed through each single rating scale was estimated using different mid- and short-term temporal windows (lag0-7, lag0-14, lag0-21 and lag0-30): the choice of limiting our analysis to the 30 days preceding recruitment was taken in accordance with the psychiatrists, since the questions underlying the severity scales investigate experiences lived relatively close in time to recruitment. Since the various investigated lags of exposure returned similar findings, for brevity we will illustrate the results with the greatest magnitude, obtained when analyzing lag0-14.

Exposure to particulate matter was not associated with severity of MDD in any of the scales (**Table 4**), while an increase of 10 $\mu g/m^3$ in NO_2 exposure was positively associated with HAMD (β=2.09, 95% CI (0.63; 3.56)), and CGI (β=0.27, 95% CI (0.02; 0.51)) scores. In GAF, lower scores indicate a more severe depression: as such, the negative association we observed with NO_2 (β=-1.96, 95% CI (-3.60; -0.33)) confirms a worsening of MDD with this scale as well.

When the effect modification of hypersusceptibility was evaluated, we observed a worsening of depression severity associated with PM_{10} exposure among hypersusceptible subjects (**Table 5**), with significant interactions detected for MADRS (p=0.019; **Figure 2**) and the sub-domains "impairment of home relationships" (p=0.041; **Figure 3**) and "perceived stress" (p=0.009) of the SDS scale. Similar results were found when considering $PM_{2.5}$ (**Table 6**), with stronger associations in hypersusceptible subjects and significant interactions for MADRS (p=0.011; **Figure 4**), CGI (p=0.035) and the sub-domains "impairment of home relationships" (p=0.029; **Figure 5**), "impairment of family responsibilities" (p=0.027; **Figure 6**) and "perceived stress" (p=0.008) of the SDS scale. On the other hand, hypersuspeptibilty did not modify the association between NO_2 exposure and severity of MDD (**Table 7**), even if associations were again stronger among hypersusceptible subjects.

We further investigated the role of apparent temperature (AT) as an effect modifier. Results are reported in **Tables 8-10**. In particular, when AT levels were below the first quartile of exposure, strong positive associations were found between PM_{10} exposure and severity scores of MDD measured with MADRS (β=2.37, 95% CI (0.18; 4.56)**),** HAMD (β=3.24, 95% CI (0.97; 5.51)**),** and GAF (-2.74, 95% CI (-5.29; -0.20)**)**. On the other hand, when AT levels were higher than the first quartile of exposure, associations followed the opposite direction and confirmed presence of interaction across most scales (**Figures 7-9**).

A similar pattern of results was observed for $PM_{2.5}$ exposure (**Table 9**, **Figures 10-12**).

When NO_2 exposure was considered, stronger estimates were observed for lower temperatures even if a formal statistical interaction was not found except for HAMD. We also investigated AT as possible environmental determinant of MDD severity (**Table 11**), finding that increasing AT levels were associated with an improvement in all the scales. Extreme heat days were not associated with more severe scores of MDD, while extreme cold days were positively associated with MADRS (β=0.79, 95%CI (0.11; 1.47)), and HAMD (β=0.80, 95%CI (0.11; 1.50)) scores, thus confirming a relevant role of low temperatures in affecting MDD severity. When sensitivity analyses were conducted, further adjusting for $PM_{2.5}$ or NO_2 exposure did not change our results.

Table 4: Estimates with corresponding confidence intervals and p-values of the association between air pollutant exposure (10 µg/m^3 increase) and Major Depressive Disorder severity rating scales

MDD Rating scale	**β (95%CI) p-value**		
	PM_{10}	**$PM_{2.5}$**	**NO_2**
MADRS	0.10 (-1.18; 1.37) p=0.883	-0.35 (-2.19; 1.51) p=0.715	0.89 (-0.53; 2.31) p=0.220
HAMD	0.51 (-0.81; 1.84) p=0.447	0.34 (-1.59; 2.26) p=0.731	**2.09 (0.63; 3.56) p=0.005**
GAF	-0.88 (-2.36; 0.59) p=0.241	-1.01 (-3.16; 1.13) p=0.355	**-1.96 (-3.60; -0.33) p=0.019**
CGI	-0.02 (-0.24; 0.21) p=0.891	-0.05 (-0.37; 0.28) p=0.778	**0.27 (0.02; 0.51) p=0.034**
SDS			
Impairment at work	0.15 (-0.21; 0.50) p=0.414	0.21 (-0.31; 0.73) p=0.435	0.27 (-0.11; 0.66) p=0.162
Impairment in home relationships	0.04 (-0.25; 0.34) p=0.773	0.05 (-0.38; 0.48) p=0.818	0.25 (-0.08; 0.57) p=0.138
Impairment in family responsibilities	-0.04 (-0.35; 0.27) p=0.802	-0.03 (-0.47; 0.42) p=0.907	0.36 (0.02; 0.70) p=0.038
Perceived stress	0.08 (-0.25; 0.42) p=0.631	0.02 (-0.47; 0.50) p=0.950	0.24 (-0.14; 0.61) p=0.216
Perceived social support	-2.74 (-5.92; 0.44) p=0.091	-2.96 (-7.58; 1.66) p=0.209	-3.99 (-7.53; -0.46) p=0.027

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; PM_{10}: particulate matter with diameter less than or equal to 10; $PM_{2.5}$: particulate matter with diameter less than or equal to 2.5; NO_2: nitrogen dioxide; β: beta estimate; 95% CI: confidence interval at 95% level

Table 5: Stratified estimates by hypersusceptibility (defined as presence of at least one of the following: obesity, hypercholesterolemia, hypertension, type II diabetes, current smoking), with corresponding confidence intervals and p-values of the association between PM_{10} exposure (10 $\mu g/m^3$ increase) and Major Depressive Disorder severity rating scales

MDD Rating scale	**β (95%CI) p-value**		**Interaction p-value**
	Hypersusceptible subjects	**Not Hypersusceptible subjects**	
MADRS	0.79 (-0.60; 2.19) p=0.265	-1.20 (-2.86; 0.47) p=0.159	**0.019**
HAMD	1.01 (-0.44; 2.48) p=0.170	-0.39 (-2.13; 1.35) p=0.661	0.111
GAF	-1.30 (-2.93; 0.32) p=0.115	-0.14 (-2.08; 1.79) p=0.884	0.237
CGI	0.07 (-0.17; 0.31) p=0.562	-0.20 (-0.49; 0.10) p=0.188	0.072
SDS			
Impairment at work	0.25 (-0.14; 0.64) p=0.201	-0.01 (-0.46; 0.43) p=0.948	0.229
Impairment in home relationships	0.19 (-0.13; 0.51) p=0.255	-0.21 (-0.59; 0.17) p=0.277	**0.041**
Impairment in family responsibilities	0.10 (-0.24; 0.43) p=0.575	-0.27 (-0.67; 0.13) p=0.182	0.070
Perceived stress	0.28 (-0.08; 0.65) p=0.128	-0.30 (-0.73; 0.14) p=0.185	**0.009**
Perceived social support	-3.32 (-6.83; 0.18) p=0.063	-1.61 (-5.78; 2.57) p=0.450	0.416

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; PM_{10}: particulate matter with diameter less than or equal to 10; β: beta estimate; 95% CI: confidence interval at 95% level

Figure 2: Association between PM_{10} exposure and MADRS scores. Red line represents the association in not hypersusceptible subjects, while blue line represents the association in hypersusceptible subjects (defined as presence of at least one of the following: obesity, hypercholesterolemia, hypertension, type II diabetes, current smoking).

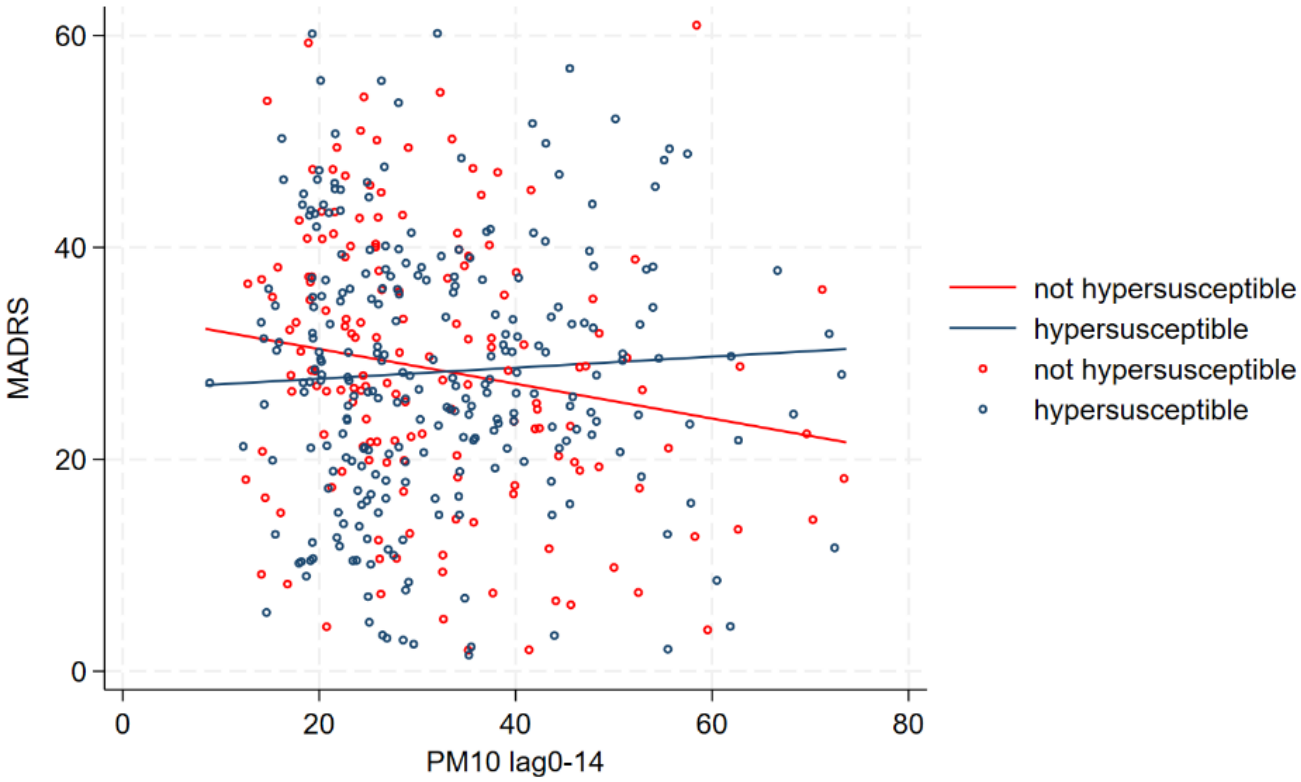


Figure 3: Association between PM_{10} exposure and Relationship domain scores from SDS scale. Red line represents the association in not hypersusceptible subjects, while blue line represents the association in hypersusceptible subjects (defined as presence of at least one of the following: obesity, hypercholesterolemia, hypertension, type II diabetes, current smoking).

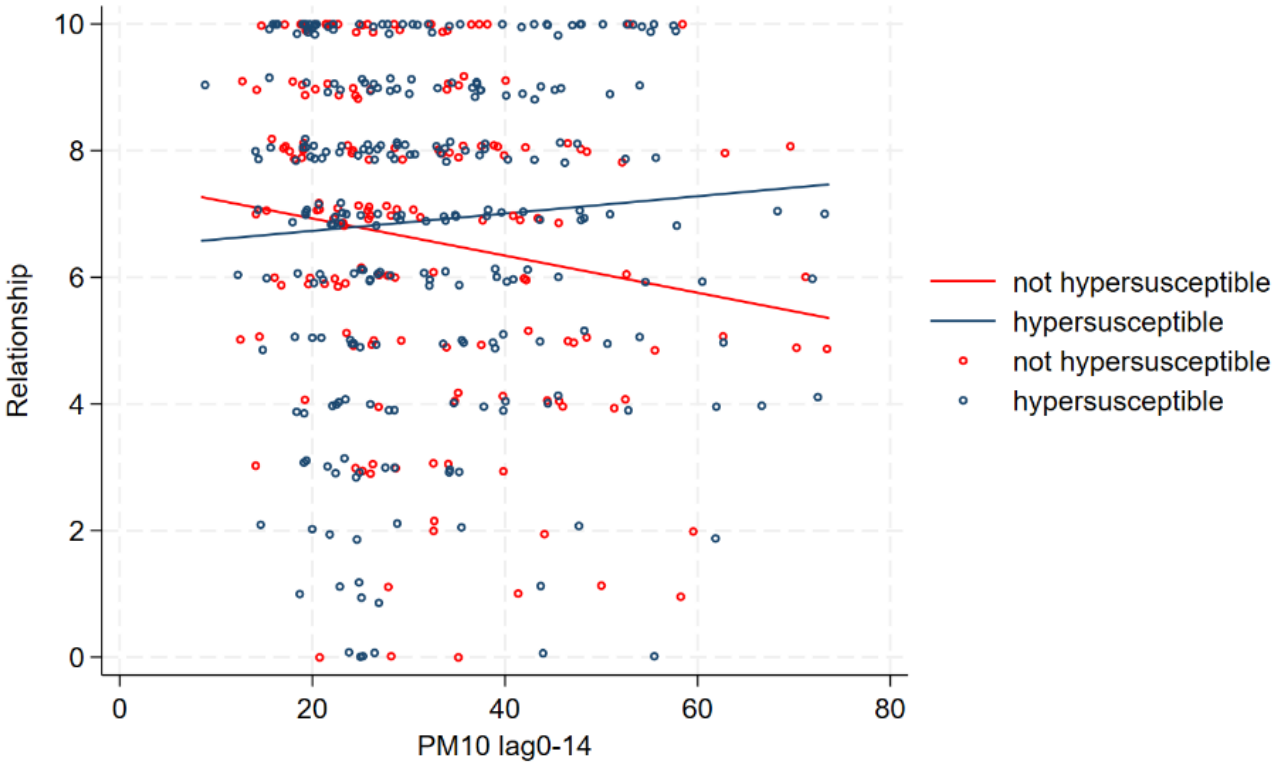


Table 6: Stratified estimates by hypersusceptibility (defined as presence of at least one of the following: obesity, hypercholesterolemia, hypertension, type II diabetes, current smoking), with corresponding confidence intervals and p-values of the association between $PM_{2.5}$ exposure (10 $\mu g/m^3$ increase) and Major Depressive Disorder severity rating scales

MDD Rating scale	**β (95%CI) p-value**		**Interaction p-value**
	Hypersusceptible subjects	**Not Hypersusceptible subjects**	
MADRS	0.65 (-1.34; 2.65) p=0.520	-2.04 (-4.30; 0.22) p=0.077	**0.011**
HAMD	1.08 (-1.00; 3.17) p=0.309	-0.91 (-3.27; 1.45) p=0.448	0.072
GAF	-1.69 (-4.01; 0.63) p=0.153	0.13 (-2.50; 2.76) p=0.924	0.141
CGI	0.08 (-0.27; 0.43) p=0.650	-0.33 (-0.74; 0.09) p=0.121	**0.035**
SDS			
Impairment at work	0.36 (-0.20; 0.93) p=0.207	-0.02 (-0.63; 0.60) p=0.960	0.183
Impairment in home relationships	0.25 (-0.21; 0.71) p=0.286	-0.28 (-0.80; 0.23) p=0.282	**0.029**
Impairment in family responsibilities	0.19 (-0.29; 0.66) p=0.446	-0.38 (-0.92; 0.16) p=0.170	**0.027**
Perceived stress	0.29 (-0.23; 0.81) p=0.277	-0.45 (-1.05; 0.14) p=0.134	**0.008**
Perceived social support	-3.99 (-9.00; 1.02) p=0.118	-1.18 (-6.85; 4.50) p=0.684	0.290

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Figure 4: Association between $PM_{2.5}$ exposure and MADRS scores. Red line represents the association in not hypersusceptible subjects, while blue line represents the association in hypersusceptible subjects (defined as presence of at least one of the following: obesity, hypercholesterolemia, hypertension, type II diabetes, current smoking).

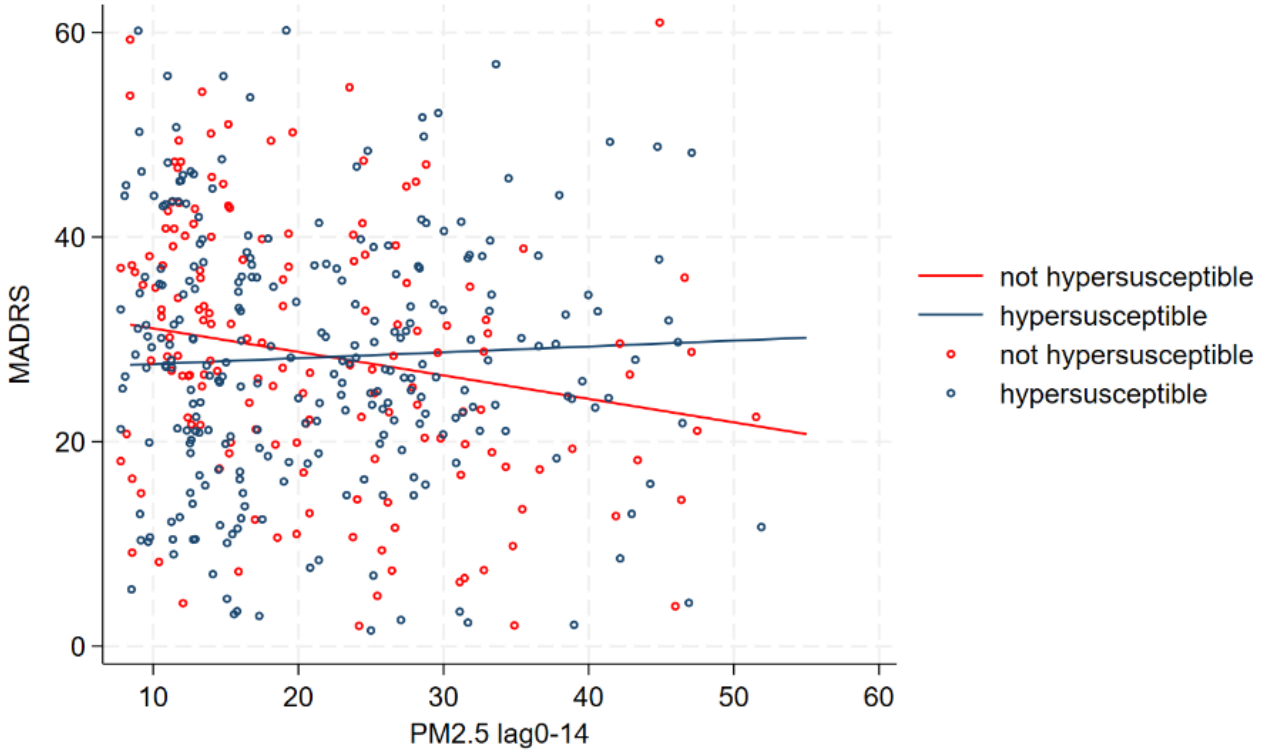


Figure 5: Association between $PM_{2.5}$ exposure and Relationship domain scores from SDS scale. Red line represents the association in not hypersusceptible subjects, while blue line represents the association in hypersusceptible subjects (defined as presence of at least one of the following: obesity, hypercholesterolemia, hypertension, type II diabetes, current smoking).

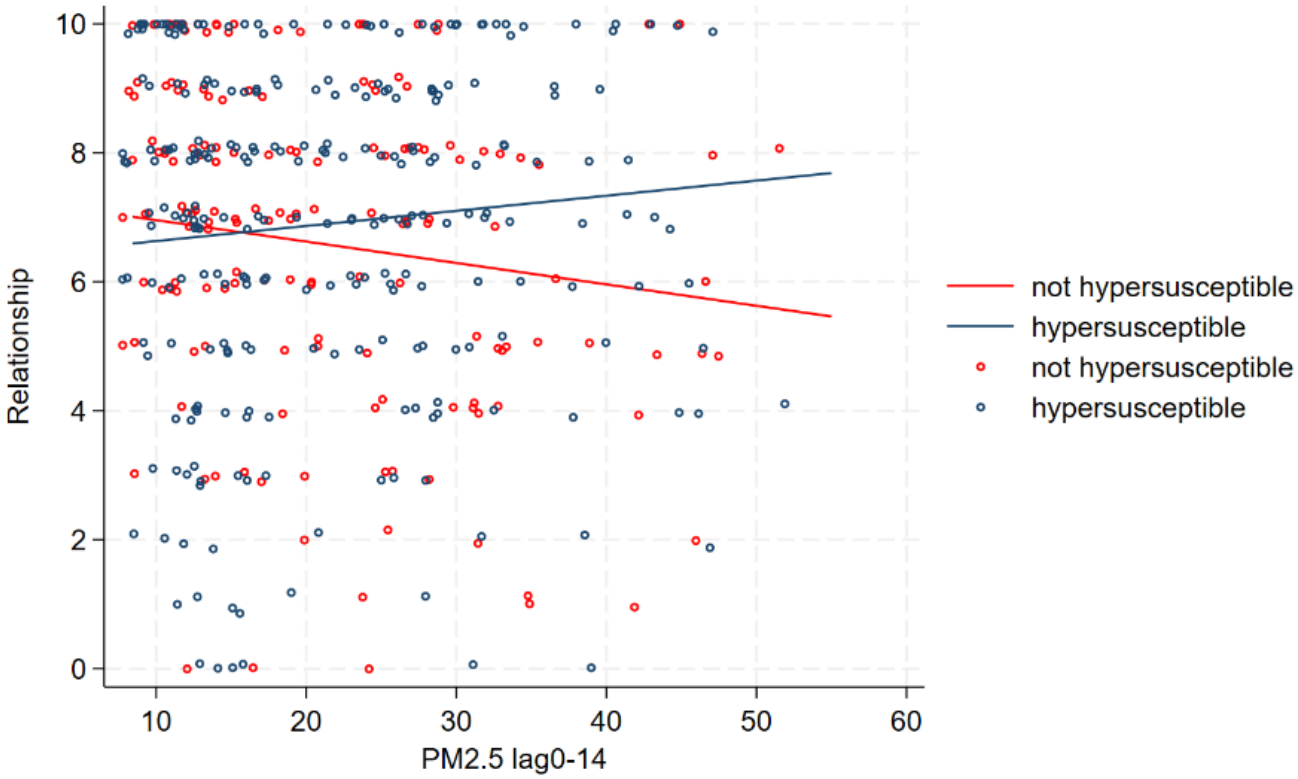


Figure 6: Association between $PM_{2.5}$ exposure and Family domain scores from SDS scale. Red line represents the association in not hypersusceptible subjects, while blue line represents the association in not hypersusceptible subjects (defined as presence of at least one of the following: obesity, hypercholesterolemia, hypertension, type II diabetes, current smoking).

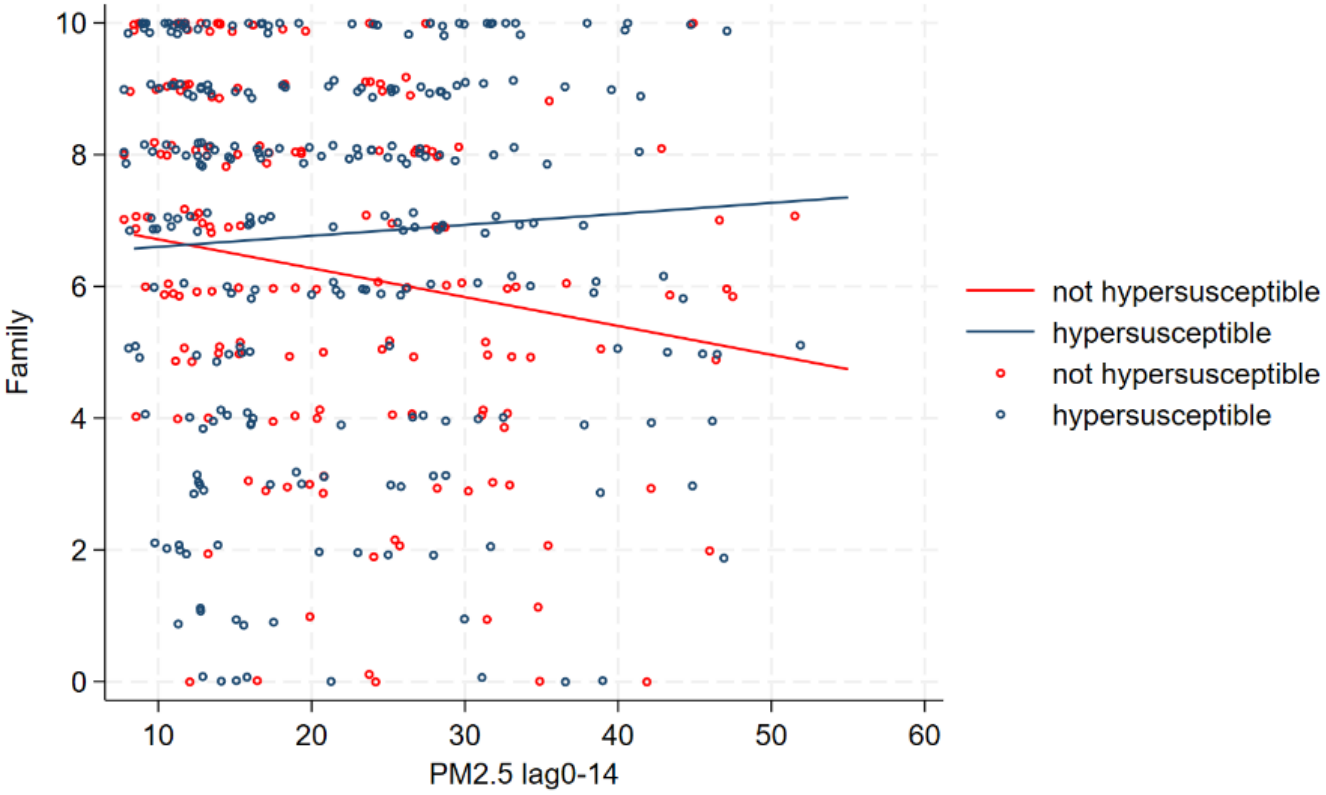


Table 7: Stratified estimates by hypersusceptibility (defined as presence of at least one of the following: obesity, hypercholesterolemia, hypertension, type II diabetes, current smoking), with corresponding confidence intervals and p-values of the association between NO_2 exposure (10 $\mu g/m^3$ increase) and MDD severity rating scales

MDD Rating scale	**β (95%CI) p-value**		**Interaction p-value**
	Hypersusceptible subjects	**Not Hypersusceptible subjects**	
MADRS	1.32 (-0.23; 2.86) p=0.094	0.03 (-1.84; 1.89) p=0.978	0.163
HAMD	**2.30 (0.70; 3.89) p=0.005**	1.75 (-0.18; 3.68) p=0.075	0.567
GAF	**-2.32 (-4.10; -0.54) p=0.011**	-1.34 (-3.50; 0.81) p=0.221	0.360
CGI	**0.34 (0.07; 0.60) p=0.013**	0.13 (-0.20; 0.47) p=0.428	0.225
SDS			
Impairment at work	0.34 (-0.08; 0.76) p=0.115	0.19 (-0.29; 0.67) p=0.438	0.534
Impairment in home relationships	0.34 (-0.01; 0.69) p=0.060	0.08 (-0.35; 0.51) p=0.704	0.226
Impairment in family responsibilities	**0.45 (0.08; 0.81) p=0.017**	0.23 (-0.22; 0.67) p=0.316	0.319
Perceived stress	0.38 (-0.03; 0.78) p=0.067	-0.06 (-0.55; 0.43) p=0.808	0.071
Perceived social support	**-4.96 (-8.79; -1.12) p=0.011**	-1.94 (-6.58; 2.71) p=0.413	0.189

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 8: Stratified estimates by apparent temperature, with corresponding confidence intervals and p-values of the association between PM_{10} exposure (10 μg/m^3 increase) and Major Depressive Disorder severity rating scales

MDD Rating scale	**β (95%CI) p-value**		**Interaction p-value**
	Apparent Temperature ≤ Ist quartile (5.86 °C)	**Apparent Temperature > Ist quartile (5.86 °C)**	
MADRS	**2.37 (0.18; 4.56) p=0.034**	**-1.53 (-3.06; -0.00) p=0.050**	**0.004**
HAMD	**3.24 (0.97; 5.51) p=0.005**	-1.33 (-2.91; 0.25) p=0.099	**0.001**
GAF	**-2.74 (-5.29; -0.20) p=0.035**	0.56 (-1.22; 2.33) p=0.537	**0.036**
CGI	0.28 (-0.11; 0.67) p=0.156	-0.20 (-0.47; 0.06) p=0.128	**0.043**
SDS			
Impairment at work	0.42 (-0.20; 1.04) p=0.186	-0.15 (-0.56; 0.27) p=0.489	0.134
Impairment in home relationships	0.38 (-0.13; 0.88) p=0.144	-0.19 (-0.54; 0.16) p=0.292	0.071
Impairment in family responsibilities	0.26 (-0.27; 0.79) p=0.337	-0.29 (-0.66; 0.08) p=0.127	0.096
Perceived stress	0.02 (-0.56; 0.59) p=0.959	0.17 (-0.23; 0.57) p=0.410	0.667
Perceived social support	-1.93 (-7.37; 3.51) p=0.487	-3.37 (-7.16; 0.42) p=0.082	0.668

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; PM_{10}: particulate matter with diameter less than or equal to 10; β: beta estimate; 95% CI: confidence interval at 95% level

Figure 7: Association between PM_{10} exposure and MADRS scores. Red line represents the association when apparent temperature (AT) is below 5.86°C (I^{st} quartile of exposure), while blue line represents the association when apparent temperature (AT) is higher 5.86°C.

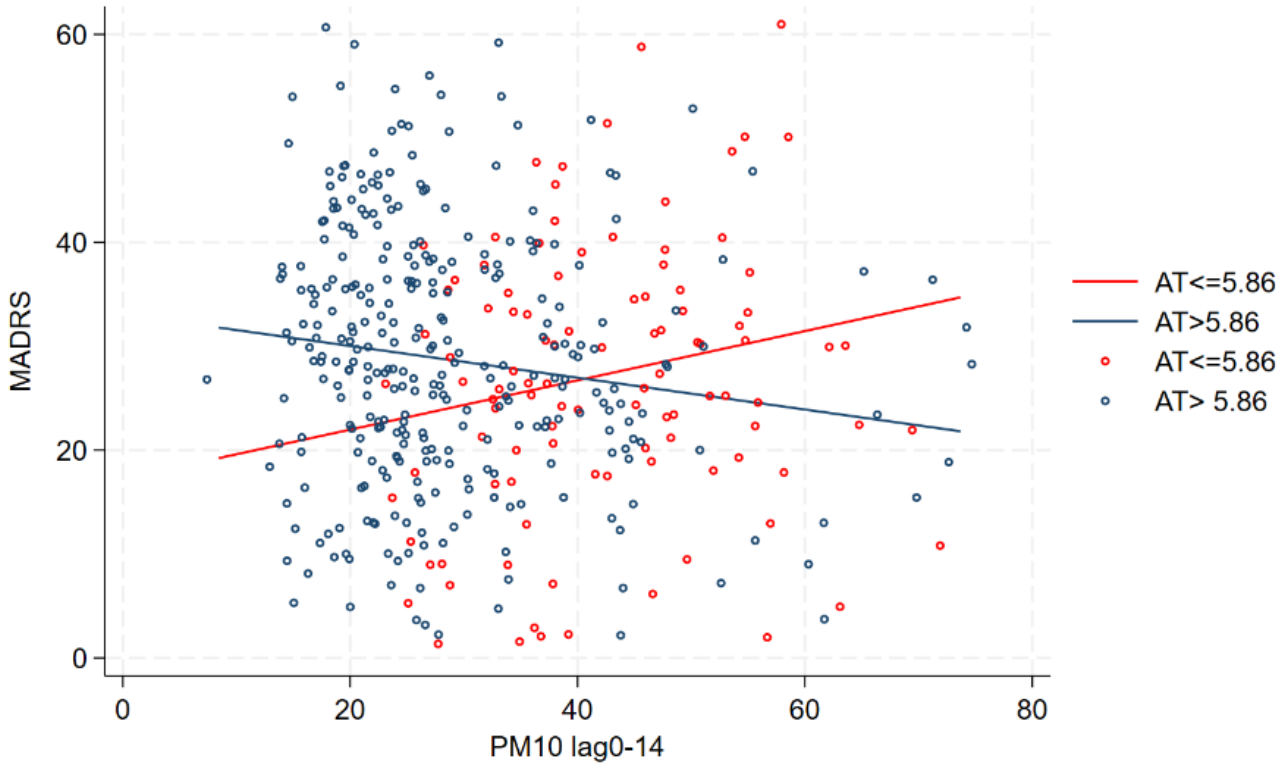


Figure 8: Association between PM_{10} exposure and HAMD scores. Red line represents the association when apparent temperature (AT) is below 5.86°C (I^{st} quartile of exposure), while blue line represents the association when apparent temperature (AT) is higher 5.86°C.

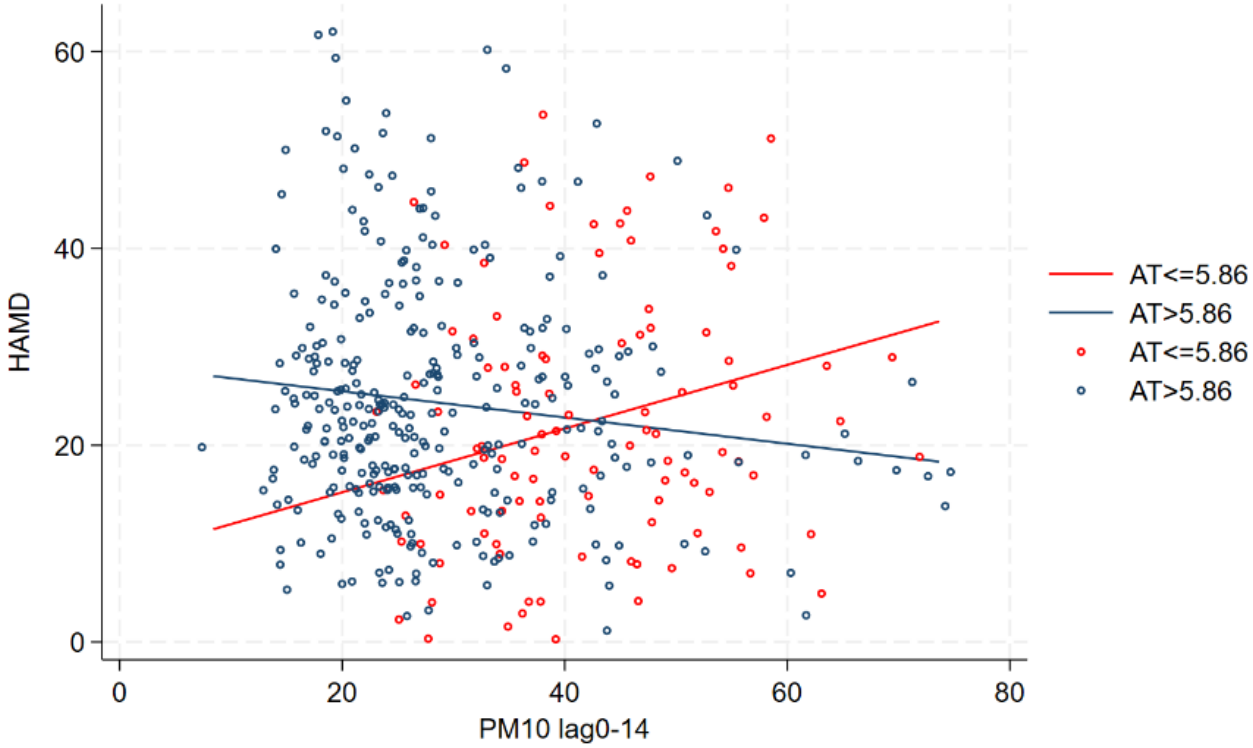


Figure 9: Association between PM_{10} exposure and GAF scores. Red line represents the association when apparent temperature (AT) is below 5.86°C (I^{st} quartile of exposure), while blue line represents the association when apparent temperature (AT) is higher 5.86°C.

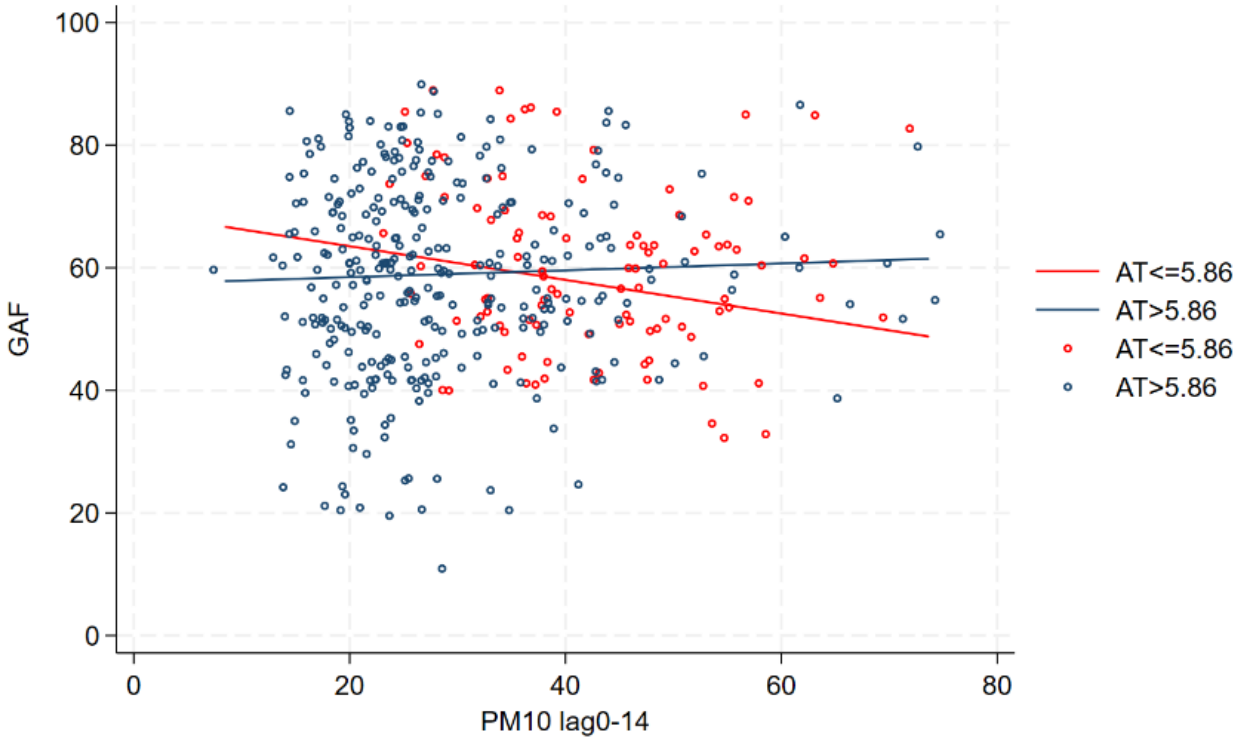


Table 9: Stratified estimates by apparent temperature, with corresponding confidence intervals and p-values of the association between $PM_{2.5}$ exposure (10 µg/m^3 increase) and Major Depressive Disorder severity rating scales

MDD Rating scale	**β (95%CI) p-value**		**Interaction p-value**
	Apparent Temperature ≤ Ist quartile (5.86 °C)	**Apparent Temperature > Ist quartile (5.86 °C)**	
MADRS	**3.00 (0.01; 5.99) p=0.049**	**-2.65 (-4.99; -0.30) p=0.027**	**0.004**
HAMD	**3.62 (0.51; 6.73) p=0.023**	-0.19 (-0.44; 0.05) p=0.119	**0.006**
GAF	-3.43 (-6.91; 0.05) p=0.054	0.70 (-2.03; 3.44) p=0.614	0.067
CGI	0.40 (-0.14; 0.93) p=0.146	-0.33 (-0.74; 0.08) p=0.118	**0.035**
SDS			
Impairment at work	0.53 (-0.31; 1.38) p=0.215	-0.11 (-0.77; 0.55) p=0.742	0.236
Impairment in home relationships	0.54 (-0.15; 1.23) p=0.122	-0.28 (-0.82; 0.26) p=0.310	0.065
Impairment in family responsibilities	0.40 (-0.33; 1.12) p=0.284	-0.33 (-0.90; 0.23) p=0.249	0.119
Perceived stress	0.05 (-0.74; 0.84) p=0.896	0.02 (-0.60; 0.64) p=0.945	0.952
Perceived social support	-2.98 (-10.41; 4.46) p=0.432	-3.25 (-9.09; 2.59) p=0.274	0.954

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale;β: beta estimate; 95% CI: confidence interval at 95% level

Figure 10: Association between $PM_{2.5}$ exposure and MADRS scores. Red line represents the association when apparent temperature (AT) is below 5.86°C (I^{st} quartile of exposure), while blue line represents the association when apparent temperature (AT) is higher 5.86°C.

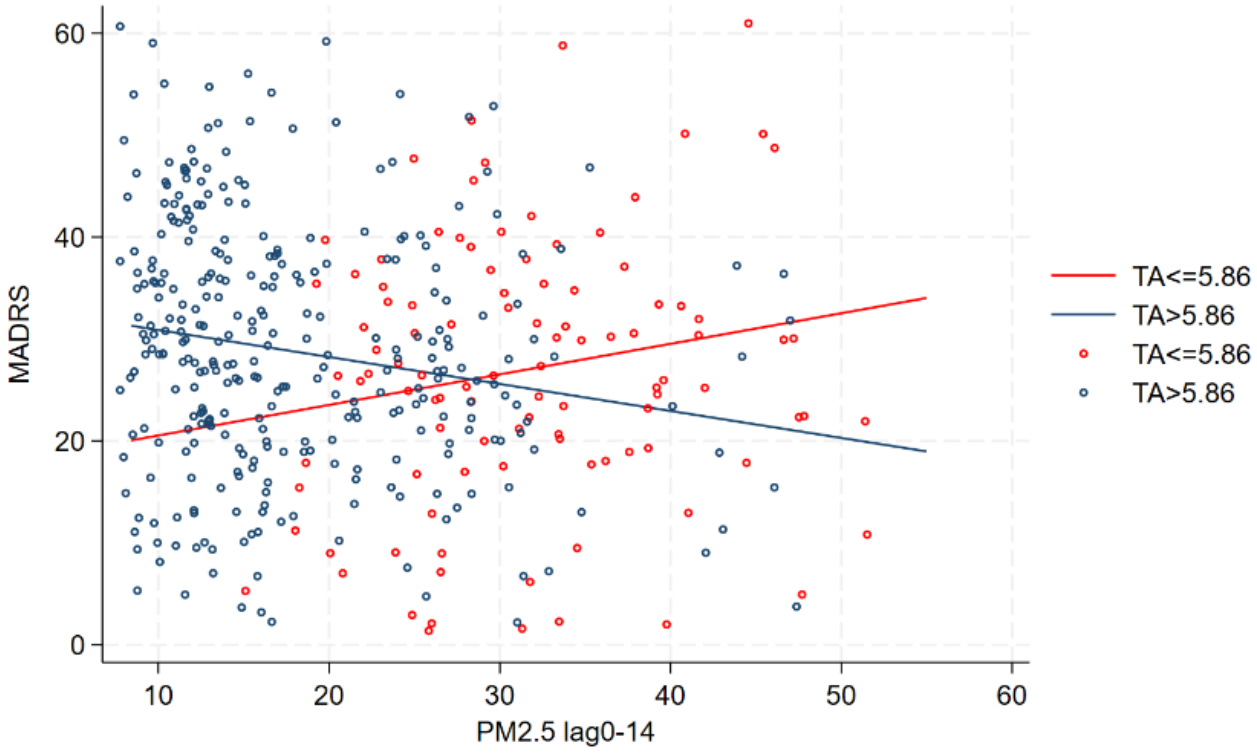


Figure 11: Association between $PM_{2.5}$ exposure and HAMD scores. Red line represents the association when apparent temperature (AT) is below 5.86°C (I^{st} quartile of exposure), while blue line represents the association when apparent temperature (AT) is higher 5.86°C.

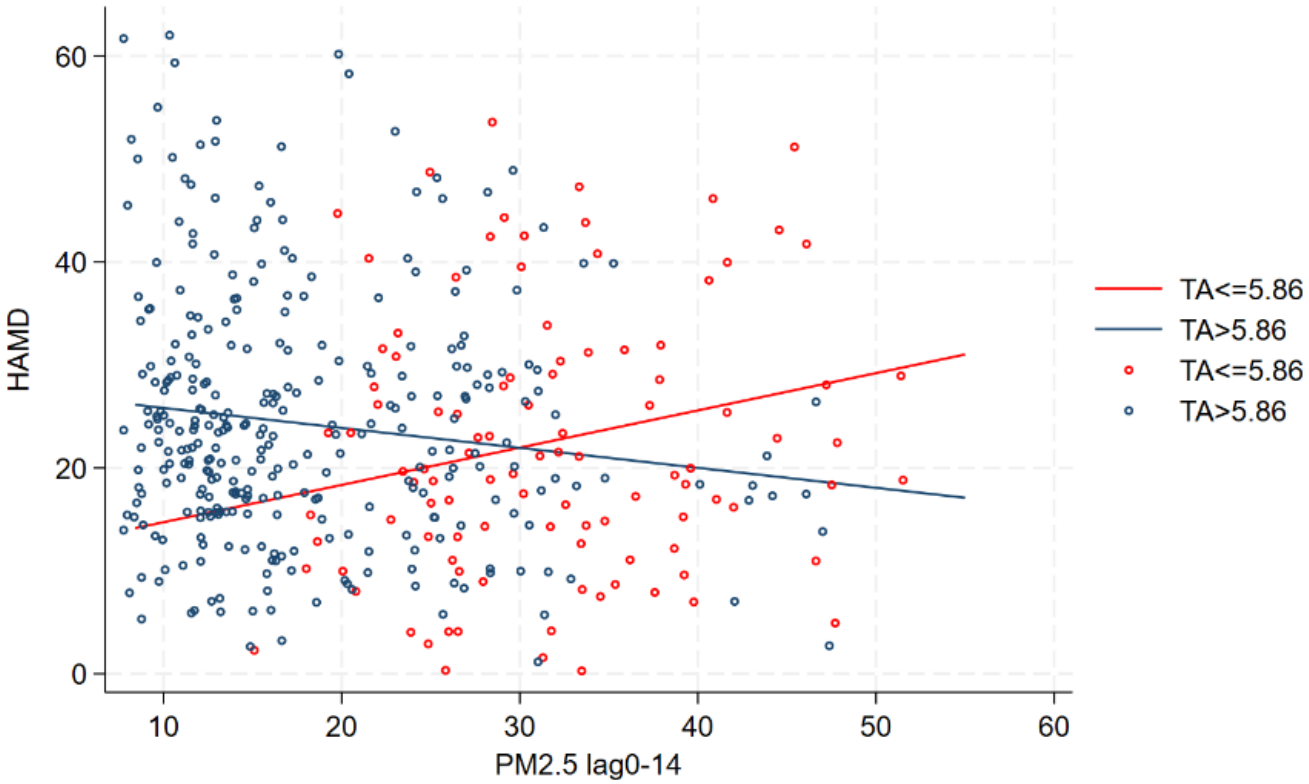


Figure 12: Association between $PM_{2.5}$ exposure and GAF scores. Red line represents the association when apparent temperature (AT) is below 5.86°C (I^{st} quartile of exposure), while blue line represents the association when apparent temperature (AT) is higher 5.86°C.

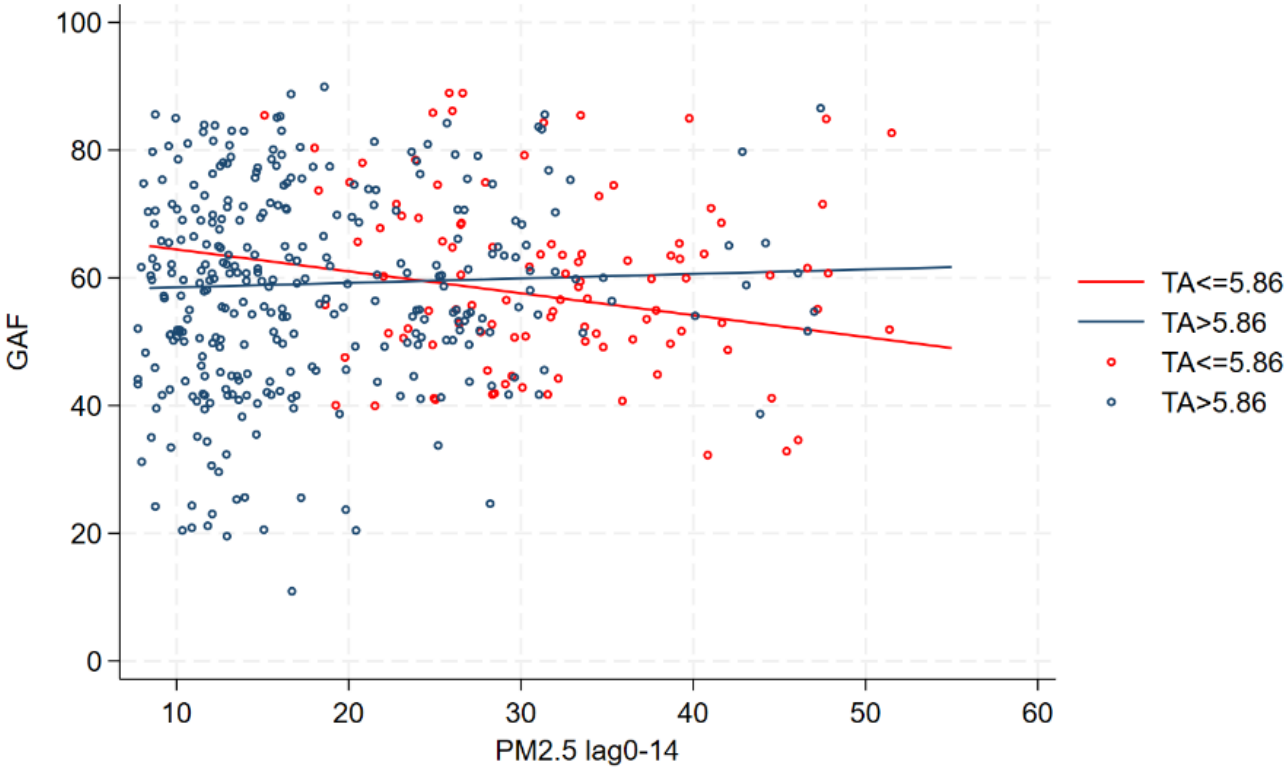


Table 10: Stratified estimates by apparent temperature, with corresponding confidence intervals and p-values of the association between NO_2 exposure (10 μg/m^3 increase) and MDD severity rating scales

MDD Rating scale	**β (95%CI) p-value**		**Interaction p-value**
	Apparent Temperature ≤ Ist quartile (5.86 °C)	**Apparent Temperature > Ist quartile (5.86 °C)**	
MADRS	**2.52 (0.24; 4.81) p=0.031**	-0.01 (-1.82; 1.80) p=0.991	0.086
HAMD	**4.07 (1.72; 6.42) p=0.001**	0.94 (-0.93; 2.81) p=0.322	**0.039**
GAF	**-3.39 (-6.02; -0.75) p=0.012**	-1.26 (-3.35; 0.84) p=0.238	0.212
CGI	**0.50 (0.10; 0.90) p=0.015**	0.16 (-0.15; 0.46) p=0.316	0.182
SDS			
Impairment at work	0.45 (-0.17; 1.08) p=0.150	0.14 (-0.35; 0.64) p=0.563	0.439
Impairment in home relationships	**0.53 (0.01; 1.05) p=0.047**	0.11 (-0.31; 0.52) p=0.610	0.212
Impairment in family responsibilities	0.48 (-0.07; 1.03) p=0.087	0.32 (-0.12; 0.76) p=0.149	0.654
Perceived stress	0.23 (-0.37; 0.83) p=0.449	0.23 (-0.25; 0.70) p=0.343	0.997
Perceived social support	-3.58 (-9.21; 2.06) p=0.213	**-4.65 (-9.12; -0.18) p=0.042**	0.768

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 11: Estimates with corresponding confidence intervals and p-values of the association between apparent temperature (1 °C increase), extreme heat, and extreme cold and Major Depressive Disorder severity rating scales

MDD Rating scale	**β (95%CI) p-value**		
	AT	**Heat**	**Cold**
MADRS	**-0.70 (-1.14; -0.26) p=0.002**	0.10 (-0.55; 0.76) p=0.752	**0.79 (0.11; 1.47) p=0.023**
HAMD	**-0.67 (-1.12; -0.21) p=0.004**	0.05 (-0.60; 0.70) p=0.875	**0.80 (0.11; 1.50) p=0.024**
GAF	**0.73 (0.21; 1.24) p=0.006**	-0.00 (-0.88; 0.87) p=0.992	-0.62 (-1.28; 0.05) p=0.068
CGI	**-0.08 (-0.16; -0.01) p=0.032**	0.10 (-0.04; 0.23) p=0.175	0.06 (-0.05; 0.17) p=0.308
SDS			
Impairment at work	**-0.16 (-0.29; -0.04) p=0.008**	0.05 (-0.12; 0.22) p=0.541	0.04 (-0.14; 0.22) p=0.683
Impairment in home relationships	**-0.11 (-0.21; -0.01) p=0.029**	0.07 (-0.09; 0.24) p=0.391	0.10 (-0.04; 0.25) p=0.155
Impairment in family responsibilities	**-0.13 (-0.23; -0.02) p=0.019**	0.05 (-0.12; 0.23) p=0.551	0.12 (-0.02; 0.27) p=0.092
Perceived stress	0.05 (-0.06; 0.17) p=0.355	-0.03 (-0.22; 0.16) p=0.751	-0.01 (-0.17; 0.16) p=0.937
Perceived social support	0.09 (-1.00; 1.20) p=0.866	-0.85 (-2.55; 0.85) p=0.320	0.10 (-1.52; 1.71) p=0.904

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; AT: apparent temperature; β: beta estimate; 95% CI: confidence interval at 95% level

3.3 Association between air pollution exposure and methylation of *CLOCK* and *CLOCK*-related genes

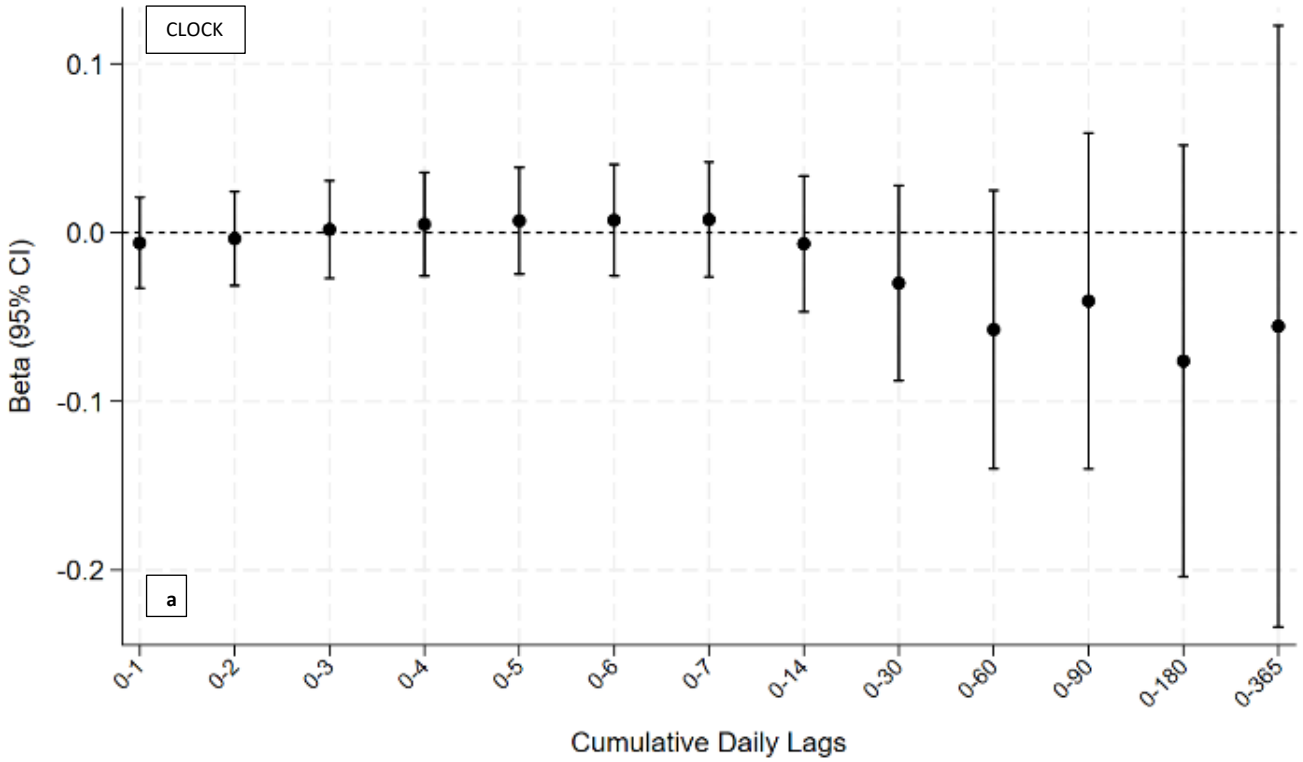
The association between air pollutants exposure and methylation of *CLOCK* and *CLOCK*-related genes was evaluated using different temporal lag of exposure representing short-, mid-, and long-term exposure. As regard to short-term exposure, cumulative lags starting from lag0-1 to lag0-7 were used, while for mid-term exposure cumulative lag0-14, lag0-30 and lag0-60 were analyzed and for long-term exposure cumulative lag0-90, lag0-180 and lag0-365. We examined also long-term exposure based on evidence form previous studies conducted in our lab which showed that long-term exposure can influence *CLOCK* gene methylation (68).

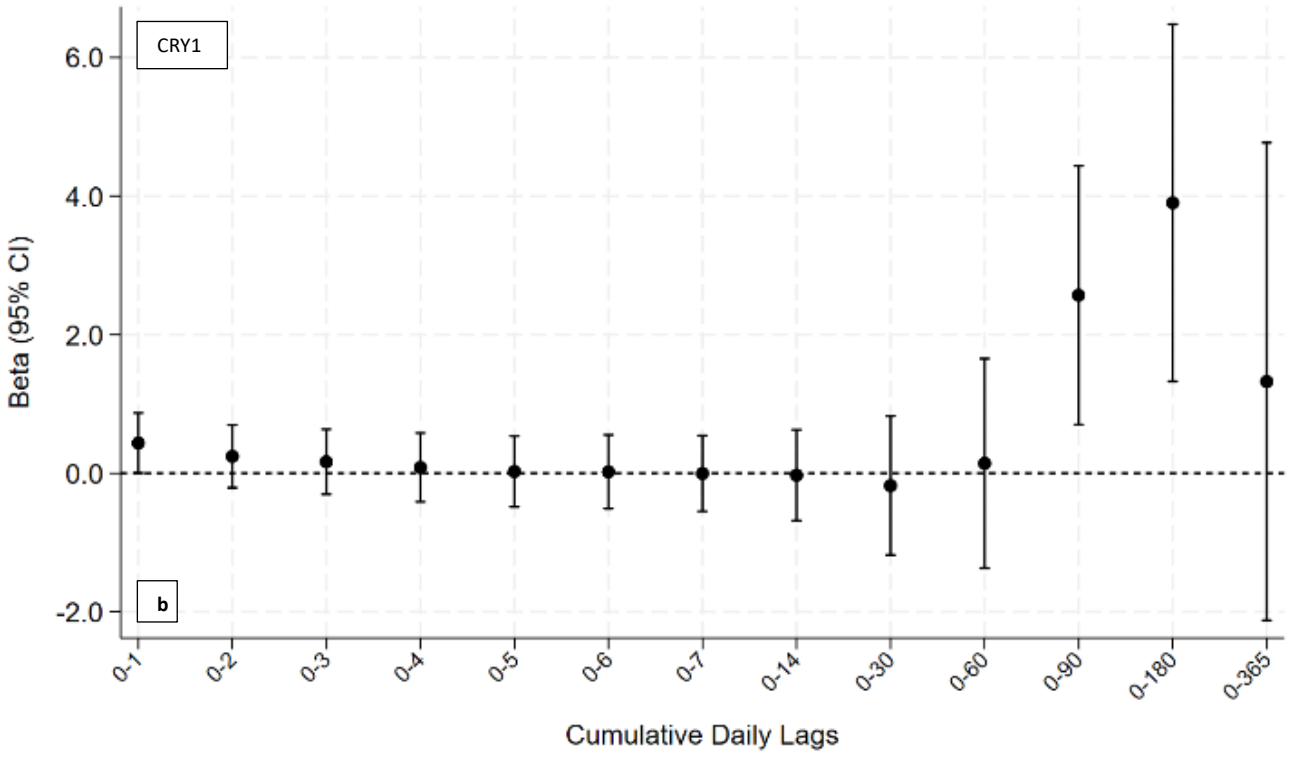
Results for PM_{10} are reported in **Figure 13**. Short-term exposure to PM_{10} was associated with hypomethylation of *CRY2* (between lag0-1 and lag0-7) and an hypermethylation of *OX1R* (between lag0-1 and lag0-14) and *CRY1* (lag0-1), whereas hypermethylation of *ARNTL* was observed in lags 0-5, 0-6, 0-7. When we evaluated long-term exposure, we observed a positive association between PM_{10} and *CRY1* (lag0-90 and lag0-180) and a negative association with *CRY2* at lag0-90 and lag0-180. No relevant associations were observed for *CLOCK*, *PER1*, *PER2*, *FOXP3*, and *HERWV* methylation.

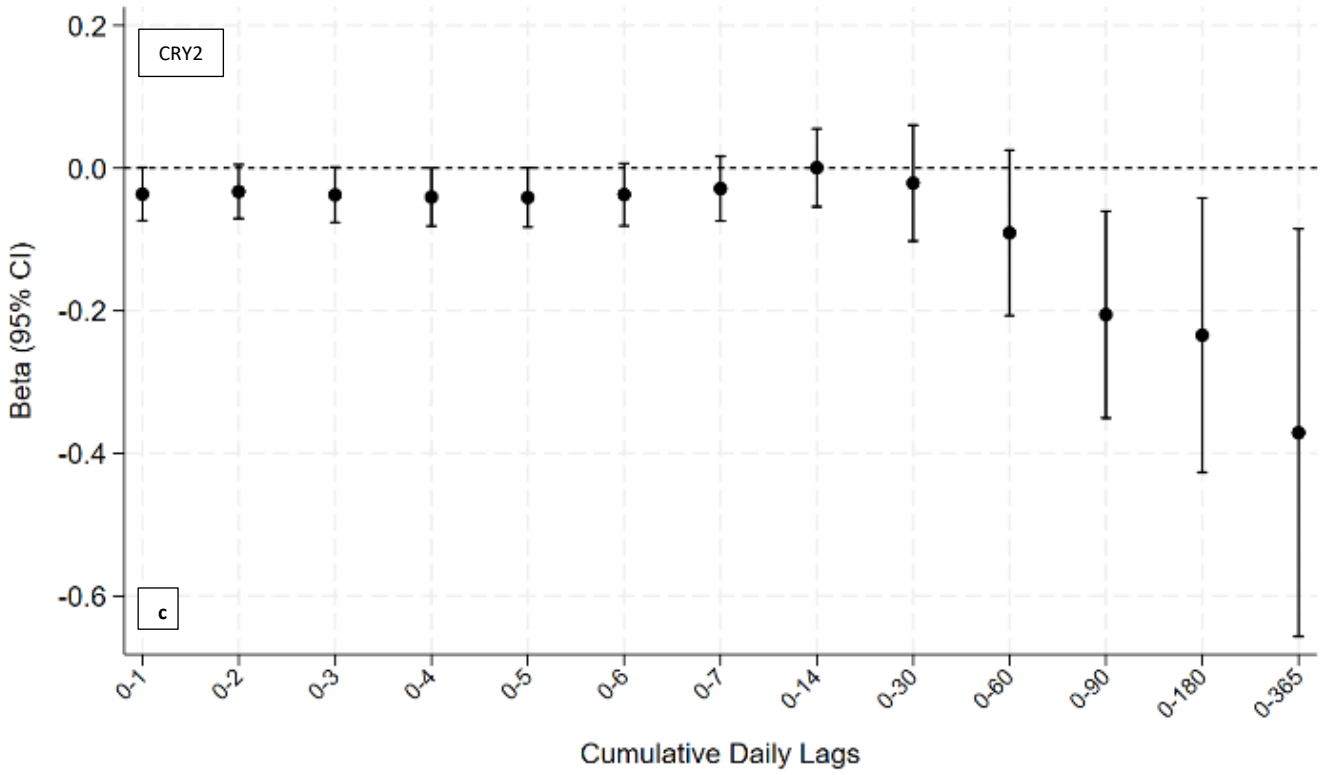
Results for $PM_{2.5}$ exposure were mostly similar (**Figure 14**), with the addition of a negative association with *CRY2* methylation at lags 0-60 and 0-365.

When exposure to NO_2 was considered (**Figure 15**), we observed an increase methylation of *CRY1* at lags 0-1, 0-2, 0-3 and hypomethylation of *CRY2* at lags 0-5, 0-6. Long-term exposure to NO_2 was positively associated to the methylation of *CRY1* at lags 0-90, 0-180 and *HERVW* at lag 0-365. A negative association was observed for *CRY2* at lags 0-60, 0-90, 0-180. In summary, both short- and long-term exposures alter the methylation of selected genes, in particular *CRY1* and *CRY2* leading to an hypermethylation of the former and an hypomethylation of the latter.

Figure 13: Association between different lag (lag 0-1, lag 0-2, lag 0-3, lag 0-4, lag 0-5, lag0-6, lag0-7, lag0-14, lag 0-30, lag 0-60, lag0-90, lag 0-180, lag 0-365) of PM_{10} exposure and log-methylation of the following *CLOCK*-related genes: a) *CLOCK*, b) *CRY1*, c) *CRY2*, d) *PER1*, e) *PER2*, f) *OXTR*, g) *OX1R*, h) *FOXP3*, i) *ARNTL*, l) *HERVW*






CRY2
c
0.2
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-0.2
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-0.6
Beta (95% CI)
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0-2
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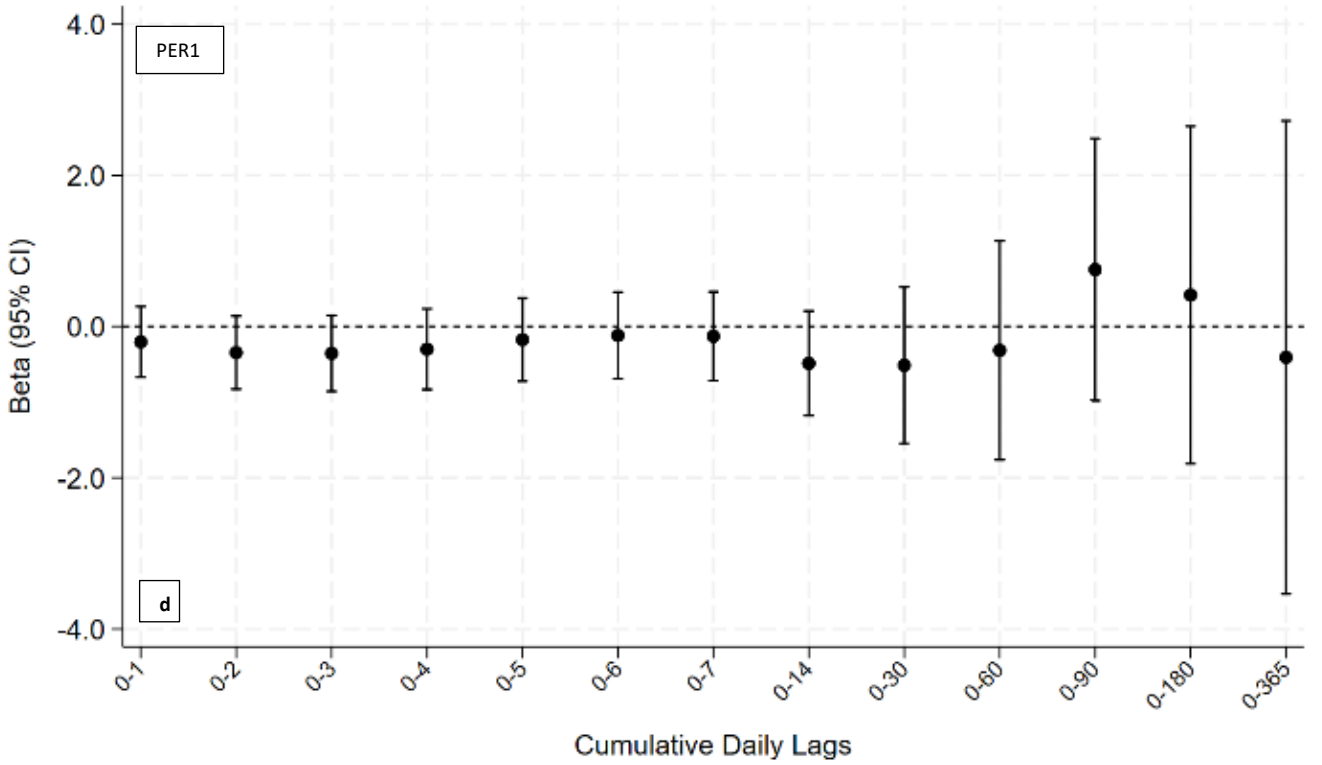
0-90
0-180
0-365
Cumulative Daily Lags


PER1
d
4.0
2.0
0.0
-2.0
-4.0
Beta (95% CI)
0-1
0-2
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0-14
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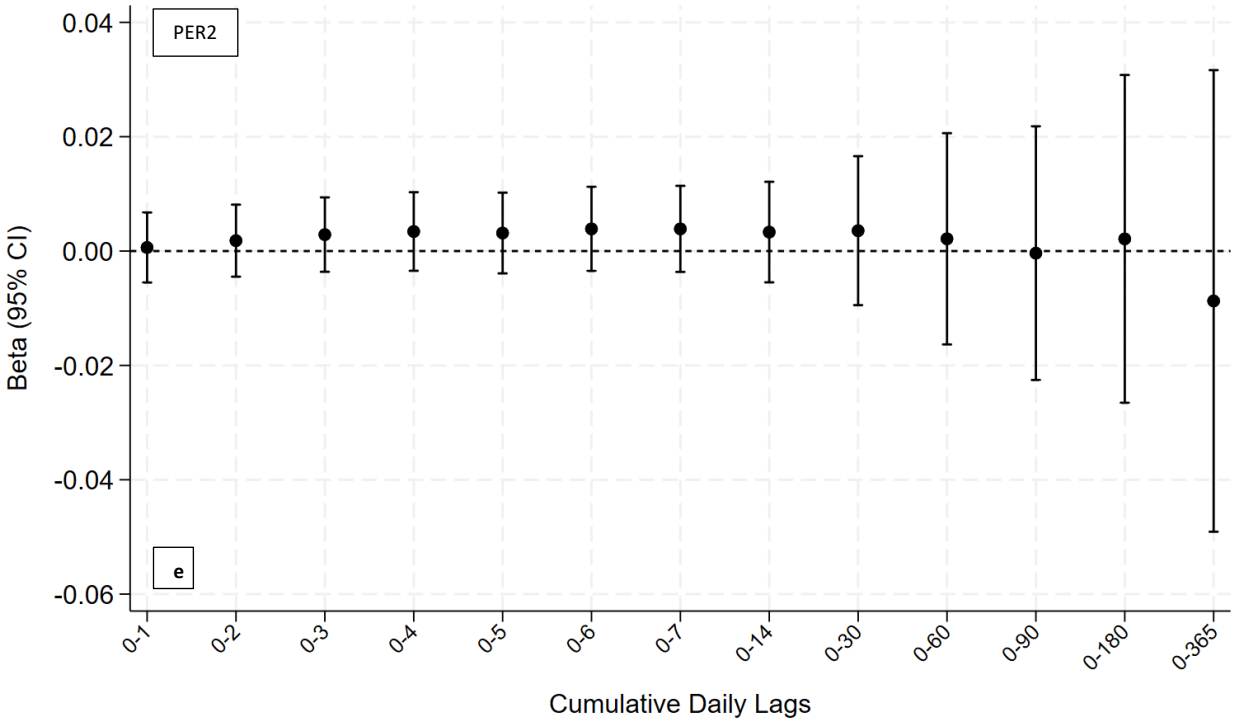
0-60
0-90
0-180
0-365
Cumulative Daily Lags


PER2
e
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0.00
-0.02
-0.04
-0.06
Beta (95% CI)
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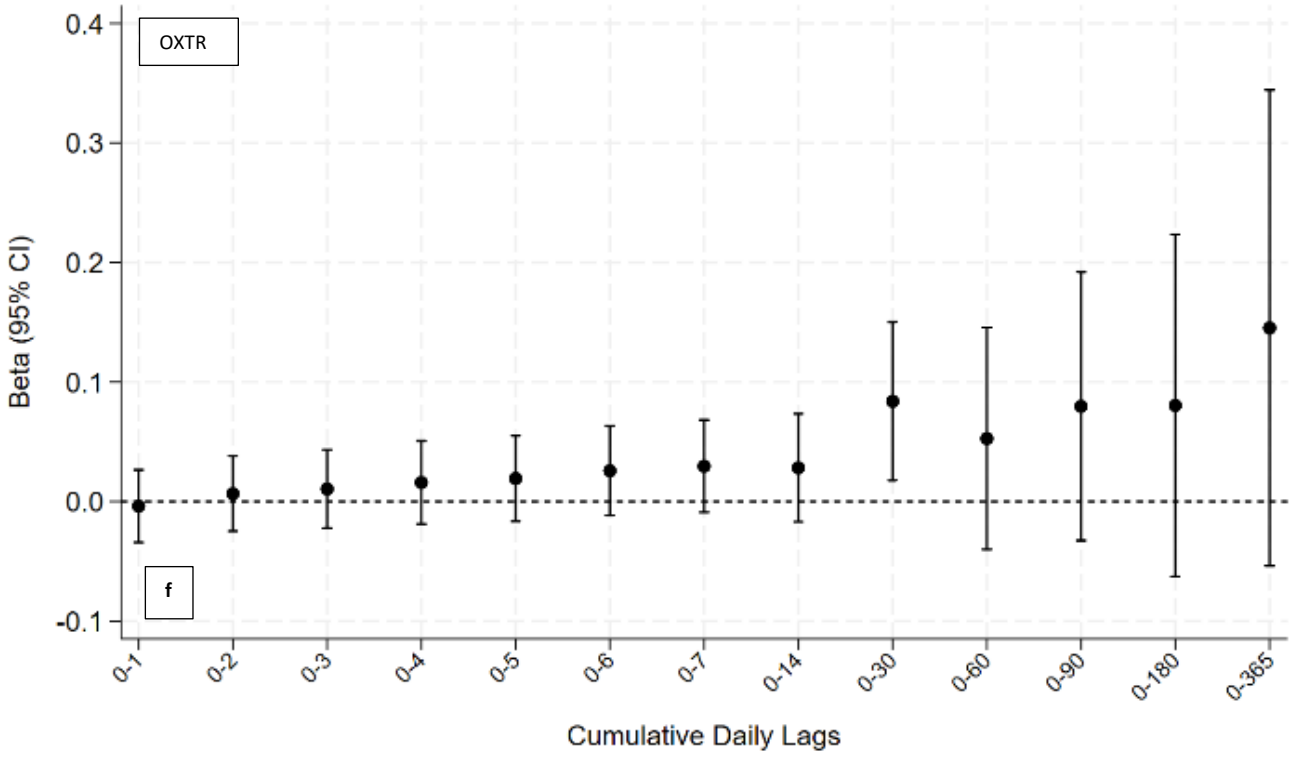
Cumulative Daily Lags


OXTR
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Beta (95% CI)
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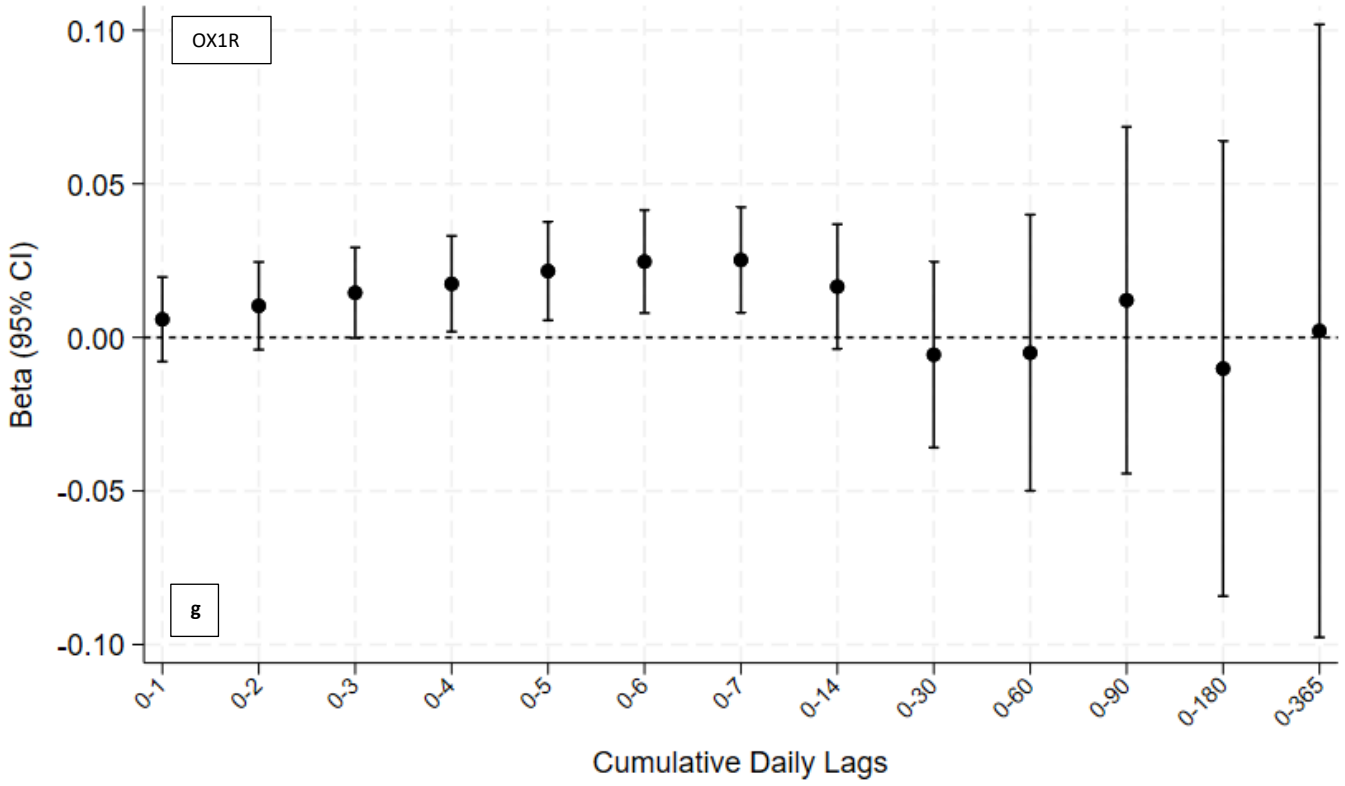
Cumulative Daily Lags


OX1R
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0.05
0.00
-0.05
-0.10
Beta (95% CI)
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0-2
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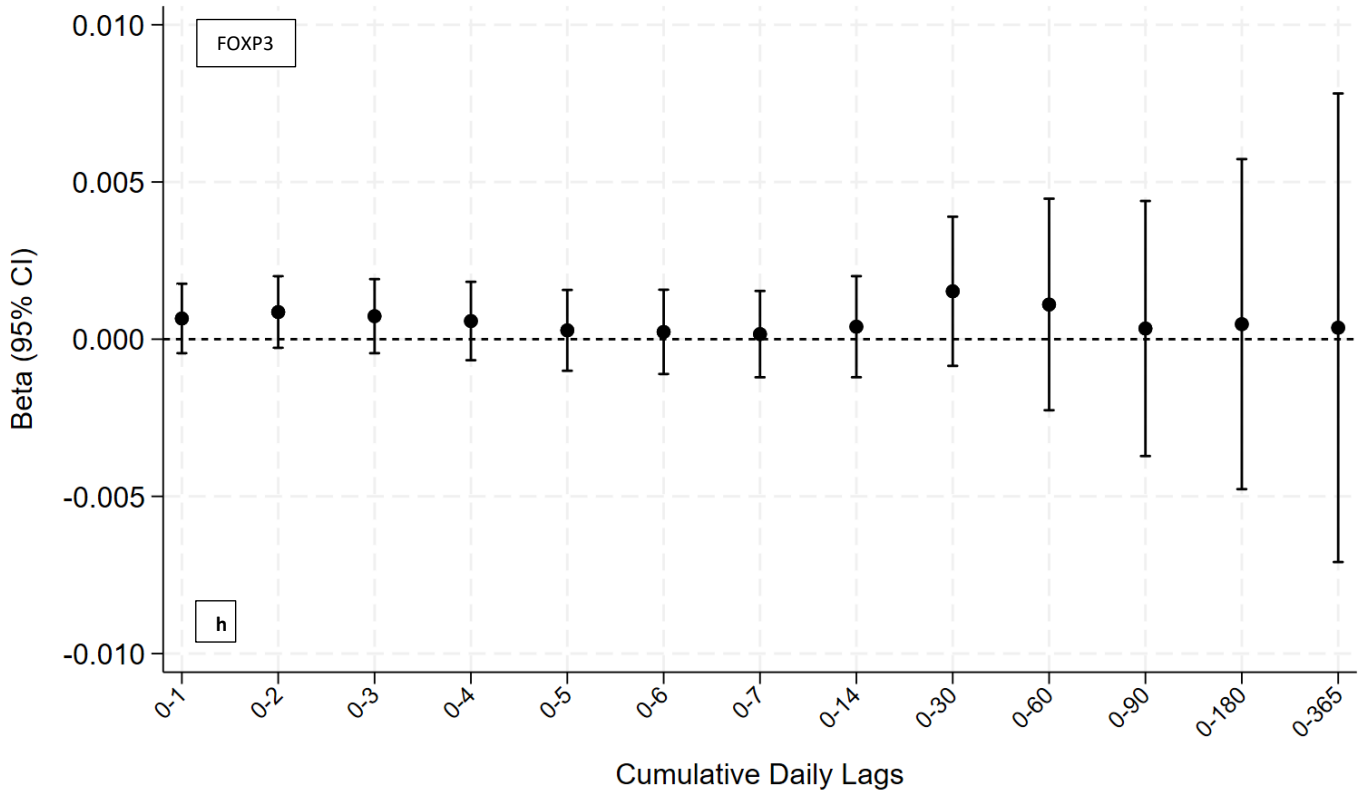
Cumulative Daily Lags


FOXP3
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0.000
-0.005
-0.010
Beta (95% CI)
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0-2
0-3
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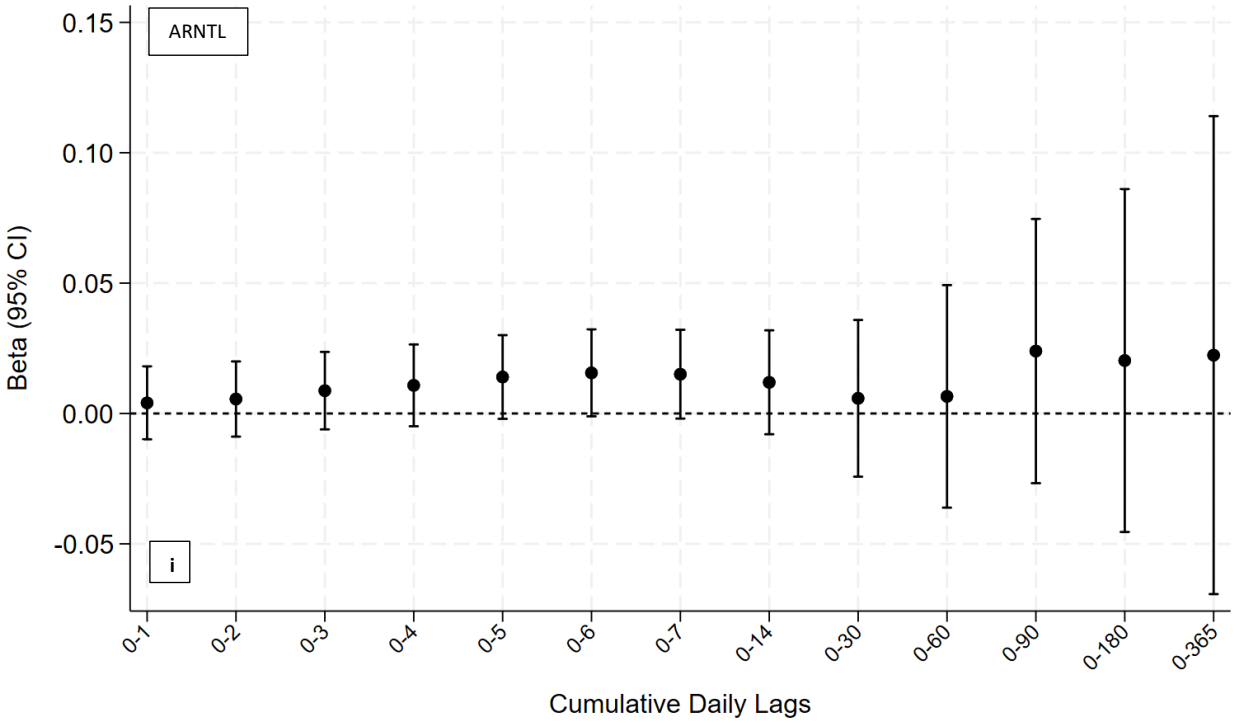
0-365
Cumulative Daily Lags


ARNTL
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Beta (95% CI)
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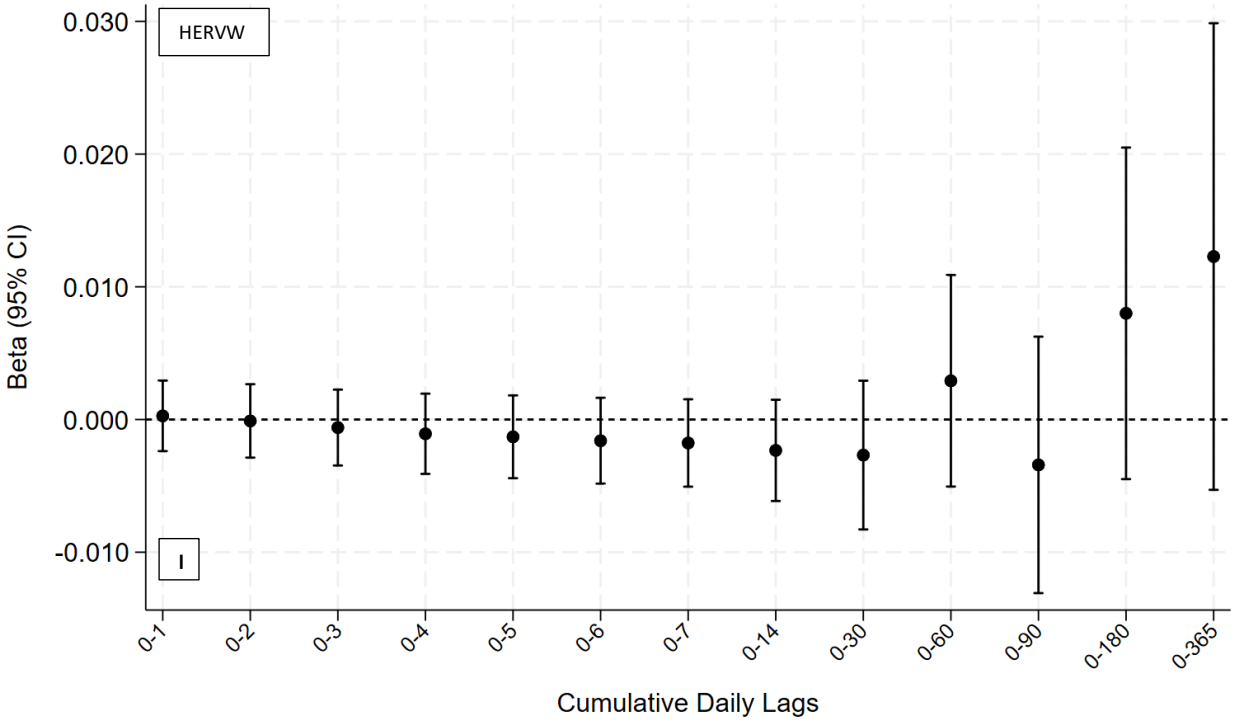
0-90
0-180
0-365
Cumulative Daily Lags


HERVW
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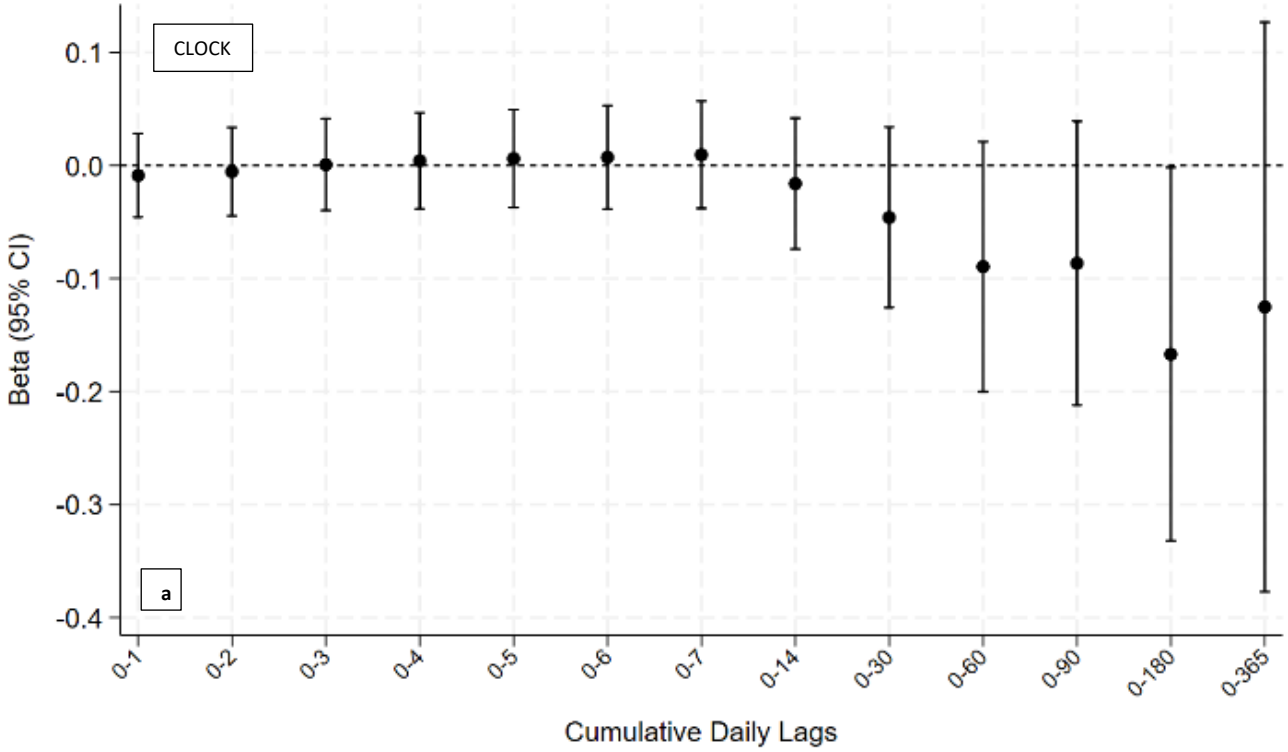
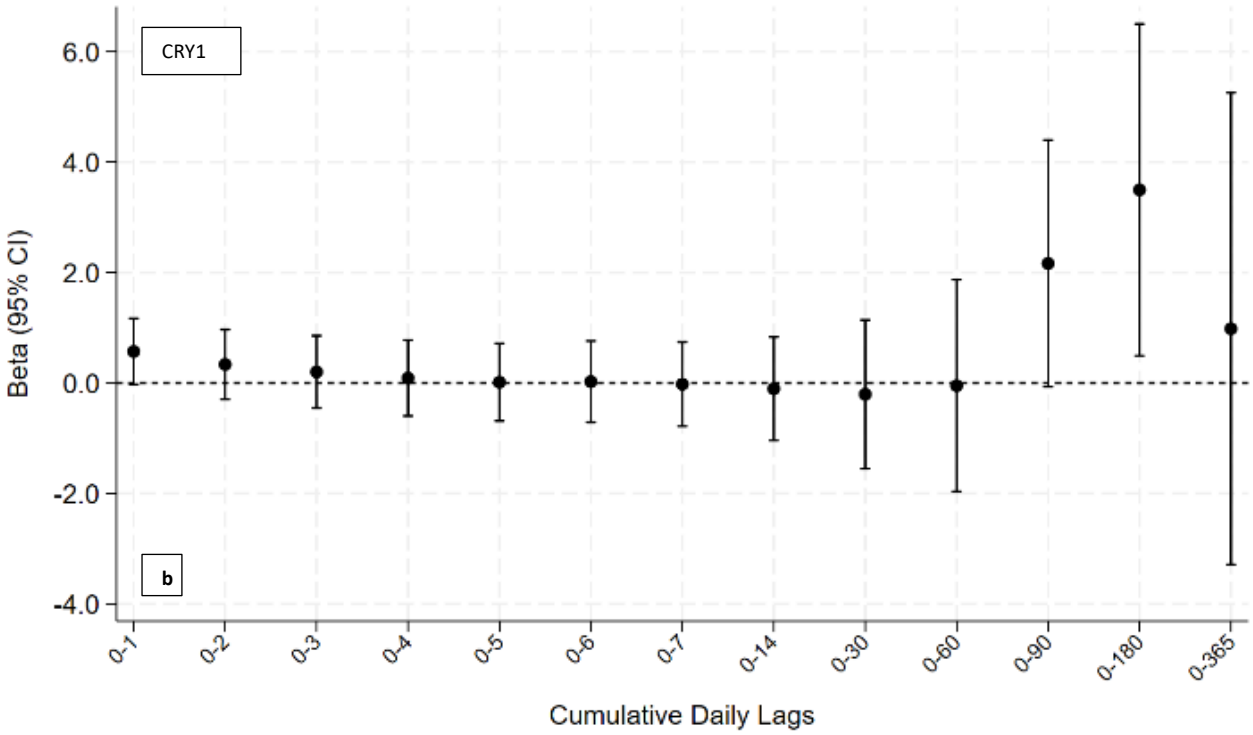
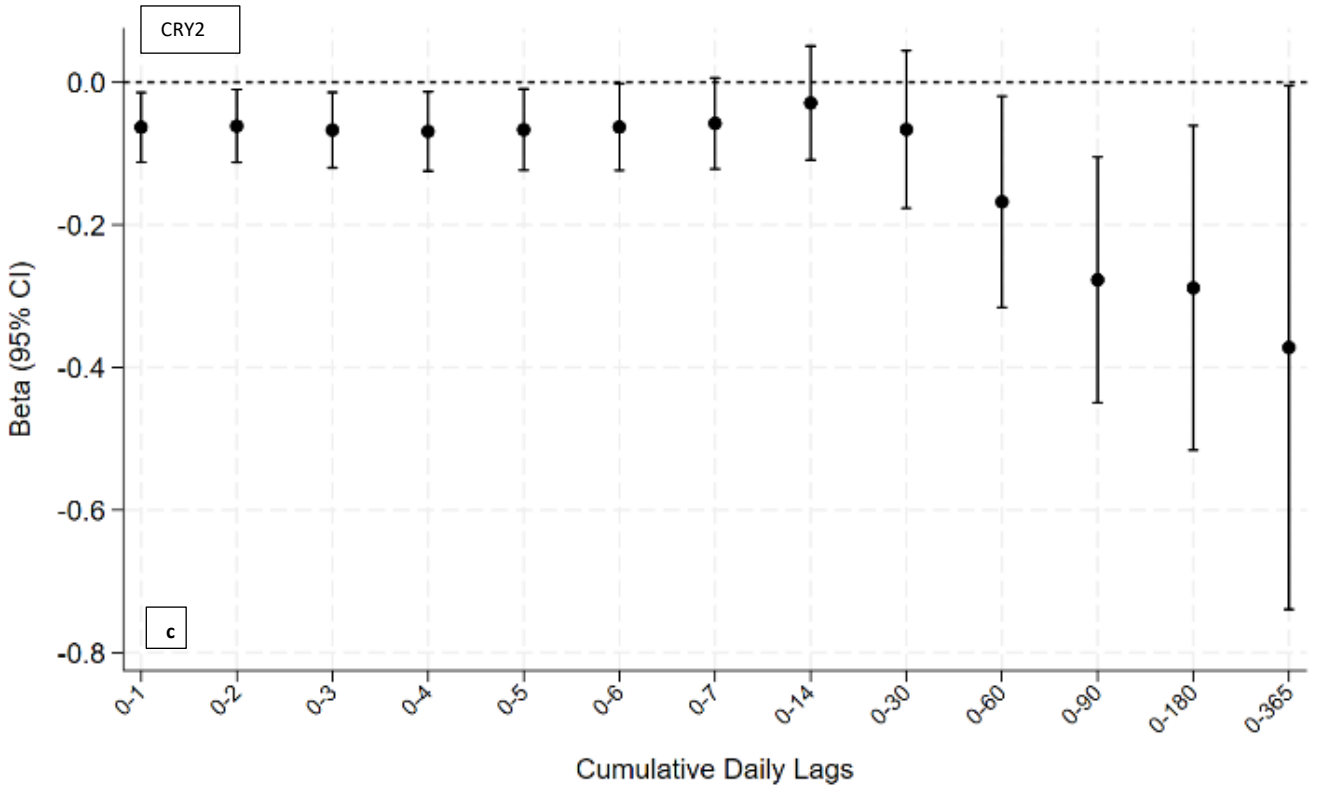
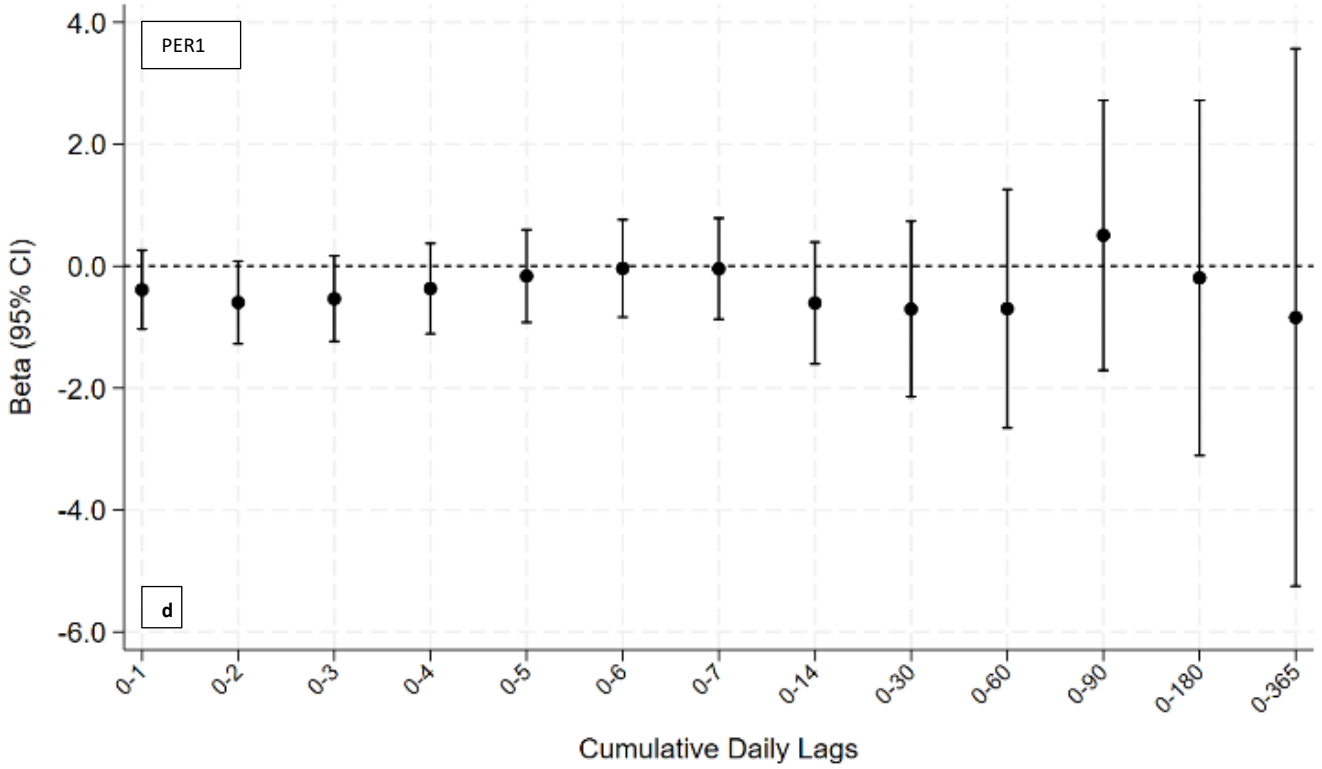
0.030
0.020
0.010
0.000
-0.010
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags

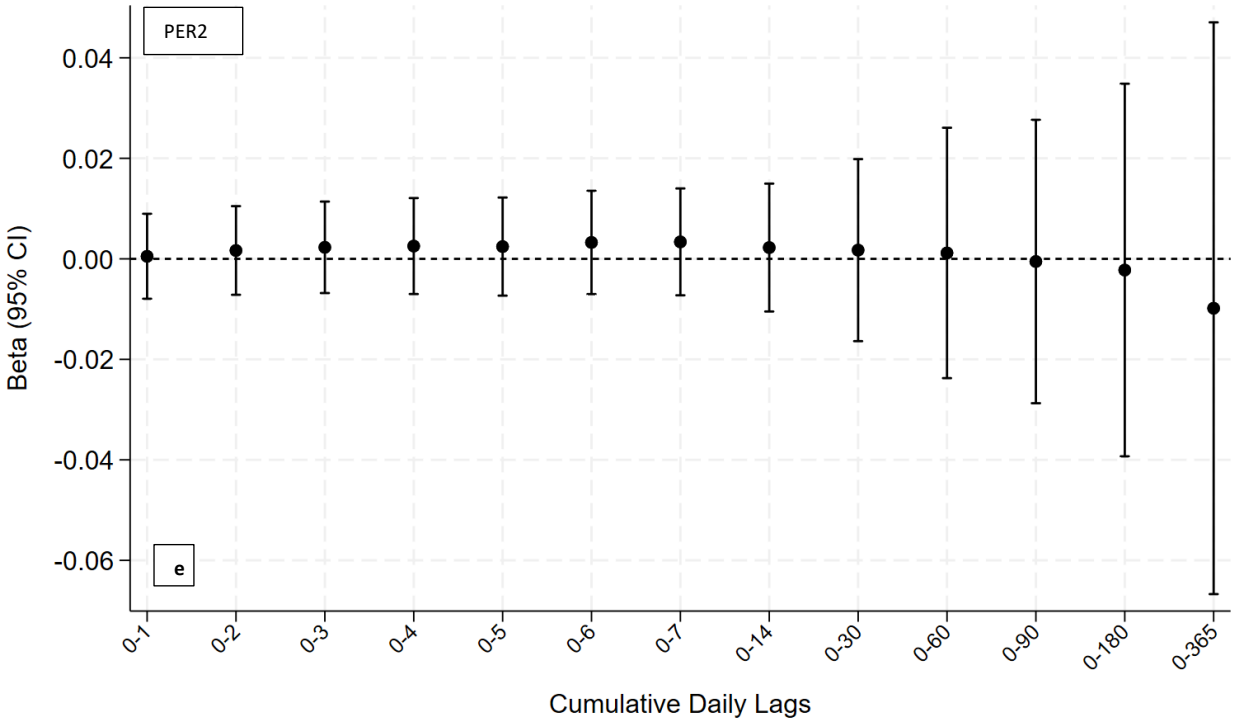
Figure 14: Association between different lag (lag 0-1, lag 0-2, lag 0-3, lag 0-4, lag 0-5, lag0-6, lag0-7, lag0-14, lag 0-30, lag 0-60, lag0-90, lag 0-180, lag 0-365) of $PM_{2.5}$ exposure and log-methylation of the following *CLOCK*-related genes: a) *CLOCK*, b) *CRY1*, c) *CRY2*, d) *PER1*, e) *PER2*, f) *OXTR*, g) *OX1R*, h) *FOXP3*, i) *ARNTL*, l) *HERVW*

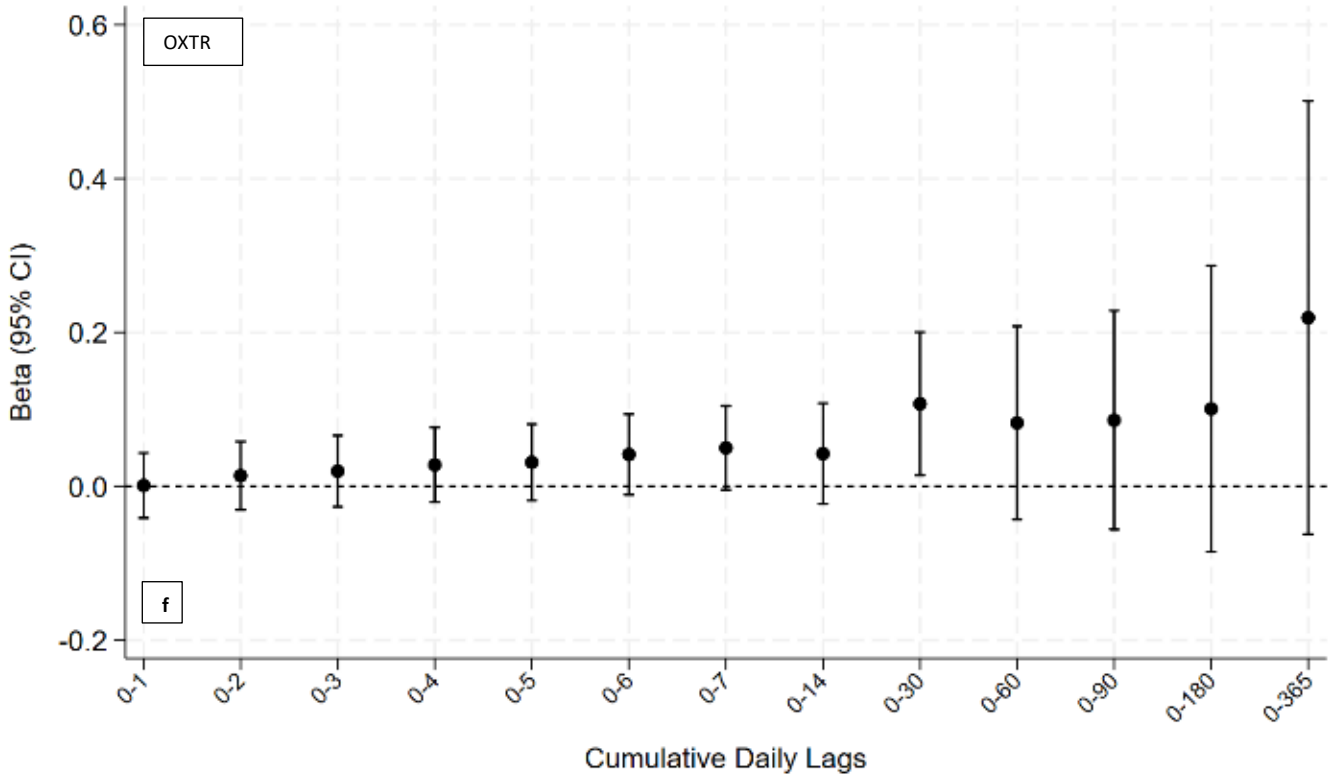


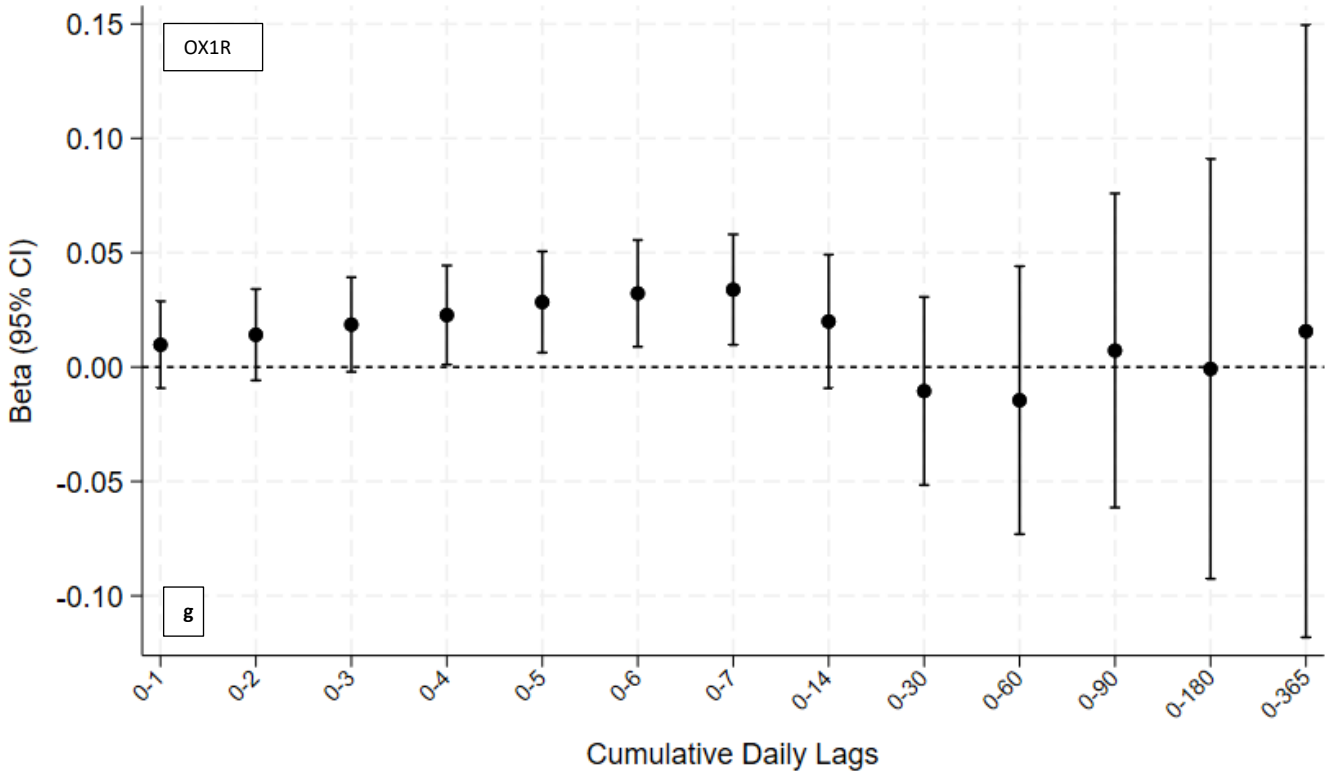


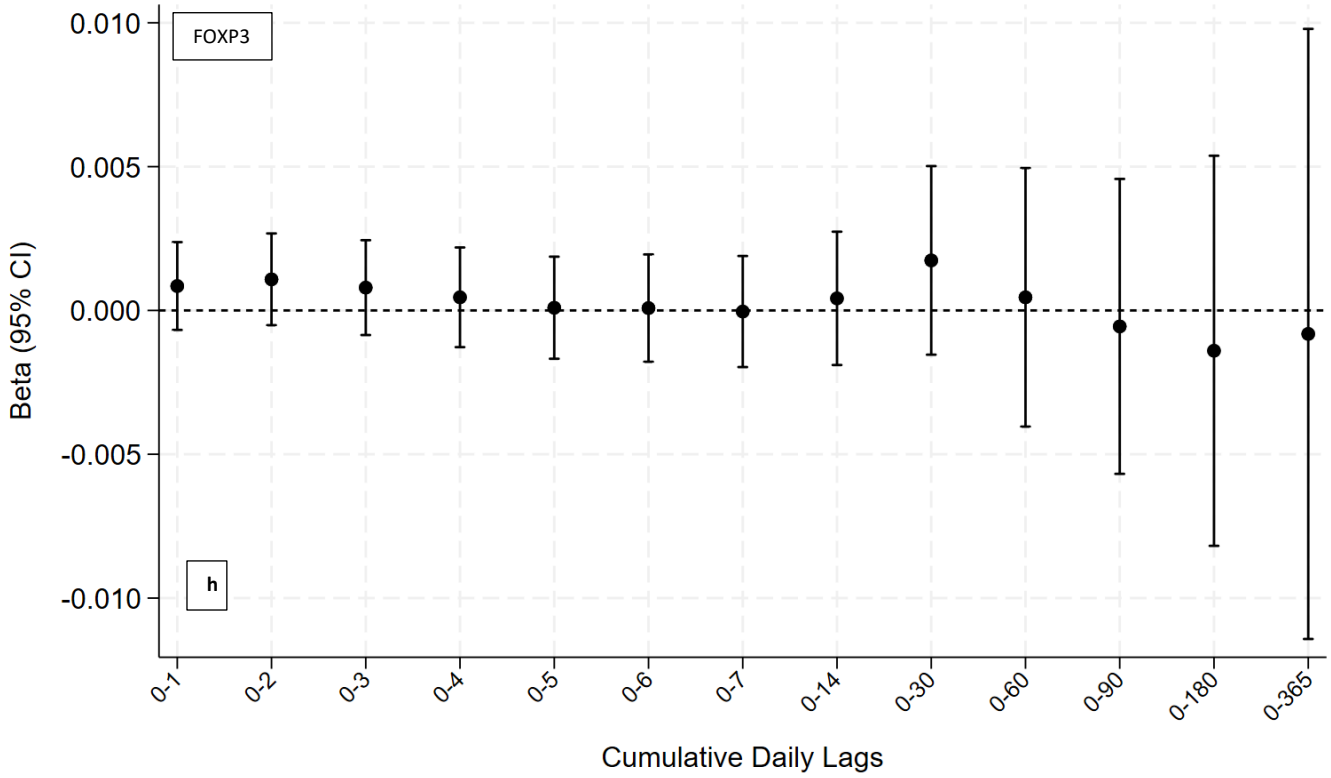

CRY2
0.0
-0.2
-0.4
-0.6
-0.8
Beta (95% CI)
c
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags

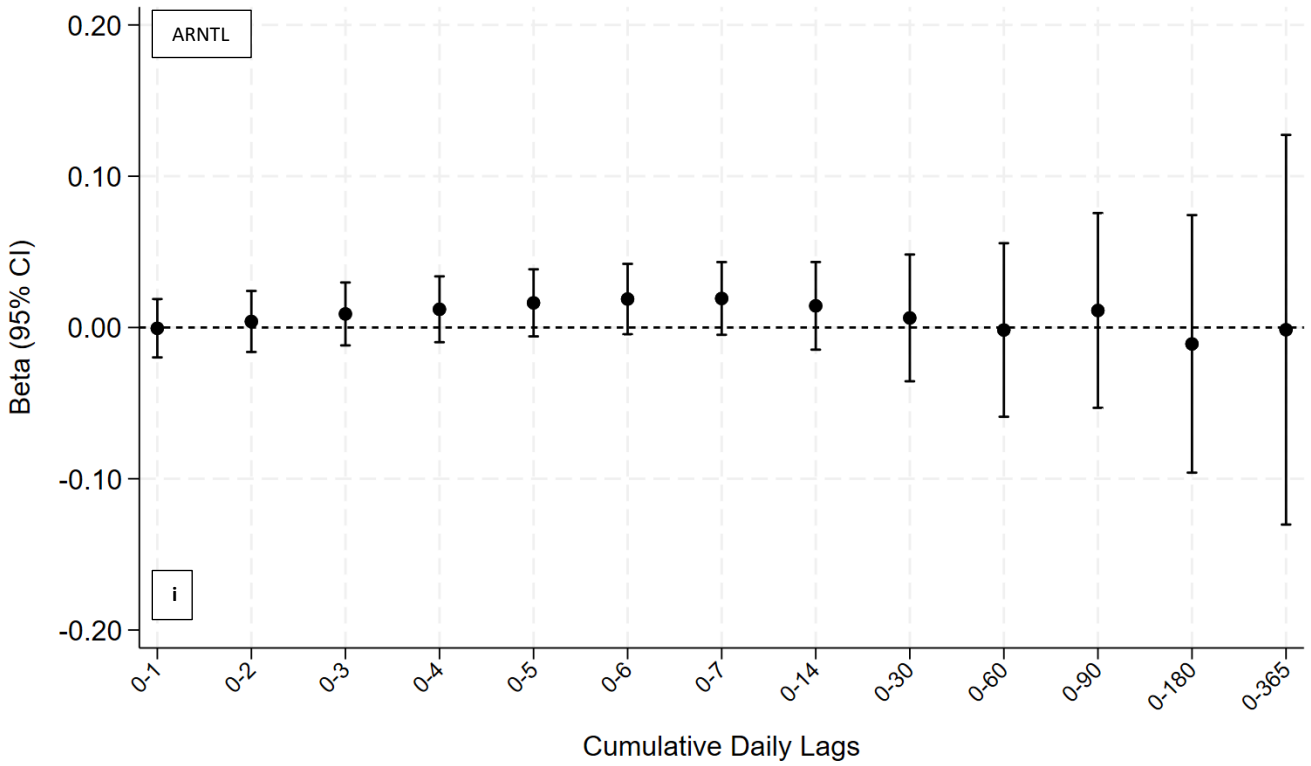

PER1
4.0
2.0
0.0
-2.0
-4.0
-6.0
Beta (95% CI)
d
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


PER2
e
Beta (95% CI)
0.04
0.02
0.00
-0.02
-0.04
-0.06
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


OXTR
f
Beta (95% CI)
0.6
0.4
0.2
0.0
-0.2
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


OX1R
g
0.15
0.10
0.05
0.00
-0.05
-0.10
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


FOXP3
h
0.010
0.005
0.000
-0.005
-0.010
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


ARNTL
i
0.20
0.10
0.00
-0.10
-0.20
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags

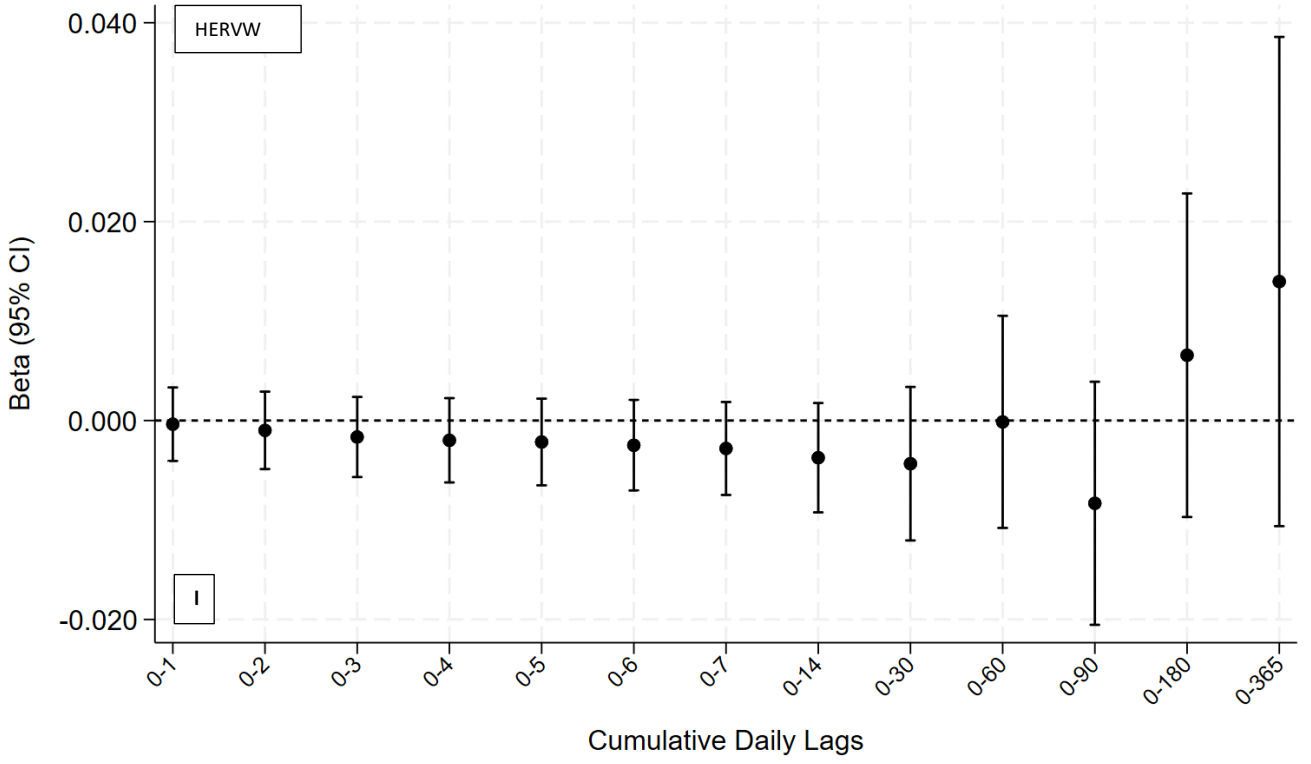
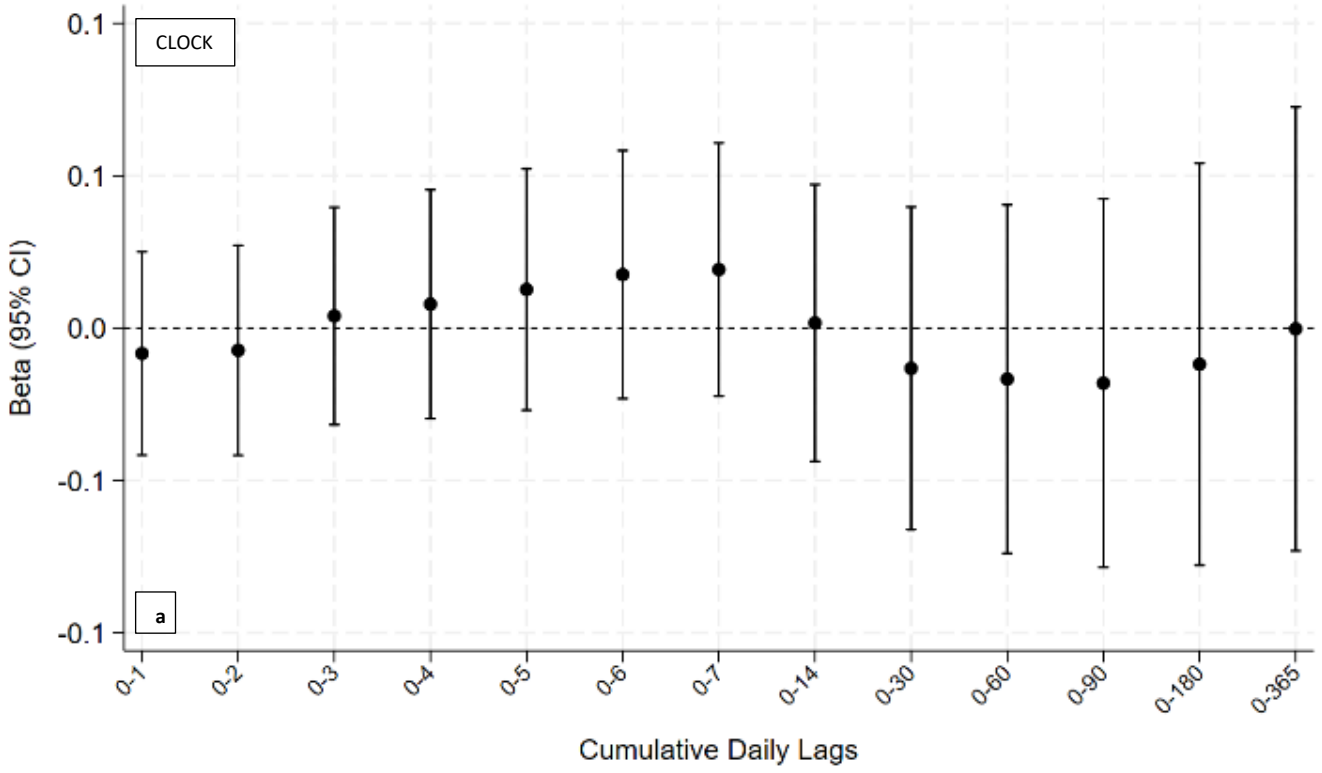
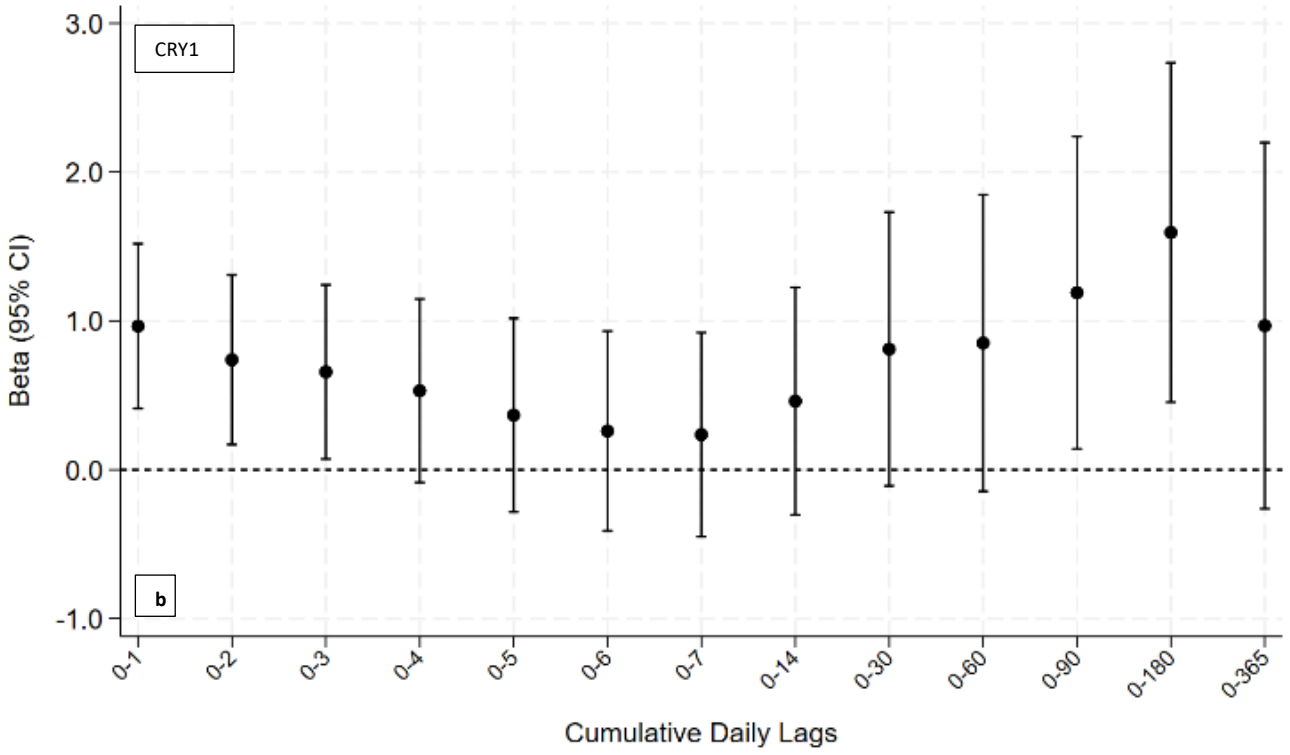
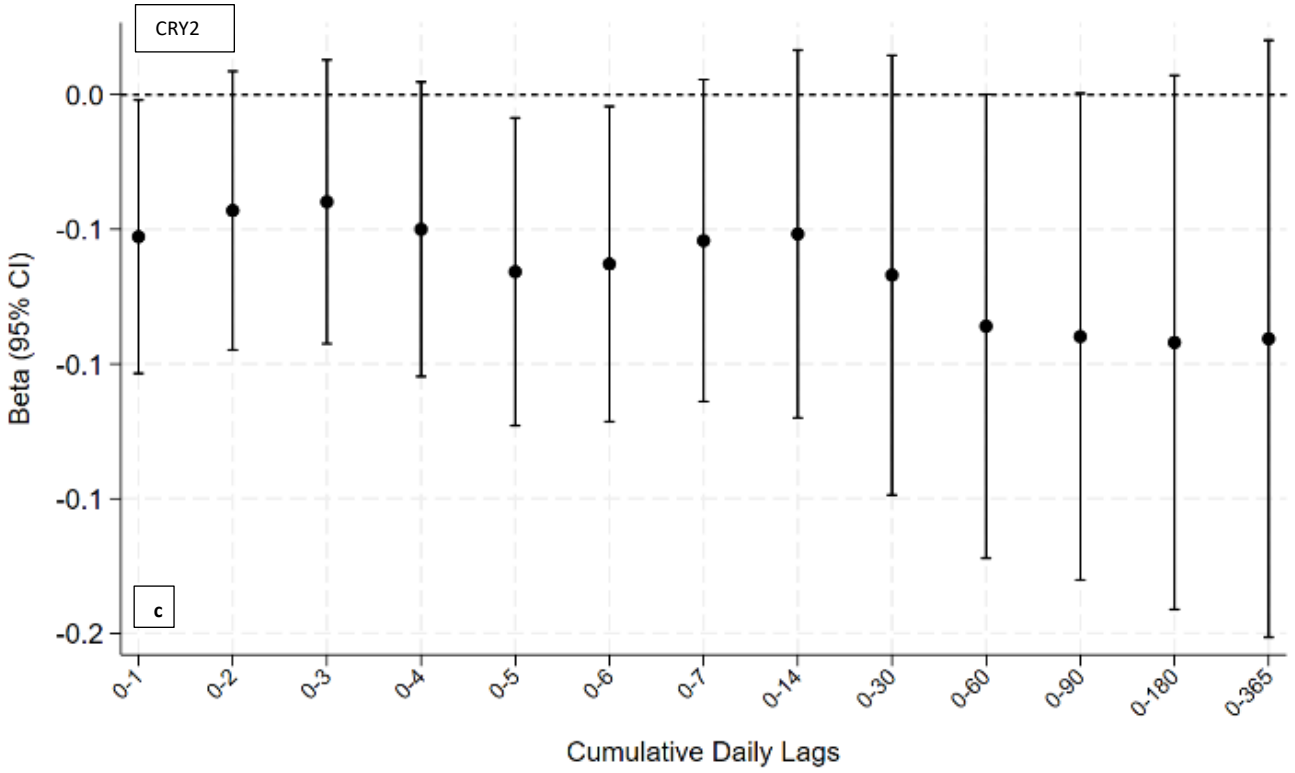
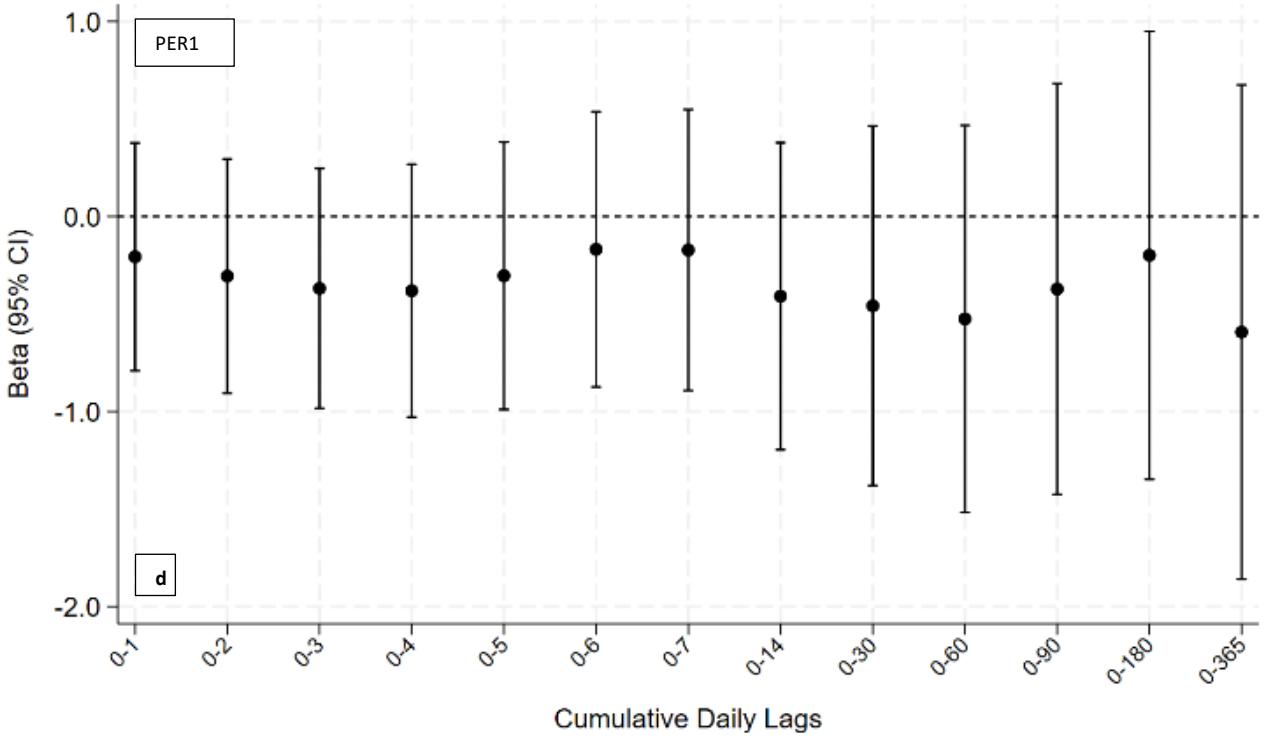

HERVW
l
0.040
0.020
0.000
-0.020
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags

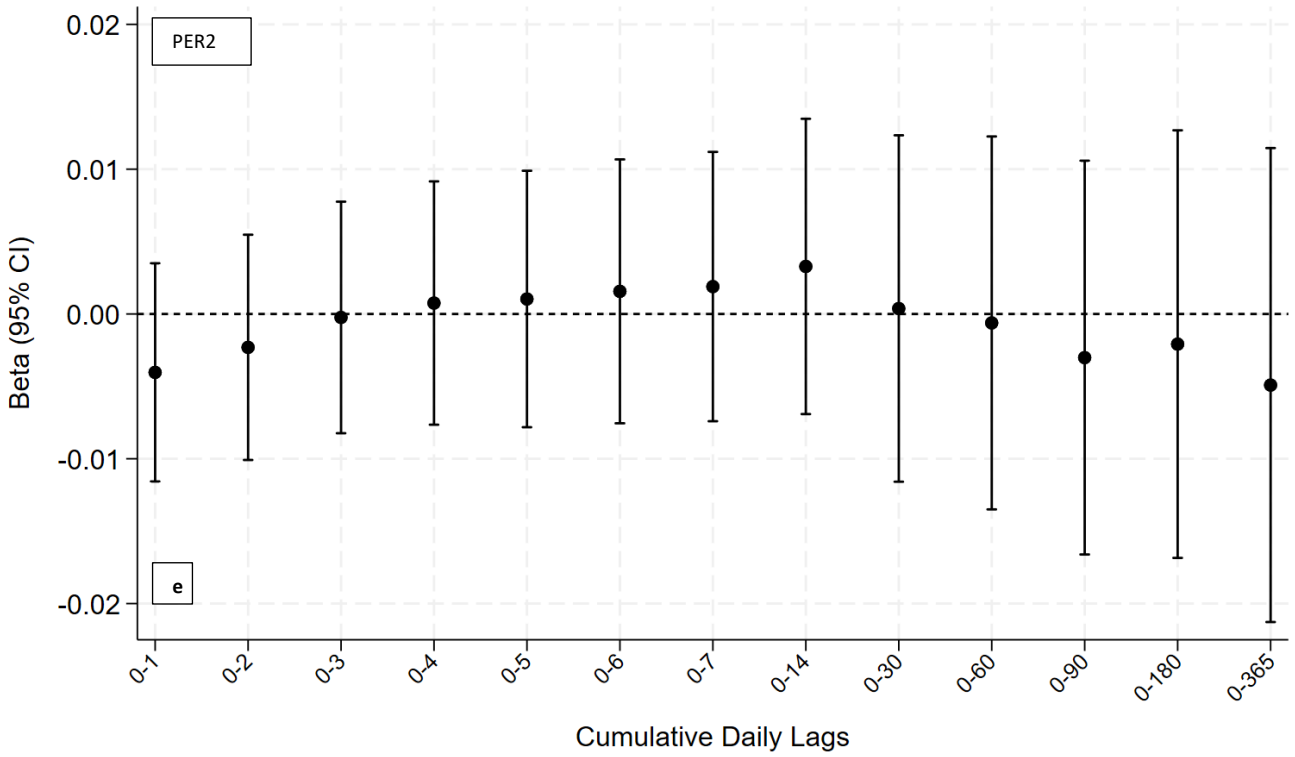
Figure 15: Association between different lag (lag 0-1, lag 0-2, lag 0-3, lag 0-4, lag 0-5, lag0-6, lag0-7, lag0-14, lag 0-30, lag 0-60, lag0-90, lag 0-180, lag 0-365) of NO_2 exposure and log-methylation of the following *CLOCK*-related genes: a) *CLOCK*, b) *CRY1*, c) *CRY2*, d) *PER1*, e) *PER2*, f) *OXTR*, g) *OX1R*, h) *FOXP3*, i) *ARNTL*, l) *HERVW*

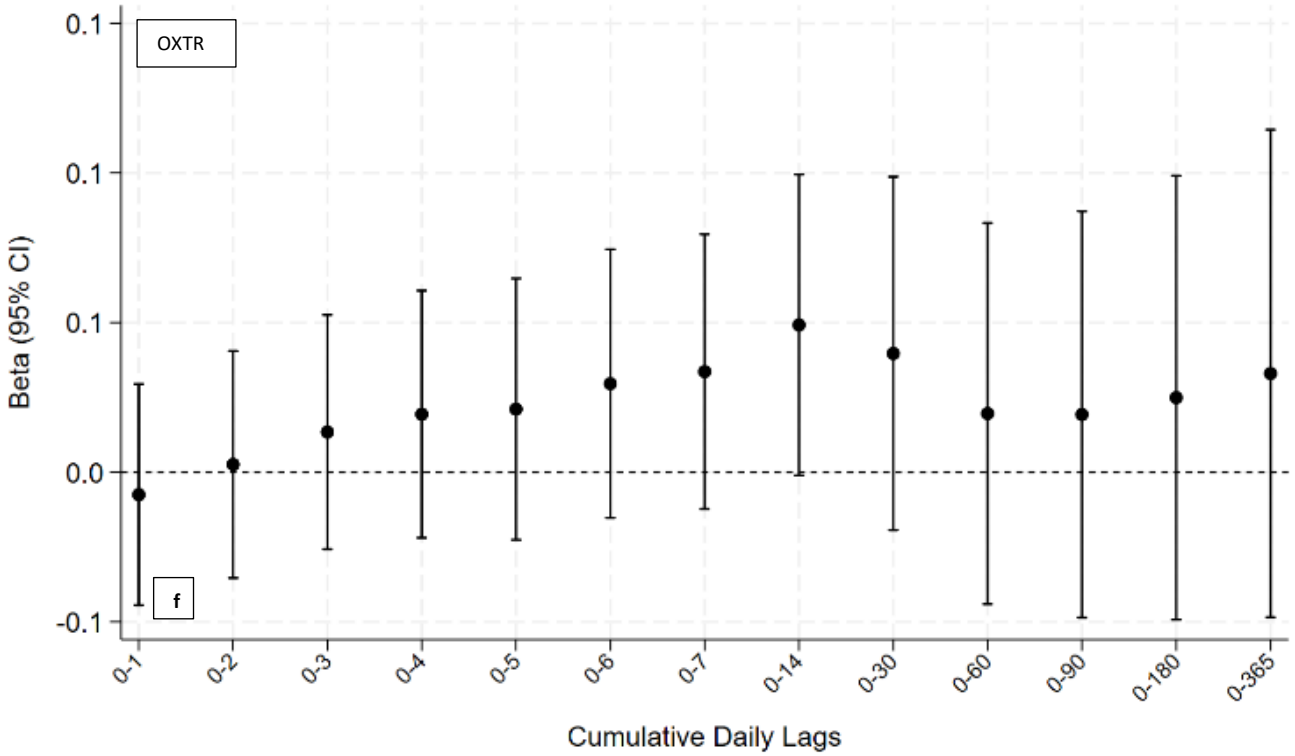


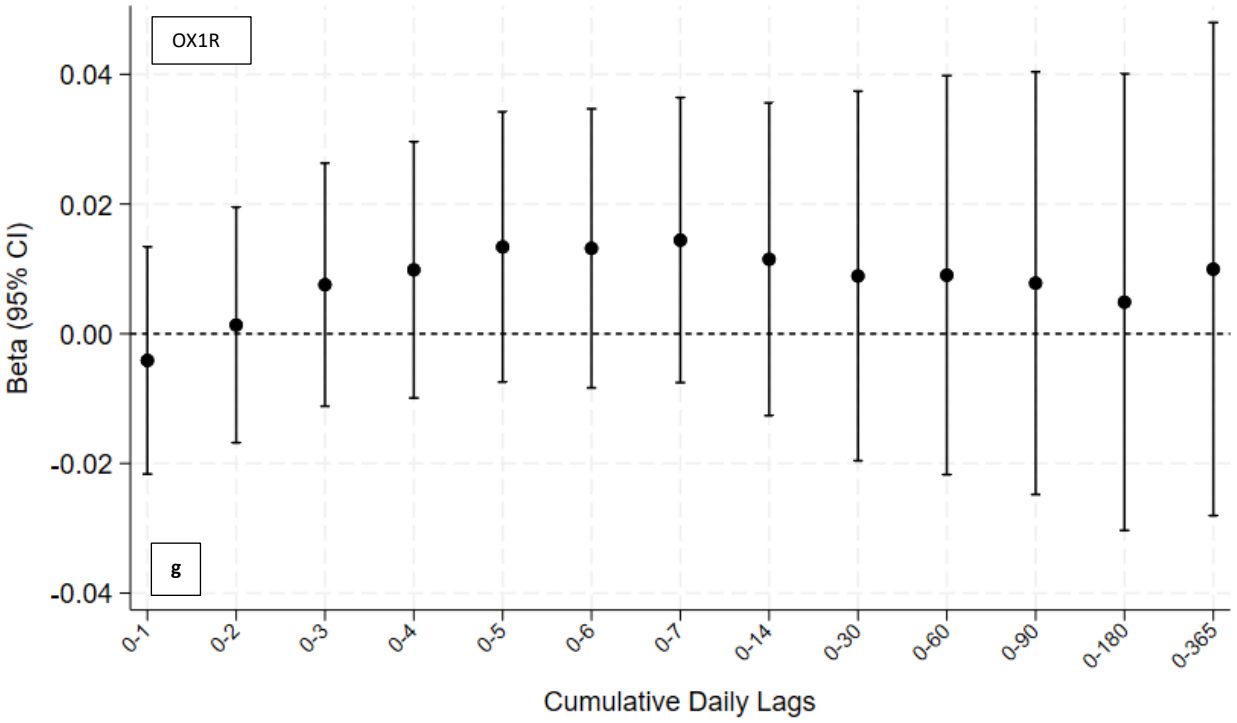


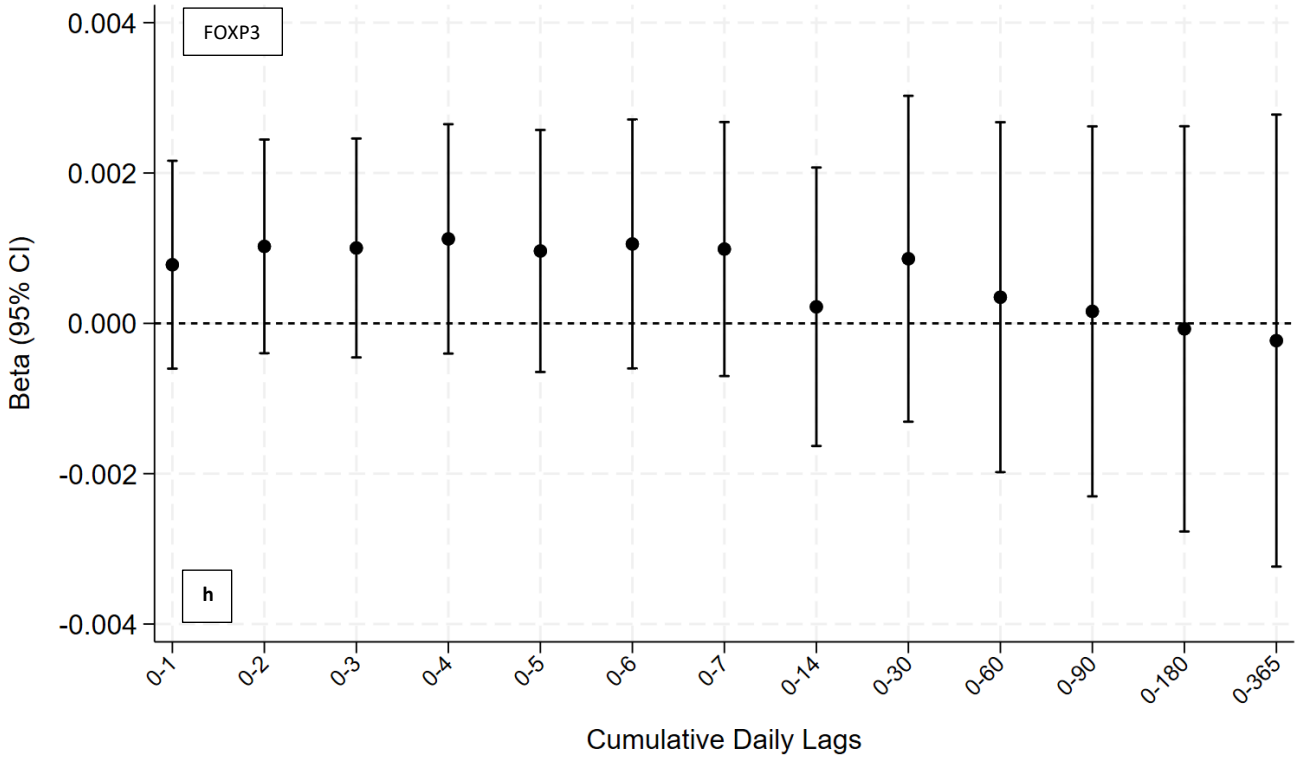

CRY2
0.0
-0.1
-0.1
-0.2
Beta (95% CI)
c
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags

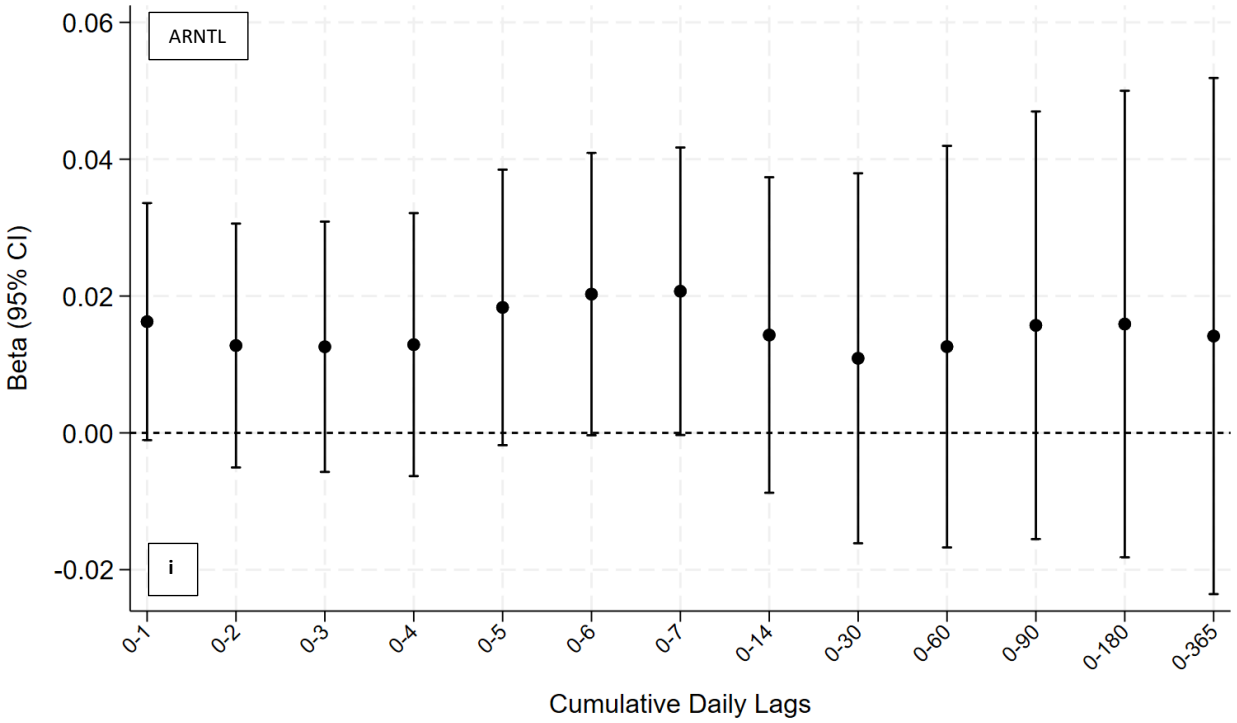

PER1
1.0
0.0
-1.0
-2.0
Beta (95% CI)
d
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags

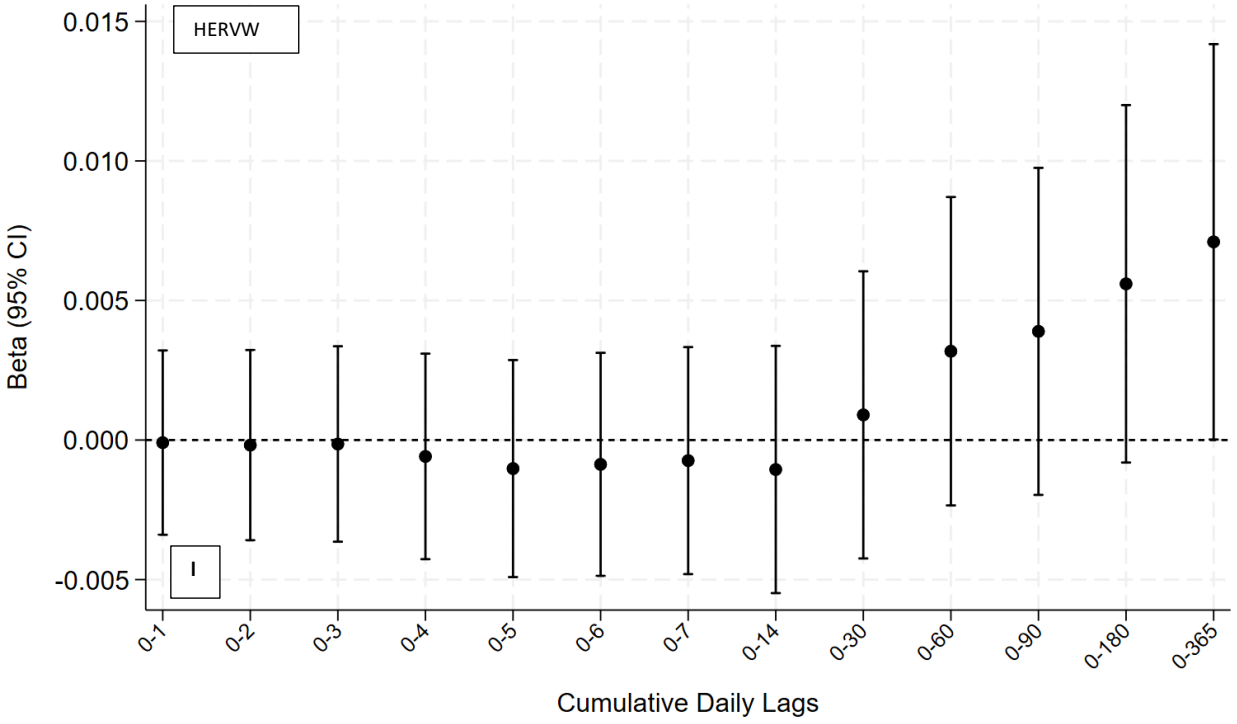

PER2
e
0.02
0.01
0.00
-0.01
-0.02
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


OXTR
f
0.1
0.1
0.1
0.0
-0.1
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


OX1R
0.04
0.02
0.00
-0.02
-0.04
Beta (95% CI)
g
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


FOXP3
0.004
0.002
0.000
-0.002
-0.004
Beta (95% CI)
h
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


ARNTL
i
0.06
0.04
0.02
0.00
-0.02
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags


HERVW
I
0.015
0.010
0.005
0.000
-0.005
Beta (95% CI)
0-1
0-2
0-3
0-4
0-5
0-6
0-7
0-14
0-30
0-60
0-90
0-180
0-365
Cumulative Daily Lags

3.4 Association between methylation of *CLOCK* and *CLOCK*-related genes and severity of MDD

We examined the association between methylation of *CLOCK* and *CLOCK*-related genes and severity of MDD. Results are shown in **Tables 12-21** for the whole population and for different subtypes of Depression categorized as "melancholic / psychotic / no prevalent type", "with strong symptoms of anxiety", and "atypical".

In the whole population (first column of each table), only the methylation of *CLOCK* was associated with MDD severity (**Table 12**). In particular, we observed negative associations between methylation of this gene and the MDD severity assessed through the HAMD scale (β=-3.40, 95% CI (-6.65; -0.15)) and some sub domains of the SDS scale ["Impairment at work" (β=-1.08, 95% CI (-1.96; -0.19)), "impairment in home relationship" (β=-0.81, 95% CI (-1.55; -0.07)) and "impairment in family responsibilities" (β=-0.99, 95% CI (-1.75; -0.22))]

When results were stratified by subtype of MDD (**Tables 12-21**), a stronger negative association between hypermethylation of *CLOCK* (**Table 12**), *CRY1* (**Table 13**) and *PER1* (**Table 15**) and MDD severity (measured through HAMD, MADRS or CGI scales) was observed mainly in the subgroup of patients with strong symptoms of anxiety.

Methylation of *OXTR* was positively associated with melancholic / psychotic / no prevalent type depression (**Table 17**), when looking at all the investigated scales.

Table 12: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between *CLOCK* gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	-2.79 (-6.03; 0.44) p=0.090	-2.87 (-6.92; 1.78) p=0.164	**-9.90 (-19.00; -0.81) p=0.033**	0.36 (-5.76; 6.48) p=0.909
HAMD	**-3.40 (-6.65; -0.15) p=0.040**	**-4.10 (-8.14; -0.06) p=0.047**	**-11.93 (-20.99; -2.87) p=0.010**	1.44 (-4.66; 7.54) p=0.643
GAF	2.30 (-1.41; 6.01) p=0.223	3.82 (-0.83; 8.47) p=0.107	3.83 (-6.61; 14.27) p=0.471	-1.91 (-8.94; 5.12) p=0.594
CGI	-0.49 (-1.05; 0.07) p=0.086	-0.43 (-1.12; 0.26) p=0.219	**-1.68 (-3.21; -0.15) p=0.031**	-0.11 (-1.19; 0.97) p=0.842
SDS				
Impairment at work	**-1.08 (-1.96; -0.19) p=0.018**	-0.93 (-2.11; 0.25) p=0.122	-0.40 (-2.62; 1.82) p=0.722	**-1.70 (-3.30; -0.10) p=0.038**
Impairment in home relationships	**-0.81 (-1.55; -0.07) p=0.032**	-0.62 (-1.55; 0.31) p=0.189	-1.21 (-3.30; 0.88) p=0.256	-1.08 (-2.49; 0.33) p=0.132
Impairment in family responsibilities	**-0.99 (-1.75; -0.22) p=0.011**	**-0.98 (-1.94; -0.03) p=0.043**	**-2.94 (-5.08; -0.80) p=0.007**	-0.26 (-1.70; 1.18) p=0.719
Perceived stress	-0.76 (-1.62; 0.09) p=0.080	-0.93 (-2.00; 0.13) p=0.086	1.57 (-0.82; 3.96) p=0.197	-1.41 (-3.01; 0.20) p=0.086
Perceived social support	3.67 (-4.49; 11.84) p=0.377	-0.16 (-10.39; 10.07) p=0.976	-0.82 (-23.79; 22.15) p=0.944	13.77 (-1.70; 29.23) p=0.081

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 13: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between CRY1 gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	-0.26 (-1.04; 0.52) p=0.517	0.19 (-0.77; 1.14) p=0.699	-1.26 (-3.00; 0.48) p=0.155	-0.60 (-1.76; 0.56) p=0.313
HAMD	-0.08 (-0.87; 0.70) p=0.834	0.40 (-0.56; 1.35) p=0.414	**-2.13 (-3.87; -0.39) p=0.017**	-0.24 (-1.40; 0.93) p=0.691
GAF	-0.08 (-0.98; 0.83) p=0.866	-0.34 (-1.45; 0.77) p=0.547	0.17 (-1.86; 2.20) p=0.872	0.19 (-1.16; 1.55) p=0.779
CGI	-0.00 (-0.14; 0.13) p=0.951	0.04 (-0.13; 0.20) p=0.679	-0.05 (-0.36; 0.26) p=0.745	-0.05 (-0.25; 0.15) p=0.629
SDS				
Impairment at work	-0.08 (-0.30; 0.14) p=0.472	-0.00 (-0.26; 0.25) p=0.983	-0.42 (-0.87; 0.03) p=0.068	-0.09 (-0.43; 0.24) p=0.579
Impairment in home relationships	-0.03 (-0.21; 0.15) p=0.727	0.05 (-0.17; 0.26) p=0.664	**-0.50 (-0.90; -0.11) p=0.013**	0.01 (-0.25; 0.27) p=0.949
Impairment in family responsibilities	-0.01 (-0.19; 0.18) p=0.945	0.10 (-0.13; 0.32) p=0.391	-0.32 (-0.73; 0.09) p=0.131	-0.07 (-0.35; 0.20) p=0.602
Perceived stress	0.03 (-0.18; 0.24) p=0.760	0.07 (-0.18; 0.33) p=0.579	0.05 (-0.42; 0.52) p=0.839	-0.05 (-0.36; 0.26) p=0.755
Perceived social support	1.04 (-0.94; 3.01) p=0.302	0.35 (-2.06; 2.76) p=0.775	4.32 (-0.08; 8.71) p=0.054	0.87 (-2.07; 3.80) p=0.561

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 14: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between CRY2 gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	-0.18 (-1.85; 1.48) p=0.830	-0.42 (-3.35; 2.51) p=0.777	-3.30 (-9.69; 3.10) p=0.311	0.17 (-1.67; 2.01) p=0.856
HAMD	0.62 (-0.96; 2.20) p=0.440	1.39 (-1.38; 4.16) p=0.322	-1.68 (-7.73; 4.36) p=0.584	0.45 (-1.30; 2.19) p=0.615
GAF	0.62 (-1.34; 2.59) p=0.532	-0.21 (-3.66; 3.23) p=0.902	-3.09 (-4.43; 10.62) p=0.419	0.64 (-1.53; 2.81) p=0.561
CGI	-0.02 (-0.31; 0.26) p=0.872	0.02 (-0.56; 0.52) p=0.941	0.15 (-1.01; 1.31) p=0.797	-0.02 (-0.32; 0.29) p=0.911
SDS				
Impairment at work	-0.09 (-0.52; 0.34) p=0.687	0.35 (-0.41; 1.11) p=0.362	-0.72 (-2.32; 0.89) p=0.380	-0.17 (-0.64; 0.31) p=0.486
Impairment in home relationships	0.04 (-0.33; 0.41) p=0.839	0.01 (-0.64; 0.67) p=0.967	-1.28 (-2.71; 0.14) p=0.077	0.15 (-0.26; 0.56) p=0.479
Impairment in family responsibilities	-0.17 (-0.55; 0.20) p=0.367	-0.40 (-1.07; 0.27) p=0.238	-0.48 (-1.93; 0.97) p=0.516	-0.10 (-0.52; 0.32) p=0.632
Perceived stress	-0.13 (-0.55; 0.30) p=0.554	0.21 (-0.53; 0.95) p=0.572	-0.03 (-1.65; 1.59) p=0.971	-0.26 (-0.73; 0.21) p=0.271
Perceived social support	3.34 (-0.94; 7.62) p=0.126	1.79 (-5.73; 9.31) p=0.639	12.16 (-4.26; 28.59) p=0.146	3.23 (-1.50; 7.96) p=0.180

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 15: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between PER1 gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	-0.15 (-1.92; 1.92) p=0.867	0.77 (-1.65; 3.19) p=0.533	**-5.84 (-10.83; -0.85) p=0.022**	0.55 (-2.38; 3.48) p=0.714
HAMD	-0.49 (-2.31; 1.33) p=0.597	0.32 (-2.16; 2.80) p=0.801	**-5.28 (-10.39; -0.18) p=0.043**	0.43 (-2.57; 3.42) p=0.780
GAF	-0.42 (-2.44; 1.60) p=0.684	-1.16 (-3.95; 1.63) p=0.415	2.86 (-2.89; 8.60) p=0.329	-0.33 (-3.70; 3.05) p=0.849
CGI	-0.01 (-0.33; 0.31) p=0.966	0.14 (-0.31; 0.59) p=0.540	-0.54 (-1.41; 0.33) p=0.225	-0.03 (-0.54; 0.49) p=0.919
SDS				
Impairment at work	-0.19 (-0.70; 0.32) p=0.458	0.14 (-0.58; 0.86) p=0.695	-0.69 (-2.12; 0.74) p=0.342	-0.45 (-1.27; 0.37) p=0.283
Impairment in home relationships	0.02 (-0.39; 0.43) p=0.910	0.24 (-0.32; 0.80) p=0.405	-0.70 (-1.86; 0.47) p=0.240	-0.01 (-0.70; 0.68) p=0.967
Impairment in family responsibilities	-0.14 (-0.56; 0.29) p=0.529	0.03 (-0.10; 0.16) p=0.633	-0.02 (-0.21; 0.16) p=0.812	-0.01 (-0.16; 0.13) p=0.868
Perceived stress	-0.17 (-0.65; 0.31) p=0.484	-0.04 (-0.70; 0.62) p=0.905	0.43 (-0.94; 1.79) p=0.538	-0.60 (-1.40; 0.21) p=0.144
Perceived social support	-0.98 (-5.51; 3.55) p=0.670	-3.75 (-9.98; 2.49) p=0.238	0.50 (-12.34; 13.35) p=0.939	2.41 (-5.13; 9.95) p=0.530

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 16: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between PER2 gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	-0.11 (-0.32; 0.10) p=0.288	-0.13 (-0.41; 0.15) p=0.362	-0.27 (-0.77; 0.23) p=0.292	-0.01 (-0.36; 0.34) p=0.958
HAMD	-0.12 (-0.33; 0.10) p=0.280	-0.23 (-0.51; 0.06) p=0.114	-0.21 (-0.72; 0.29) p=0.407	0.08 (-0.27; 0.43) p=0.655
GAF	0.17 (-0.07; 0.41) p=0.168	0.26 (-0.06; 0.59) p=0.115	-0.02 (-0.60; 0.56) p=0.954	0.09 (-0.31; 0.50) p=0.653
CGI	-0.03 (-0.07; 0.01) p=0.098	**-0.06 (-0.11; -0.01) p=0.018**	0.01 (-0.08; 0.09) p=0.868	0.00 (-0.06; 0.06) p=0.992
SDS				
Impairment at work	-0.02 (-0.08; 0.04) p=0.530	-0.06 (-0.15; 0.02) p=0.157	0.09 (-0.04; 0.23) p=0.160	-0.02 (-0.13; 0.08) p=0.656
Impairment in home relationships	-0.01 (-0.06; 0.04) p=0.751	-0.00 (-0.07; 0.06) p=0.941	0.06 (-0.05; 0.17) p=0.298	-0.05 (-0.13; 0.03) p=0.261
Impairment in family responsibilities	-0.01 (-0.06; 0.03) p=0.562	-0.01 (-0.07; 0.06) p=0.824	0.01 (-0.11; 0.13) p=0.863	-0.04 (-0.12; 0.04) p=0.368
Perceived stress	-0.02 (-0.07; 0.04) p=0.584	-0.00 (-0.08; 0.07) p=0.962	0.02 (-0.11; 0.16) p=0.723	-0.06 (-0.15; 0.03) p=0.219
Perceived social support	-0.18 (-0.70; 0.35) p=0.505	-0.57 (-1.27; 0.13) p=0.111	-0.41 (-1.66; 0.84) p=0.520	0.51 (-0.36; 1.38) p=0.251

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 17: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between OXTR gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	0.06 (-0.06; 0.17) p=0.330	**0.16 (0.01; 0.31) p=0.031**	-0.02 (-0.34; 0.31) p=0.919	-0.05 (-0.20; 0.11) p=0.569
HAMD	0.09 (-0.03; 0.20) p=0.130	**0.17 (0.02; 0.32) p=0.030**	-0.01 (-0.34; 0.33) p=0.974	0.03 (-0.13; 0.19) p=0.741
GAF	-0.10 (-0.23; 0.03) p=0.143	**-0.21 (-0.39; -0.04) p=0.018**	-0.16 (-0.55; 0.23) p=0.424	0.04 (-0.15; 0.23) p=0.681
CGI	0.02 (-0.00; 0.04) p=0.121	**0.03 (0.00; 0.06) p=0.023**	0.00 (-0.06; 0.06) p=0.909	0.00 (-0.03; 0.03) p=0.821
SDS				
Impairment at work	0.01 (-0.02; 0.05) p=0.398	0.01 (-0.03; 0.06) p=0.548	0.03 (-0.06; 0.12) p=0.503	0.01 (-0.04; 0.06) p=0.675
Impairment in home relationships	0.02 (-0.01; 0.04) p=0.245	**0.04 (0.00; 0.07) p=0.039**	0.03 (-0.04; 0.11) p=0.405	-0.01 (-0.05; 0.03) p=0.531
Impairment in family responsibilities	0.01 (-0.02; 0.04) p=0.570	**0.04 (-0.00; 0.07) p=0.052**	-0.00 (-0.08; 0.08) p=0.998	-0.02 (-0.06; 0.01) p=0.219
Perceived stress	-0.02 (-0.05; 0.01) p=0.304	-0.02 (-0.06; 0.02) p=0.393	0.01 (-0.08; 0.10) p=0.810	-0.03 (-0.07; 0.02) p=0.244
Perceived social support	0.08 (-0.21; 0.36) p=0.602	**0.02 (-0.37; 0.40) p=0.938**	0.44 (-0.41; 1.29) p=0.307	0.04 (-0.36; 0.45) p=0.830

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 18: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between OX1R gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	0.08 (-0.19; 0.34) p=0.569	0.10 (-0.25; 0.44) p=0.585	-0.05 (-0.75; 0.65) p=0.895	0.09 (-0.33; 0.52) p=0.661
HAMD	0.10 (-0.16; 0.37) p=0.446	0.05 (-0.30; 0.41) p=0.760	0.04 (-0.66; 0.75) p=0.901	0.19 (-0.23; 0.62) p=0.373
GAF	-0.07 (-0.37; 0.24) p=0.677	-0.06 (-0.46; 0.35) p=0.784	0.10 (-0.71; 0.92) p=0.804	-0.15 (-0.64; 0.35) p=0.561
CGI	0.00 (-0.04; 0.05) p=0.844	0.00 (-0.06; 0.06) p=0.971	0.02 (-0.09; 0.14) p=0.700	0.01 (-0.06; 0.08) p=0.827
SDS				
Impairment at work	-0.05 (-0.13; 0.03) p=0.262	-0.02 (-0.13; 0.09) p=0.723	-0.08 (-0.26; 0.10) p=0.401	-0.07 (-0.20; 0.06) p=0.271
Impairment in home relationships	-0.00 (-0.06; 0.06) p=0.918	0.02 (-0.06; 0.09) p=0.699	-0.09 (-0.25; 0.07) p=0.275	-0.00 (-0.10; 0.09) p=0.969
Impairment in family responsibilities	-0.01 (-0.07; 0.05) p=0.790	-0.01 (-0.09; 0.07) p=0.837	-0.06 (-0.23; 0.10) p=0.443	0.01 (-0.09; 0.11) p=0.842
Perceived stress	-0.06 (-0.13; 0.01) p=0.105	-0.09 (-0.18; 0.01) p=0.068	0.04 (-0.14; 0.23) p=0.647	-0.05 (-0.17; 0.06) p=0.351
Perceived social support	-0.17 (-0.83; 0.50) p=0.622	-0.05 (-0.93; 0.82) p=0.909	-0.45 (-2.21; 1.31) p=0.613	-0.27 (-1.34; 0.79) p=0.616

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 19: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between FOXP3 gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	0.40 (-0.47; 1.28) p=0.364	0.27 (-0.89; 1.44) p=0.646	-0.39 (-2.52; 1.73) p=0.716	0.76 (-0.52; 2.04) p=0.242
HAMD	0.04 (-0.85; 0.93) p=0.926	0.30 (-0.88; 1.48) p=0.619	-0.41 (-2.56; 1.74) p=0.709	-0.02 (-1.31; 1.27) p=0.976
GAF	-0.21 (-1.21; 0.79) p=0.677	-0.00 (-1.33; 1.33) p=1.000	0.19 (-2.24; 2.62) p=0.878	-0.50 (-1.96; 0.95) p=0.497
CGI	0.03 (-0.12; 0.18) p=0.668	0.00 (-0.20; 0.20) p=0.994	0.07 (-0.30; 0.44) p=0.717	0.05 (-0.16; 0.27) p=0.634
SDS				
Impairment at work	0.21 (-0.03; 0.44) p=0.087	0.23 (-0.09; 0.54) p=0.159	0.03 (-0.53; 0.58) p=0.928	0.23 (-0.11; 0.58) p=0.179
Impairment in home relationships	**0.21 (0.01; 0.41) p=0.043**	**0.33 (0.06; 0.60) p=0.015**	0.13 (-0.36; 0.61) p=0.606	0.08 (-0.21; 0.37) p=0.586
Impairment in family responsibilities	0.18 (-0.02; 0.39) p=0.083	**0.30 (0.03; 0.58) p=0.030**	-0.00 (-0.50; 0.50) p=0.994	0.10 (-0.20; 0.40) p=0.506
Perceived stress	0.14 (-0.09; 0.37) p=0.235	0.18 (-0.13; 0.49) p=0.249	0.16 (-0.40; 0.72) p=0.574	0.11 (-0.23; 0.45) p=0.536
Perceived social support	-0.03 (-2.24; 2.18) p=0.979	0.36 (-2.58; 3.30) p=0.809	-1.70 (-3.66; 7.05) p=0.534	-0.83 (-4.05; 2.39) p=0.611

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 20: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between ARNTL gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	-0.12 (-1.27; 1.03) p=0.836	-0.24 (-1.58; 1.10) p=0.728	-0.99 (-4.23; 2.25) p=0.548	1.07 (-1.85; 3.99) p=0.471
HAMD	-0.06 (-1.25; 1.13) p=0.918	0.15 (-1.22; 1.52) p=0.832	-2.48 (-5.79; 0.84) p=0.143	0.80 (-2.19; 3.80) p=0.598
GAF	-0.49 (-1.82; 0.85) p=0.474	-0.49 (-2.04; 1.06) p=0.536	1.52 (-2.24; 5.28) p=0.427	-2.03 (-5.42; 1.36) p=0.241
CGI	-0.02 (-0.24; 0.20) p=0.845	0.03 (-0.22; 0.28) p=0.809	-0.46 (-1.04; 0.13) p=0.127	0.08 (-0.44; 0.60) p=0.765
SDS				
Impairment at work	-0.23 (-0.53; 0.07) p=0.137	-0.31 (-0.65; 0.04) p=0.079	-0.11 (-0.99; 0.76) p=0.797	0.22 (-0.66; 1.10) p=0.622
Impairment in home relationships	0.10 (-0.17; 0.36) p=0.466	0.12 (-0.19; 0.42) p=0.448	-0.47 (-1.20; 0.27) p=0.216	0.44 (-0.23; 1.10) p=0.197
Impairment in family responsibilities	-0.03 (-0.30; 0.24) p=0.838	0.06 (-0.26; 0.37) p=0.727	**-0.90 (-1.66; -0.14) p=0.020**	0.26 (-0.42; 0.95) p=0.453
Perceived stress	-0.11 (-0.42; 0.20) p=0.490	-0.00 (-0.36; 0.36) p=0.993	-0.82 (-1.69; 0.05) p=0.064	-0.04 (-0.82; 0.74) p=0.916
Perceived social support	-0.83 (-3.76; 2.09) p=0.574	-2.34 (-5.73; 1.05) p=0.175	5.26 (-2.93; 13.46) p=0.208	1.78 (-5.61; 9.17) p=0.636

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

Table 21: Overall and stratified estimates by subtype of Major Depressive Disorder (MDD), with corresponding confidence intervals and p-values of the association between HERVW gene methylation and MDD severity rating scales

MDD severity scale	**β (95%CI) p-value**			
	Whole population	**MDD subtype**		
		Melancholic / psychotic / no prevalent type	**With strong symptoms of anxiety**	**Atypical**
MADRS	0.03 (-0.35; 0.42) p=0.864	0.21 (-0.32; 0.75) p=0.436	-0.04 (-0.82; 0.75) p=0.929	-0.18 (-0.80; 0.43) p=0.562
HAMD	-0.18 (-0.57; 0.20) p=0.351	-0.12 (-0.66; 0.42) p=0.666	-0.17 (-0.96; 0.63) p=0.678	-0.21 (-0.83; 0.41) p=0.507
GAF	0.07 (-0.37; 0.51) p=0.751	-0.05 (-0.66; 0.57) p=0.875	0.56 (-0.35; 1.46) p=0.225	-0.02 (-0.73; 0.69) p=0.953
CGI	0.01 (-0.06; 0.07) p=0.814	0.01 (-0.08; 0.11) p=0.762	0.01 (-0.13; 0.14) p=0.934	-0.00 (-0.11; 0.10) p=0.977
SDS				
Impairment at work	-0.03 (-0.13; 0.07) p=0.584	0.08 (-0.07; 0.22) p=0.288	-0.17 (-0.39; 0.04) p=0.114	-0.07 (-0.22; 0.09) p=0.399
Impairment in home relationships	0.01 (-0.08; 0.09) p=0.879	0.08 (-0.04; 0.20) p=0.210	-0.07 (-0.25; 0.11) p=0.429	-0.04 (-0.18; 0.10) p=0.611
Impairment in family responsibilities	0.00 (-0.09; 0.09) p=0.947	0.03 (-0.10; 0.16) p=0.633	-0.02 (-0.21; 0.16) p=0.812	-0.01 (-0.16; 0.13) p=0.868
Perceived stress	-0.03 (-0.13; 0.07) p=0.583	-0.07 (-0.22; 0.07) p=0.304	0.13 (-0.08; 0.34) p=0.228	-0.06 (-0.22; 0.10) p=0.466
Perceived social support	0.03 (-0.94; 1.00) p=0.954	0.52 (-0.83; 1.87) p=0.447	-1.10 (-3.08; 0.89) p=0.278	0.14 (-1.41; 1.69) p=0.861

Legend: MDD: Major Depressive Disorder; MADRS: Montgomery Asberg Depression Rating Scale; HAMD: Hamilton Depression Rating Scale; GAF: Global Assessment of Functioning; CGI: Clinical Global Impression; SDS: Sheehan Disability Scale; β: beta estimate; 95% CI: confidence interval at 95% level

4 DISCUSSION

To the best of my knowledge, DeprAir is the first study whose aim is to detangle the complex interplay between pollution, alterations in biological markers, and depressive symptoms. In particular, in this thesis, results are focused on the interplay between PM_{10}, $PM_{2.5}$, and NO_2 pollution, DNA methylation of selected genes, and MDD severity.

Population characteristics were in line with those described in the literature on MDD (69–71), since about two-thirds of the patients were females and about one-third had a family history of depression. In addition, a significant proportion suffered from obesity and attempted suicide at least once. As regard to gender differences, in accordance with previous studies, women reported predominantly anxiety depression (72,73), whereas the largest majority of men had a melancholic depression and they were more likely to experience suicidal and addictive behaviors (74). Moreover, on average, the subjects included in our sample had moderate current depression according to both the MADRS and HAM-D scales, and according to the CGI scale about one-third were moderately ill.

As first step, it was evaluated whether pollution by PM_{10}, $PM_{2.5}$, and NO_2 had an impact on depressive symptoms, with an in-depth evaluation of temperature. Exposure to PM_{10} and $PM_{2.5}$ did not seem to exert a direct effect on variations in the severity of depressive symptoms, although hypersusceptible subjects seemed to be more negatively affected by these pollutants. Of note, all conditions we identified to define hypersusceptibility (defined as the presence of at least one of the following: type II diabetes, obesity, hypertension, hypercholesterolemia, and current smoking) are characterized by higher baseline levels of chronic inflammation and might thus represent a flourishing soil for PMs to exert their noxious effects.

In addition, particulate matter had a greater significant impact on MDD severity when temperatures were very low (below first quartile of exposure). This finding could have several explanations. First, it could uncover the potential synergistic effects of air pollution and temperature on human health, as it has been documented for other health outcomes (75). Second, low temperatures could be considered a proxy for the winter season (notoriously associated with a higher incidence of depression (76)) as well as a surrogate measure of irradiance, thus suggesting how light could play a role in depressive symptoms (77,78). This latter interpretation is also supported by several studies showing an association between low sunlight, low temperatures and high levels of $PM_{2.5}$, deriving from the capacity of solid particles to reflect and refract sunlight (79,80). The possible role of temperature is also supported by our findings showing a decrease in MDD severity for increasing temperatures levels, and, on the other hand, a worsening of depressive symptoms during cold days.

NO_2 exposure was strongly associated with MDD severity in the whole population. Notwithstanding the absence of formal statistical interactions, also NO_2 exposure showed higher effects among hypersusceptible subjects and with concomitant exposure to low temperatures. Indeed, NO_2 can more directly affect MDD severity since, being a gas, it can cross more easily the alveolar-capillary barrier and reach the bloodstream.

Short- and long-term exposure to particulate matter resulted associated with hypermethylation of *CRY1* and hypomethylation of *CRY2*. *CRY1* and *CRY2* (82) are different from other transcriptional factors taking part in the circadian rhythms, as they have no Per-Arnt-Sim domain (83). They both operate in the retina and non-visual light detection pathways in a way that is important for the internal alignment (84,85). Of these two, *CRY2* is particularly highly expressed in the brain and has a dose-dependent inhibitory effect on the activated *ARNTL*, whereas both *CRY2* and *CRY1* repress all four combinations of the *ARNTL* (ARNTL2) – *CLOCK* (NPAS2) protein heterodimers (86). The altered expression of both genes has been associated with MDD through a possible influence on sleep deprivation (a condition predominantly present in MDD patients (87)) and a delay in circadian rhythms (81).

OX1R methylation levels were increased by short-term exposure to particulate matter. *OX1R* (orexin receptor type 1) gene encodes for one of the orexin receptors (82) which is a neuropeptide released from neurons located in the hypothalamus and prefrontal cortex; the former are associated with the regulation of reward functions, while the latter contribute to arousal functions. In addition, this hormone stimulates appetite. It is known in the literature that patients with depression exhibit reduced levels of orexin compared with normal individuals, and that treatment with antidepressants improves serum levels of this neuropeptide. Higher expression of this gene results in activation and vigilance, while a lower expression is associated with sedation as well as reduced appetite that are all typical symptoms of MDD (83).

As regard the association between the degree of *CLOCK*-related genes methylation and severity of MDD, significant results emerged for *CLOCK*, *CRY1*, *PER1* and *OXTR*. For a proper interpretation of our findings, we must recall that, most of the times, increased methylation of a given gene corresponds to a decrease in its expression, and *vice versa*. In particular, we observed that an increase in the methylation of *CLOCK* was associated with lower scores of MDD severity scales, suggesting (conversely) that the hypomethylation of *CLOCK* could be associated with a worsening of depressive symptoms. This association is consistent with preliminary data from the literature, which have highlighted an increased expression of *CLOCK* in subjects affected by MDD (1). Furthermore, knockout mice for *CLOCK* showed a lengthening of circadian rhythms (84); vice versa, other studies have highlighted how subjects suffering from MDD showed an overexpression of

CLOCK with consequent shortening of circadian rhythms, typical of the more classic forms of depression characterized by an early onset of sleep and terminal insomnia (16).

Other genes involved in circadian rhythms are *CRY1* and *PER1*, coding for proteins that dimerize and block the activity of CLOCK-BMAL, leading to a lengthening of circadian rhythms (85). For these two genes, the association between an increase in their expression and a worsening of MDD was found only for the subtype with marked anxious symptoms, in which, as a matter of fact, initial or central insomnia tends to prevail.

Another result concerns the association between *OXTR* methylation and MDD severity. *OXTR* encodes for the oxytocin receptor, a neuropeptide that plays a fundamental role in the regulation of emotions, social behavior and the HPA axis. The role of oxytocin in modulating human behavior is supported by several studies that have shown an association between low oxytocin levels and more severe depressive symptoms (86,87). Other research has suggested that the protective role of oxytocin against depression could also derive from its effect favoring a methylation profile characterized by reduced expression of *CRY1* and *CRY2* (16). In our study, we observed how an increase in *OXTR* methylation, indicative of a lower functionality of oxytocin "signaling", was associated with a global worsening of depressive symptoms among subjects with the melancholic and psychotic subtypes of depression, i.e., those forms where social behavior is more deteriorated.

The present study has several strengths. This is the first study investigating whether exposure to air pollution could be an important modifiable environmental factor associated with MDD severity and which biological mechanisms might mediate the negative effect of air pollution on mental health. Moreover, this is the first study evaluating the combined effects of air pollution and epigenetics factors on MDD severity.

However, the present study has also several limitations. First, it is a cross-sectional study, so it is not possible to establish a cause-and-effect relationship or analyze longitudinal time trends. Second, the study has a limited sample size, which, however, is an intrinsic constraint of studies examining preliminary hypotheses with collection of biological data.

5 CONCLUSIONS

The present study shows that air pollution is associated with worsening of depressive symptoms in MDD patients. In addition, the observed alterations in methylation levels in specific genes related to both air pollution exposure and to higher MDD severity suggest a possible role of *CLOCK* and *CLOCK*-related genes in the pathway linking air pollution exposure to MDD severity. The inclusion of other biological indicators, such as inflammatory and hormonal markers, could contribute to shed further light on the biological mechanisms potentially underlying this association.

6 FURTHER STEPS OF THE DEPRAIR PROJECT

Hormonal and inflammatory biomarkers will be analyzed independently and combined to assess their single and interactive or combined role on severity of Major Depressive Disorder. The role of greenness exposure and extracellular vesicles on severity of depression will also be investigated during the next year. Finally, all the results will be published in peer-reviewed journals.

7 REFERENCES

1. Gouin J-P, Connors J, Kiecolt-Glaser JK, Glaser R, Malarkey WB, Atkinson C, et al. Altered expression of circadian rhythm genes among individuals with a history of depression. J Affect Disord [Internet]. 2010 Oct;126(1–2):161–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20471092
2. Otte C, Gold SM, Penninx BW, Pariante CM, Etkin A, Fava M, et al. Major depressive disorder. Nat Rev Dis Prim [Internet]. 2016;2(September):1–21. Available from: http://dx.doi.org/10.1038/nrdp.2016.65
3. Pedersen CB, Mors O, Bertelsen A, Waltoft BL, Agerbo E, McGrath JJ, et al. A comprehensive nationwide study of the incidence rate and lifetime risk for treated mental disorders. JAMA psychiatry [Internet]. 2014 May;71(5):573–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24806211
4. Seedat S, Scott KM, Angermeyer MC, Berglund P, Bromet EJ, Brugha TS, et al. Cross-national associations between gender and mental disorders in the World Health Organization World Mental Health Surveys. Arch Gen Psychiatry [Internet]. 2009 Jul;66(7):785–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19581570
5. Albert PR. Why is depression more prevalent in women? J Psychiatry Neurosci [Internet]. 2015 Jul;40(4):219–21. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26107348
6. Vos T, Barber RM, Bell B, Bertozzi-Villa A, Biryukov S, Bolliger I, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013. Lancet. 2015;386(9995):743–800.
7. Whooley MA, Wong JM. Depression and cardiovascular disorders. Annu Rev Clin Psychol. 2013;9:327–54.
8. Dong M, Wang S-B, Li Y, Xu D-D, Ungvari GS, Ng CH, et al. Prevalence of suicidal behaviors in patients with major depressive disorder in China: A comprehensive meta-analysis. J Affect Disord [Internet]. 2018 Jan 1;225:32–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28779680
9. Dong M, Zeng L-N, Lu L, Li X-H, Ungvari GS, Ng CH, et al. Prevalence of suicide attempt in individuals with major depressive disorder: a meta-analysis of observational surveys. Psychol Med [Internet]. 2019 Jul;49(10):1691–704. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30178722
10. Chesney E, Goodwin GM, Fazel S. Risks of all-cause and suicide mortality in mental disorders: a meta-review. World Psychiatry [Internet]. 2014 Jun;13(2):153–60. Available

from: http://www.ncbi.nlm.nih.gov/pubmed/24890068

11. Flint J, Kendler KS. The genetics of major depression. Neuron [Internet]. 2014 Feb 5;81(3):484–503. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24507187
12. Lorant V, Deliège D, Eaton W, Robert A, Philippot P, Ansseau M. Socioeconomic inequalities in depression: a meta-analysis. Am J Epidemiol [Internet]. 2003 Jan 15;157(2):98–112. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12522017
13. Risch N, Herrell R, Lehner T, Liang K-Y, Eaves L, Hoh J, et al. Interaction between the serotonin transporter gene (5-HTTLPR), stressful life events, and risk of depression: a meta-analysis. JAMA [Internet]. 2009 Jun 17;301(23):2462–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19531786
14. Li M, D'Arcy C, Meng X. Maltreatment in childhood substantially increases the risk of adult depression and anxiety in prospective cohort studies: systematic review, meta-analysis, and proportional attributable fractions. Psychol Med [Internet]. 2016 Mar;46(4):717–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26708271
15. Blier P. Neurobiology of depression and mechanism of action of depression treatments. J Clin Psychiatry [Internet]. 2016 Mar;77(3):e319. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27046319
16. Iodice S, Ceresa A, Esposito CM, Mucci F, Conti DM, Pergoli L, et al. The Independent Role of Body Mass Index (BMI) and Severity of Depressive Symptoms on Biological Changes of Women Affected by Overweight/Obesity. Int J Environ Res Public Health [Internet]. 2021 Mar 12;18(6). Available from: http://www.ncbi.nlm.nih.gov/pubmed/33809270
17. Pitsillou E, Liang J, Hung A, Karagiannis TC. The circadian machinery links metabolic disorders and depression: A review of pathways, proteins and potential pharmacological interventions. Life Sci [Internet]. 2021 Jan 15;265:118809. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33249097
18. Wirz-Justice A. Biological rhythm disturbances in mood disorders. Int Clin Psychopharmacol [Internet]. 2006 Feb;21 Suppl 1:S11-5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16436934
19. Boivin DB. Influence of sleep-wake and circadian rhythm disturbances in psychiatric disorders. J Psychiatry Neurosci [Internet]. 2000 Nov;25(5):446–58. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11109296
20. Pacchierotti C, Iapichino S, Bossini L, Pieraccini F, Castrogiovanni P. Melatonin in psychiatric disorders: a review on the melatonin involvement in psychiatry. Front

Neuroendocrinol [Internet]. 2001 Jan;22(1):18–32. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11141317

21. Morin LP. Neuroanatomy of the extended circadian rhythm system. Exp Neurol [Internet]. 2013 May;243:4–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22766204
22. McCarthy MJ, Welsh DK. Cellular circadian clocks in mood disorders. J Biol Rhythms [Internet]. 2012 Oct;27(5):339–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23010657
23. Bollinger T, Schibler U. Circadian rhythms - from genes to physiology and disease. Swiss Med Wkly [Internet]. 2014;144:w13984. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25058693
24. Alegría-Torres JA, Baccarelli A, Bollati V. Epigenetics and lifestyle. Epigenomics [Internet]. 2011 Jun;3(3):267–77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22122337
25. Nader N, Chrousos GP, Kino T. Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. FASEB J [Internet]. 2009 May;23(5):1572–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19141540
26. Horowitz MA, Zunszain PA. Neuroimmune and neuroendocrine abnormalities in depression: two sides of the same coin. Ann N Y Acad Sci [Internet]. 2015 Sep;1351:68–79. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25943397
27. Maes M, Bosmans E, Suy E, Vandervorst C, DeJonckheere C, Raus J. Depression-related disturbances in mitogen-induced lymphocyte responses and interleukin-1 beta and soluble interleukin-2 receptor production. Acta Psychiatr Scand [Internet]. 1991 Oct;84(4):379–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1746291
28. Maes M, Bosmans E, Suy E, Vandervorst C, De Jonckheere C, Raus J. Immune disturbances during major depression: upregulated expression of interleukin-2 receptors. Neuropsychobiology [Internet]. 24(3):115–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2135065
29. Smith RS. The macrophage theory of depression. Med Hypotheses [Internet]. 1991 Aug;35(4):298–306. Available from: http://www.ncbi.nlm.nih.gov/pubmed/1943879
30. Maes M. Evidence for an immune response in major depression: a review and hypothesis. Prog Neuropsychopharmacol Biol Psychiatry [Internet]. 1995 Jan;19(1):11–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7708925
31. Capuron L, Raison CL, Musselman DL, Lawson DH, Nemeroff CB, Miller AH. Association of exaggerated HPA axis response to the initial injection of interferon-alpha with

development of depression during interferon-alpha therapy. Am J Psychiatry [Internet]. 2003 Jul;160(7):1342–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12832253

32. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, et al. A meta-analysis of cytokines in major depression. Biol Psychiatry [Internet]. 2010 Mar 1;67(5):446–57. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20015486
33. Gimeno D, Kivimäki M, Brunner EJ, Elovainio M, De Vogli R, Steptoe A, et al. Associations of C-reactive protein and interleukin-6 with cognitive symptoms of depression: 12-year follow-up of the Whitehall II study. Psychol Med [Internet]. 2009 Mar;39(3):413–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18533059
34. Howren MB, Lamkin DM, Suls J. Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. Psychosom Med [Internet]. 2009 Feb;71(2):171–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19188531
35. Kivimäki M, Shipley MJ, Batty GD, Hamer M, Akbaraly TN, Kumari M, et al. Long-term inflammation increases risk of common mental disorder: a cohort study. Mol Psychiatry [Internet]. 2014 Feb;19(2):149–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23568195
36. Liu Y, Ho RC-M, Mak A. Interleukin (IL)-6, tumour necrosis factor alpha (TNF-α) and soluble interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: a meta-analysis and meta-regression. J Affect Disord [Internet]. 2012 Aug;139(3):230–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21872339
37. Raison CL, Dantzer R, Kelley KW, Lawson MA, Woolwine BJ, Vogt G, et al. CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-alpha: relationship to CNS immune responses and depression. Mol Psychiatry [Internet]. 2010 Apr;15(4):393–403. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19918244
38. Valkanova V, Ebmeier KP, Allan CL. CRP, IL-6 and depression: a systematic review and meta-analysis of longitudinal studies. J Affect Disord [Internet]. 2013 Sep 25;150(3):736–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23870425
39. Capuron L, Miller AH. Immune system to brain signaling: neuropsychopharmacological implications. Pharmacol Ther [Internet]. 2011 May;130(2):226–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21334376
40. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci [Internet]. 2008 Jan;9(1):46–56. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18073775

41. Haroon E, Raison CL, Miller AH. Psychoneuroimmunology meets neuropsychopharmacology: translational implications of the impact of inflammation on behavior. Neuropsychopharmacology [Internet]. 2012 Jan;37(1):137–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21918508
42. Jones KA, Thomsen C. The role of the innate immune system in psychiatric disorders. Mol Cell Neurosci [Internet]. 2013 Mar;53:52–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23064447
43. McCusker RH, Kelley KW. Immune-neural connections: how the immune system's response to infectious agents influences behavior. J Exp Biol [Internet]. 2013 Jan 1;216(Pt 1):84–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23225871
44. Mills NT, Scott JG, Wray NR, Cohen-Woods S, Baune BT. Research review: the role of cytokines in depression in adolescents: a systematic review. J Child Psychol Psychiatry [Internet]. 2013 Aug;54(8):816–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24027786
45. Mitchell RHB, Goldstein BI. Inflammation in children and adolescents with neuropsychiatric disorders: a systematic review. J Am Acad Child Adolesc Psychiatry [Internet]. 2014 Mar;53(3):274–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24565356
46. Quan N, Banks WA. Brain-immune communication pathways. Brain Behav Immun [Internet]. 2007 Aug;21(6):727–35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17604598
47. Raedler TJ. Inflammatory mechanisms in major depressive disorder. Curr Opin Psychiatry [Internet]. 2011 Nov;24(6):519–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21897249
48. Rivest S. Regulation of innate immune responses in the brain. Nat Rev Immunol [Internet]. 2009 Jun;9(6):429–39. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19461673
49. Sher L, Oquendo MA, Burke AK, Cooper TB, Mann JJ. Combined dexamethasone suppression-corticotrophin-releasing hormone stimulation test in medication-free major depression and healthy volunteers. J Affect Disord [Internet]. 2013 Dec;151(3):1108–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23866302
50. Carniglia L, Ramírez D, Durand D, Saba J, Turati J, Caruso C, et al. Neuropeptides and Microglial Activation in Inflammation, Pain, and Neurodegenerative Diseases. Mediators Inflamm [Internet]. 2017;2017:5048616. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28154473
51. Gong P, Liang S, Carlton EJ, Jiang Q, Wu J, Wang L, et al. Urbanisation and health in

China. Lancet (London, England) [Internet]. 2012 Mar 3;379(9818):843–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22386037

52. Altug H, Hüls A, Fuks K, Suguri D, Krämer U, Schikowski T. Ambient air pollution and depressive symptoms in elderly women: Results from the salia study. Occup Environ Med [Internet]. 2018;75:A1. Available from: https://www.embase.com/search/results?subaction=viewrecord&id=L623863492&from=export

53. Kim H, Cho J, Isehunwa O, Noh J, Noh Y, Oh SS, et al. Marriage as a social tie in the relation of depressive symptoms attributable to air pollution exposure among the elderly. J Affect Disord [Internet]. 2020 Jul;272:125–31. Available from: https://www.embase.com/search/results?subaction=viewrecord&id=L2005785481&from=export

54. Petkus AJ, Younan D, Wang X, Serre M, Vizuete W, Resnick S, et al. Particulate Air Pollutants and Trajectories of Depressive Symptoms in Older Women. Am J Geriatr psychiatry Off J Am Assoc Geriatr Psychiatry. 2019 Oct;27(10):1083–96.

55. Hergenhan S, Holtkamp S, Scheiermann C. Molecular Interactions Between Components of the Circadian Clock and the Immune System. J Mol Biol [Internet]. 2020 May 29;432(12):3700–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31931006

56. Kang YJ, Tan H-Y, Lee CY, Cho H. An Air Particulate Pollutant Induces Neuroinflammation and Neurodegeneration in Human Brain Models. Adv Sci (Weinheim, Baden-Wurttemberg, Ger [Internet]. 2021 Nov;8(21):e2101251. Available from: http://www.ncbi.nlm.nih.gov/pubmed/34561961

57. Cugurra A, Mamuladze T, Rustenhoven J, Dykstra T, Beroshvili G, Greenberg ZJ, et al. Skull and vertebral bone marrow are myeloid cell reservoirs for the meninges and CNS parenchyma. Science [Internet]. 2021 Jul 23;373(6553). Available from: http://www.ncbi.nlm.nih.gov/pubmed/34083447

58. Quilty LC, Robinson JJ, Rolland J-P, Fruyt F De, Rouillon F, Bagby RM. The structure of the Montgomery-Åsberg depression rating scale over the course of treatment for depression. Int J Methods Psychiatr Res [Internet]. 2013 Sep;22(3):175–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24038301

59. Sharp R. The Hamilton Rating Scale for Depression. Occup Med (Lond) [Internet]. 2015 Jun;65(4):340. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25972613

60. Busner J, Targum SD. The clinical global impressions scale: applying a research tool in clinical practice. Psychiatry (Edgmont) [Internet]. 2007 Jul;4(7):28–37. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/20526405

61. Coles T, Coon C, DeMuro C, McLeod L, Gnanasakthy A. Psychometric evaluation of the Sheehan Disability Scale in adult patients with attention-deficit/hyperactivity disorder. Neuropsychiatr Dis Treat [Internet]. 2014;10:887–95. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24899807
62. Aas IHM. P-580 - Information basis for rating Global Assessment of Functioning (GAF). Eur Psychiatry. 2012;27:1.
63. Silibello C, Calori G, Brusasca G, Giudici A, Angelino E, Fossati G, et al. Modelling of PM10 concentrations over Milano urban area using two aerosol modules. Environ Model Softw. 2008;23(3):333–43.
64. Mircea M, Ciancarella L, Briganti G, Calori G, Cappelletti A, Cionni I, et al. Assessment of the AMS-MINNI system capabilities to simulate air quality over Italy for the calendar year 2005. Atmos Environ [Internet]. 2014;84(September 2016):178–88. Available from: http://dx.doi.org/10.1016/j.atmosenv.2013.11.006
65. Bessagnet B, Pirovano G, Mircea M, Cuvelier C, Aulinger A, Calori G, et al. Presentation of the EURODELTA III intercomparison exercise-evaluation of the chemistry transport models' performance on criteria pollutants and joint analysis with meteorology. Vol. 16, Atmospheric Chemistry and Physics. 2016. 12667–12701 p.
66. Kukkonen J, Olsson T, Schultz DM, Baklanov A, Klein T, Miranda AI, et al. A review of operational, regional-scale, chemical weather forecasting models in Europe. Atmos Chem Phys. 2012;12(1):1–87.
67. Brokamp C, Brandt EB, Ryan PH. Assessing exposure to outdoor air pollution for epidemiological studies: Model-based and personal sampling strategies. J Allergy Clin Immunol [Internet]. 2019 Jun;143(6):2002–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31063735
68. Cantone L, Tobaldini E, Favero C, Albetti B, Sacco RM, Torgano G, et al. Particulate Air Pollution, Clock Gene Methylation, and Stroke: Effects on Stroke Severity and Disability. Int J Mol Sci [Internet]. 2020 Apr 27;21(9). Available from: http://www.ncbi.nlm.nih.gov/pubmed/32349365
69. Fava M, Kendler KS. Major depressive disorder. Neuron [Internet]. 2000 Nov;28(2):335–41. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11144343
70. Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: review and meta-analysis. Am J Psychiatry [Internet]. 2000 Oct;157(10):1552–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11007705

71. Kendler KS, Gatz M, Gardner CO, Pedersen NL. A Swedish national twin study of lifetime major depression. Am J Psychiatry [Internet]. 2006 Jan;163(1):109–14. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16390897
72. Vetter JS, Spiller TR, Cathomas F, Robinaugh D, Brühl A, Boeker H, et al. Sex differences in depressive symptoms and their networks in a treatment-seeking population - a cross-sectional study. J Affect Disord [Internet]. 2021 Jan 1;278:357–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33002727
73. Cavanagh A, Wilson CJ, Kavanagh DJ, Caputi P. Differences in the Expression of Symptoms in Men Versus Women with Depression: A Systematic Review and Meta-analysis. Harv Rev Psychiatry [Internet]. 2017;25(1):29–38. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28059934
74. Shi P, Yang A, Zhao Q, Chen Z, Ren X, Dai Q. A Hypothesis of Gender Differences in Self-Reporting Symptom of Depression: Implications to Solve Under-Diagnosis and Under-Treatment of Depression in Males. Front psychiatry [Internet]. 2021;12:589687. Available from: http://www.ncbi.nlm.nih.gov/pubmed/34759845
75. Carugno M, Consonni D, Randi G, Catelan D, Grisotto L, Bertazzi PA, et al. Air pollution exposure, cause-specific deaths and hospitalizations in a highly polluted Italian region. Environ Res [Internet]. 2016 May;147:415–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26969808
76. Henríquez-Sánchez P, Doreste-Alonso J, Martínez-González MA, Bes-Rastrollo M, Gea A, Sánchez-Villegas A. Geographical and climatic factors and depression risk in the SUN project. Eur J Public Health. 2014;24(4):626–31.
77. Golden RN, Gaynes BN, Ekstrom RD, Hamer RM, Jacobsen FM, Suppes T, et al. The efficacy of light therapy in the treatment of mood disorders: a review and meta-analysis of the evidence. Am J Psychiatry [Internet]. 2005 Apr;162(4):656–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15800134
78. Karrouri R, Hammani Z, Benjelloun R, Otheman Y. Major depressive disorder: Validated treatments and future challenges. World J Clin cases [Internet]. 2021 Nov 6;9(31):9350–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/34877271
79. Chen T, He J, Lu X, She J, Guan Z. Spatial and Temporal Variations of PM2.5 and Its Relation to Meteorological Factors in the Urban Area of Nanjing, China. Int J Environ Res Public Health [Internet]. 2016 Sep 16;13(9). Available from: http://www.ncbi.nlm.nih.gov/pubmed/27649227
80. Chong W, Lyu W, Zhang J, Liang J, Yang X, Zhang G. Effects of Air Pollution on Sunshine

Duration Trends in Typical Chinese Cities. Atmosphere (Basel). 2022;13(6):1–14.

81. Buoli M, Grassi S, Iodice S, Carnevali GS, Esposito CM, Tarantini L, et al. The role of clock genes in perinatal depression: the light in the darkness. Acta Psychiatr Scand [Internet]. 2019 Oct;140(4):382–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31400146
82. Wang C, Wang Q, Ji B, Pan Y, Xu C, Cheng B, et al. The Orexin/Receptor System: Molecular Mechanism and Therapeutic Potential for Neurological Diseases. Front Mol Neurosci [Internet]. 2018;11:220. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30002617
83. Al-Kuraishy HM, Abdulhadi MH, Hussien NR, Al-Niemi MS, Rasheed HA, Al-Gareeb AI. Involvement of orexinergic system in psychiatric and neurodegenerative disorders: A scoping review. Brain Circ [Internet]. 2020;6(2):70–80. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33033776
84. Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, et al. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science [Internet]. 1994 Apr 29;264(5159):719–25. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8171325
85. Benazzi S, Gorini S, Feraco A, Caprio M. Ritmi circadiani e variabili metaboliche. L'Endocrinologo [Internet]. 2021;22(6):533–43. Available from: http://dx.doi.org/10.1007/s40619-021-00983-5
86. Moura D, Canavarro MC, Figueiredo-Braga M. Oxytocin and depression in the perinatal period—a systematic review. Arch Womens Ment Health [Internet]. 2016 Aug 13;19(4):561–70. Available from: http://link.springer.com/10.1007/s00737-016-0643-3
87. Serati M, Grassi S, Redaelli M, Pergoli L, Cantone L, La Vecchia A, et al. Is There an Association Between Oxytocin Levels in Plasma and Pregnant Women's Mental Health? J Am Psychiatr Nurses Assoc [Internet]. 2021 May 29;27(3):222–30. Available from: http://journals.sagepub.com/doi/10.1177/1078390319890400

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