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Prenatal exposure to PM_{10} and changes in DNA methylation and telomere length in cord blood

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ABSTRACT

Background: Air pollution exposure in pregnancy can cause molecular level alterations that might influence later disease susceptibility.
Objectives: We investigated DNA methylation (DNAm) and telomere length (TL) in the cord blood in relation to gestational PM_{10} exposure and explored potential gestational windows of susceptibility.
Methods: Cord blood epigenome-wide DNAm (N = 384) and TL (N = 500) were measured in children of the Italian birth cohort Piccolipiù, using the Infinium Methylation EPIC BeadChip and qPCR, respectively. PM_{10} daily exposure levels, based on maternal residential address, were estimated for different gestational periods using models based on satellite data. Epigenome-wide analysis to identify differentially methylated probes (DMPs) and regions (DMRs) was conducted, followed by a pathway analysis and replication analysis in an second Piccolipiù dataset. Distributed lag models (DLMs) using weekly exposures were used to study the association of PM_{10} exposure across pregnancy with telomere length, as well as with the DMPs that showed robust associations.
Results: Gestational PM_{10} exposure was associated with the DNA methylation of more than 250 unique DMPs, most of them identified in early gestation, and 1 DMR. Out of 151 DMPs available in the replication dataset, ten DMPs showed robust associations: eight were associated with exposure during early gestation and 2 with exposure during the whole pregnancy. These exposure windows were supported by the DLM analysis. The PM_{10} exposure between 15th and 20th gestational week seem to be associated with shorter telomeres at birth, while exposure between 24th and 29th was associated with longer telomeres.
Discussion: The early pregnancy period is a potential critical window during which PM_{10} exposure can influence cord blood DNA methylation. The results from the TL analysis were consistent with previous findings and merit further exploration in future studies. The study underlines the importance of considering gestational windows outside of the predefined trimesters that may not always overlap with biologically relevant windows of exposure.

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1. Introduction

Air pollution exposure during pregnancy can cause molecular level changes that might activate (mal)adaptive fatal responses and influence cellular regulation of biological and genetic processes (Alvarado-Cruz et al., 2018; Entringer et al., 2018; Martin and Fry, 2018; Ferrari et al., 2019; Luyten et al., 2018). Several studies have reported an association between exposure to ambient air pollutants during pregnancy and molecular signatures in newborns, such as changes in DNA methylation and telomere length (TL) (Isaevska et al., 2021). These findings are of relevance due to the ubiquitous presence of ambient air pollutants (World Health Organization, 2016) and, considering that DNA methylation and TL both undergo fetal programming (Entringer et al., 2018; Stevenson et al., 2020), the potential effect of adverse intrauterine exposures across the lifespan (Gluckman et al., 2008).

DNA methylation typically represents the addition of methyl-groups to a cytosine base followed by guanine (CpGs) and is involved in regulation of gene expression, development, and response to stressors (Dor and Cedar, 2018). Telomeres are repetitive nucleotide sequences at the end of chromosomes that ensure complete chromosomal replication and progressively shorten with each cell division (Blackburn et al., 2015).

DNA methylation and TL have common characteristics that make them suitable for studying prenatal exposures, as well as future health trajectories. First, both DNA methylation and TL are known to be shaped by genetics and environmental exposures, including air pollution, the latter generally believed to act through mechanisms of oxidative stress and inflammation (Singh et al., 2019; Menezo et al., 2016). Second, considering the inter-individual stability and the clear mechanism of post-mitotic inheritance, changes in newborn DNA methylation and TL caused by air pollution might be retained and measured long time after the exposure took place, making them a potential biomarkers of prenatal exposure. In the same time, changes in DNA methylation patterns and TL are important for studying and understanding the mechanisms behind air pollution-related health effects, due to their involvement in many cellular processes and their established association with life-course health trajectories (Nwanaji-Enwerem and Colicino, 2020; Fasching, 2018). Furthermore, in a recent study cord blood methylation was found to be involved in the initial setting of the TL and, to a lesser extent, in TL attrition rate in early life, implying that factors influencing early life methylation patterns may also influence the baseline telomere setting (Wang et al., 2021).

Almost all methylation patterns in the early embryo are erased in order to ensure totipotency, followed by a wave of *de novo* methylation that occurs around the time of implantation in a process called "epigenetic reprogramming". This process allows DNA methylation to coordinate cell differentiation through regulation of gene expression, sex chromosome dosage compensation, expression of imprinted genes and repression of retrotransposons ensuring genomic stability (Dor and Cedar, 2018;Cedar and Bergman, 2012;Reizel et al., 2018). Although the process is less studied, it is believed that telomeres also undergo fetal programming. Following fertilization, the embryonic cells undergo dramatic telomere lengthening to support embryonal growth. The variability of newborn TL indicates that heritability and the *in utero* environment are most likely the primary determinants of TL during adulthood (Hjelmborg et al., 2015). Furthermore, the stable and fixed TL ranking from birth onwards also points toward the importance of studying factors that might influence the TL setting before birth (Martens et al., 2021; Benetos et al., 2013). For these reasons, the pregnancy, and in particular the beginning of the pregnancy, may represent a biologically relevant window of exposure for both DNA methylation and TL.

Previous studies on air pollutants and DNA methylation averaged pollutants across pregnancy or studied trimester specific exposures, while some studies on air pollution and TL also analyzed weekly exposures with inconsistent results regarding sensitive windows of exposure. Therefore, our objective was to study changes in cord blood DNA methylation and TL in relation to maternal exposure to PM_{10} (particulate matter <10 μm) in different time windows during pregnancy, with particular focus on the beginning of the pregnancy, that includes the time around conception and implantation – a period during which epigenetic and telomere reprogramming are believed to take place (Cedar and Bergman, 2012). By studying two molecular markers sensitive to environmental exposures in cord blood, we aimed to capture cellular DNA responses to prenatal PM_{10} exposure, which would help elucidating the mechanisms behind their involvement in future disease risk trajectories.

2. Methods

1. Study population

Piccolipiù is a multicentric Italian birth cohort set up to investigate the effects of exposures acting during pre-natal and early post-natal life on infant and child health (Farchi et al., 2014). Between 2011 and 2015, around 3300 mother-child pairs were recruited in five Italian centers (Turin, Trieste, Florence, Viareggio and Rome). Pregnant women with singleton pregnancy were eligible for inclusion if they were at least 18 years old, were scheduled to give birth in one of the selected hospitals participating in the study, had residence in the catchment area of the maternity center, ability to fill out the informed consent and the questionnaire in Italian and had a telephone number to be reached at. Ethical approvals were obtained from the Ethics committees of the Local Health Unit Roma E (management center), of the Istituto Superiore di Sanità (National Institute of Public Health) and of each local center. All parents provided written informed consent. At recruitment, women completed a baseline questionnaire including information on several sociodemographic, lifestyle, environmental and medical factors. For each newborn, whole blood was withdrawn from cord vessels and collected in a tube with EDTA (Ethylene Diamine Tetra Acetic acid), fractionated in buffy coat, plasma and erythrocytes and stored in a bio-bank at −80 °C.

2. Air pollution exposure assessment

Daily PM_{10} concentrations were estimated at 1-km^2 grid using the Random Forest (RF) method, as described in details elsewhere (Stafoggia et al., 2019). Briefly, the RF method combined ground-level PM_{10} measurement with finely resolved data on Aerosol Optical Depth (AOD) from the Multi Angle Implementation of Atmospheric Correction (MAIAC) algorithm, land-use variables and meteorology indicators. The models showed good predictive performance (R^2~0.95) with small prediction errors (root mean squared prediction error ~0.02) and negligible bias (intercepts = 0 and slopes ~ 1). Predictions were equally good in capturing annual and daily PM_{10} variability.

We used daily data to obtain reliable exposure estimates within different gestational windows of exposure. The pregnancy duration (in gestational weeks and days, as estimated by last menstrual period or ultrasound) was taken from the mother's obstetric medical record. We obtained the mean PM_{10} exposure levels for each gestational week (1–40 weeks), first trimester (1–13 gestational week), second trimester (14–26 gestational week), third trimester (27 gestational week-delivery), first month of pregnancy (first 30 days of pregnancy - counting from the first day of the first gestational week) and last month of pregnancy (30 days before delivery) and whole pregnancy. Since gestational age is not based on the date of conception (typically difficult to estimate both in clinical practice and in large population-based studies), but rather on the beginning of the woman's last menstrual period (or less commonly on fetal size when gestational age is based on ultrasound measurements), we defined as "the beginning of pregnancy" the period spanning from gestational weeks 2–5. We hypothesized that this period could be of particular interest as it covers the period around conception and implantation. Although ovulation timing is expected to occur around the 14th day in an average 28-day menstrual cycle, it can shift as early as the

second gestational week and as late as the third (with consequent implantation after 8–10 days) in women with shorter or longer menstrual cycle (21–35 days) (Grieger and Norman, 2020).

3. Molecular analyses

3.1. Selection of samples for molecular analyses

The DNA methylation analyses included a set of 384 children of the Piccolipiù cohort, that were selected from each center from children that satisfied the following criteria: extracted DNA with sufficient quality and quantity, mother with georeferenced address at enrollment, European origin (defined as having 4 grandparents born in Europe) and a complete follow up of at least 24 months. A total of 500 children (that also included the 384 children in the DNA methylation analyses) were included in the TL analysis. DNA was extracted using the kit QIAsymphony DSP DNA (Qiagen, Hilden, Germany). Twenty-five samples from the Turin center (included in the TL analyses only) had DNA extracted within the framework of the EXPOsOMICS project (Vineis et al., 2017) by using the QIAamp 96 DNA Blood Kit (Qiagen, Hilden, Germany). Details regarding the sample type collection, storage, extraction and DNA quality metrics of the extracted DNA can be found in the Supplementary material (eMethods S1).

3.2. DNA methylation analyses

To assess DNA methylation levels, we used the Infinium MethylationEPIC BeadChip Kit (Illumina Inc., San Diego, CA, USA), (IlluminaEPIC) that targets over 850,000 methylation sites across the genome at single-nucleotide resolution (Pidsley et al., 2016). To control for possible batch effect (Price and Robinson, 2018), we randomized the samples by study center and newborn's sex. After quality control and filtering (eMethods S1) 370 samples and 768856 CpGs remained for further analysis. Methylation levels were expressed as beta values, ranging from 0 to 1, that represent the ratio of the intensity of the methylated-probe signal to the total locus signal intensity, methylated and unmethylated. To minimize the impact of influential data points, for each probe we removed the outliers (if present) that were defined as methylation values three times smaller or greater than the inter-quantile range.

For cell type deconvulsion we used the filtered reference dataset "FlowSorted.CordBloodCombined.450k" (Gervin et al., 2019) and using the function *estimateCellCounts2* from the *FlowSorted.Blood.EPIC* R-package (Salas et al., 2018) according to the authors' instructions. We estimated cord blood proportions of 7 cell types: CD8 T cells, CD4 T cells, Natural Killer cells, B cells, monocytes, granulocytes and nucleated red blood cells.

3.3. Telomere length

The average relative TL was measured using the monochrome multiplex quantitative PCR (MMQPCR) (protocol modified from Cawthon (2009) for detailed protocol see eMethods S3. Each sample was run in triplicate on Rotor-Gene Q (Qiagen, Hilden, Germany). All samples were randomized by sex and study center. On each run, a five-point serial dilution of pooled DNA was run to assess the PCR efficiency and same pooled DNA was used as inter-run calibrator. Negative controls without a sample were included in each run. We assessed TL by each child by calculating the mean relative T/S ratio using the method $2^{-\Delta\Delta Ct}$, that represents the ratio of telomere repeat copy number (T) to single copy gene (S) copy number, relative to average T/S ratio of the entire sample set. The intra-assay interclass correlation coefficient (ICC) was 0.964 (0.962–0.972), 0.950 (0.942–0.956) and 0.964 (0.958–0.969) for CtT, CtA and T/S ratio, respectively. The inter-assay ICC based on the 10 repeated samples was 0.987 (0.952–0.997), while the extrapolated repeatability was 0.996.

3.4. Statistical analysis

The analysis plan is presented in Fig. 1. We examined the associations between exposure to PM_{10} during the exposure windows of interest (2nd, 3rd, 4th and 5th gestational week, first and last month of pregnancy, each trimester and the whole pregnancy period) and epigenome-wide methylation levels to discover differentially methylated probes (DMPs or "top CpGs") and differentially methylated regions (DMRs). We used robust linear regression to identify DMPs (using the R packages *MASS* and *sandwich*) and the R package *DMRcate* (Peters et al., 2015) to identify DMRs by calling the following specifications: a DMR should contain $\geq$2 CpGs and lambda set to 1000. Benjamini-Hochberg's false discovery rate (FDR) was used to adjust for multiple testing in both analyses. The FDR threshold was set to 0.05.

When DMPs or a DMRs were identified when analyzing a specific exposure window of interest, a formal gene set enrichment analysis was conducted by using the functions *GOmeth/GOregion* from the *missMetyl* (de Goede et al., 2017) package and performing gene set testing using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The enrichment analysis took into account two main sources of bias of methylation data: the number of CpGs per gene (genes with more CpG sites are more likely to be flagged as DMPs) and the fact that approximately 10% of gene-annotated CpGs are assigned to more than one gene thus violating assumptions of independently measured genes. The enrichment analysis was performed using only CpGs/DMRs that passed the FDR threshold (when the number was larger than 10) as well as a sensitivity analysis using all CpG sites with an adjusted p-value $<$0.0001. The FDR threshold was set to 0.05.

We performed replication of the top findings (DMPs and CpGs mapped to DMRs) in an independent Piccolipiù dataset. DNA methylation in the replication dataset was measured within the context of the EU-funded EXPOsOMICS project using Infinium HumanMethylation 450 BeadChip (Illumina Inc., San Diego, USA), while we used the same methods to estimate PM_{10} concentrations and conduct quality, control, normalization and filtering of the raw methylation data as in the discovery dataset (eMethods S4).

For the replicated CpGs we performed look-up analysis in both datasets (discovery and replication) using distributed lag-models (DLM) that include both the exposure-response relationship and the lag-response relationship in the same model by creating a cross basis function. We used the week-specific estimates extracted from the DLM analysis for four reasons: i) to jointly model the effect of temporal autocorrelated lagged exposures ii) to add flexibility in identifying vulnerable exposure windows, iii) to assess whether the effect of PM_{10} on a DMP identified in a narrow window of exposure (ex. individual gestational week) is confined to the discovery window or the effect persists outside of the window of interest (for DMPs identified in larger exposure windows,ex. the entire pregnancy, the estimates could be driven by strong effects of exposures in smaller windows) and iv) to assess the robustness of the association using a different statistical approach than the discovery analysis (DLMs allow to remove random noise from week-specific estimates). Since the DLM models do not allow missing values in the exposure matrix and the duration of gestation varied across pregnancies, ranging from 32 to 42 gestational weeks, we studied exposures from week 1 to week 32 to avoid exclusion of prematurely born children. For DNA methylation analysis we repeated the DLM analysis using daily data from weeks 2–5. We used basis cubic spline with 2 internal and 2 boundary knots for the DNA methylation analysis that allow a flexible modelling of the lagged structure avoiding random noise that might be present in analyzing independent week-specific estimates. In the analysis restricted to weeks 2–5, we used basis cubic spline with 1 internal knot.

As part of the methylation analysis, we also studied the association between 6 candidate CpGs found to be differentially methylated in relation to PM_{10} exposure in a previous meta-analysis (Gruzieva et al., 2019) to see if they showed similar strength and direction of association

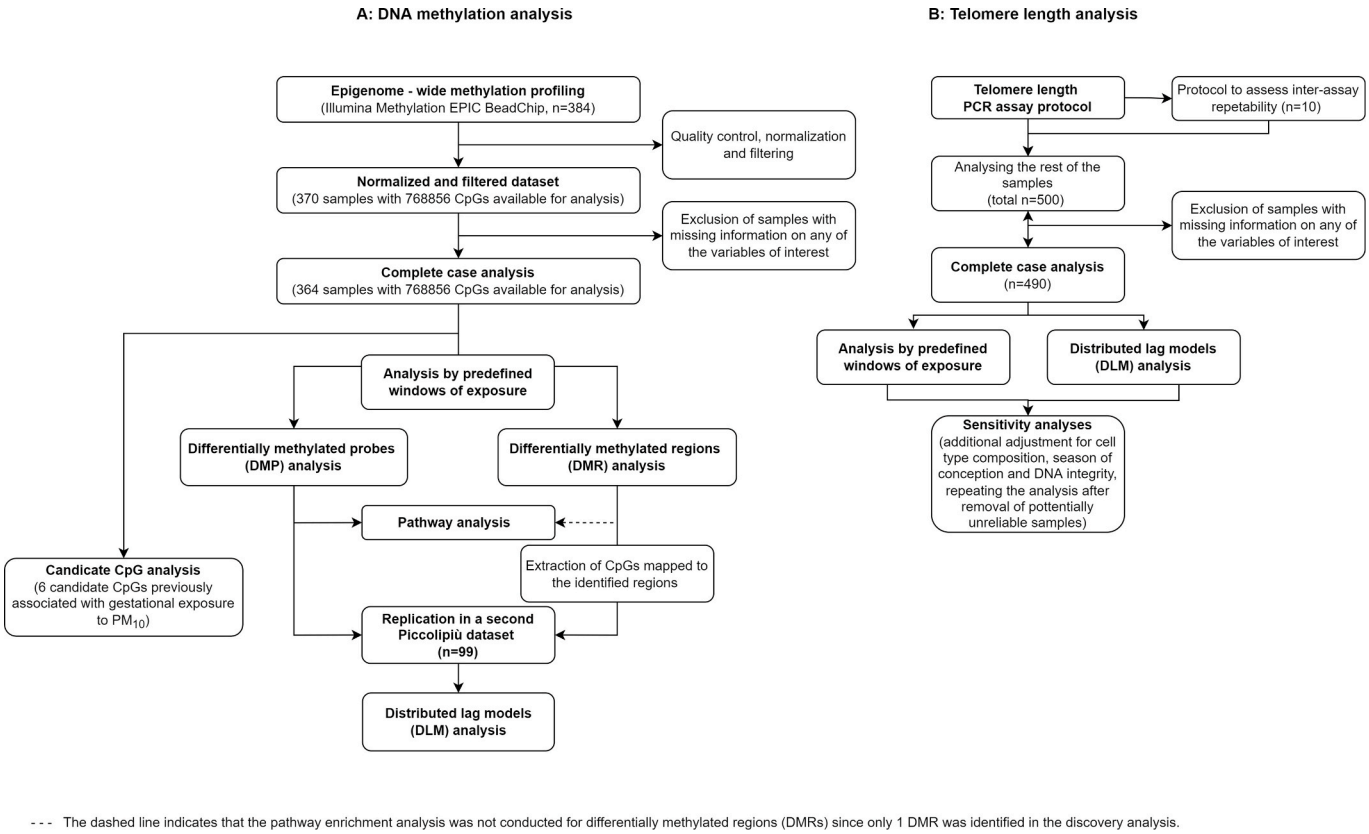

Fig. 1. Flow chart describing the analysis plan.

in our main dataset.

In the TL analysis, we used both predefined windows of exposure and lag-distributed models with cubic spline with 2 boundary and two internal knots. For comparison purposes with previous studies on TL, we conducted additional TL sensitivity analyses restricting the population to children born with completed 39 weeks of gestation instead of 40 weeks (used in previous studies) (Martens et al., 2017) since this analysis would exclude only 11% of the children, in comparison to 29% when using weeks 1–40.

All analyses were adjusted for an a priori selected set of covariates: technical variables (plate and DNA extraction time/buffer), study center, maternal age (continuous), education (high-tertiary education, medium-upper secondary education, low-lower secondary or less), parity (nulliparous vs multiparous), pre-pregnancy BMI (continuous), smoking during pregnancy (yes/no) and child's sex. The methylation analyses were additionally adjusted for cell heterogeneity. We decided not to adjust for gestational duration and pregnancy complications since they may lay on the causal pathway between the exposure and the outcome. We also did not adjust for season of conception, which strongly affects the exposure of interest, with weak or null expected effects on DNA methylation. As sensitivity analyses for TL we further adjusted for season of conception and DNA integrity of the cord blood sample, variables that might be relevant for TL. We also conducted sensitivity analysis adjusting for cell type composition (estimated based on methylation data) and removing potentially unreliable samples (samples with coefficient of variation within triplicates >20)

After excluding samples with missing data on any of the covariates, the final number of children included in the main methylation and TL analysis was 365 and 490, respectively. The replication analysis was performed on 92 children from Turin and was adjusted for the same set of confounders.

Finally, to assess the possible inter-relatedness between DNA methylation and TL we assessed the correlation between DMRs/DMPs with TL and we looked if the identified DMPs/DMRs were among the CpG/genes known to be involved in TL setting or attrition (Wang et al., 2021).

4. Results

The characteristics of the study population are described in Table 1 and eTableS 1. The mean (standard deviation) PM_{10} levels through the course of the pregnancy were 32.2 μg/m^3 (10.1) and 32.2 μg/m^3 (10.0) in the newborns included in the methylation and TL analyses respectively, Table 1. In both analyses, for around 97% of children, the average maternal exposure during the whole pregnancy exceeded 20 μg/m^3 and for 17% of the children mean exposure levels during the pregnancy were above 40 μg/m^3. The smaller the exposure windows (weeks, month, trimester, whole pregnancy), the more variable were the PM_{10} levels (eFigure S2 and eTable S1) due to the number of days from which the average concentration was estimated. PM_{10} concentrations from gestational weeks within each trimester correlated well, while the correlation between first trimester and mid and late-pregnancy PM_{10} concentrations was low (eFigure S3).

1. DNA methylation analyses

PM_{10} exposure levels during the second, third, fourth and fifth gestational weeks, the first and last month of pregnancy, the first and third trimester and the last month of pregnancy were associated with DNA methylation in 158, 26, 49, 2, 12, 14, 3, 25 and 19 CpGs, respectively, amounting to 284 unique CpGs, Fig. 2. No CpGs were associated with PM_{10} exposure during the second trimester (eFigure S4). The lambda values for each window of exposure can be found in the eTable S3. Nineteen CpGs were identified in more than one exposure window (eTableS 4), with consistent direction and strength of association, except for one CpG (cg10938586) which association with PM_{10}

Table 1
Characteristics of the participants included in the complete-case analyses.

	DNA methylation (n = 365)		Telomere length (n = 490)	
	N	**Mean (SD) or %**	**N**	**Mean (SD) or %**
Study center				
Florence	89	24.38%	97	19.80%
Rome	20	5.48%	99	20.20%
Trieste	94	25.75%	97	19.80%
Turin	71	19.45%	98	20.00%
Viareggio	91	24.93%	99	20.20%
Maternal age	365	34.63 (4.67)	490	34.74 (4.70)
Maternal education				
High	178	48.77%	250	51.02%
Medium	150	41.10%	190	38.78%
Low	37	10.14%	50	10.20%
Maternal pre-pregnancy BMI (in kg/m^2)	365	22.36 (3.50)	490	22.37 (3.49)
Parity				
Nulliparous	216	59.18%	301	61.43%
Multiparous	149	40.82%	189	38.57%
Smoking in pregnancy				
No	292	80.00%	379	77.35%
Yes	73	20.00%	111	22.65%
Child's sex				
Female	194	53.15%	258	52.65%
Male	171	46.85%	232	47.35%
PM_{10} concentrations (in μg/m^3)				
Whole pregnancy		32.15 (10.07)		32.21 (9.98)
Second gestational week		28.66 (15.30)		29.82 (15.90)
Third gestational week		29.25 (15.09)		29.97 (15.21)
Fourth gestational week		29.72 (17.02)		30.45 (17.12)
Fifth gestational week		29.64 (16.13)		30.20 (16.86)
First month of pregnancy		29.35 (14.50)		30.24 (14.76)
First trimester		30.17 (14.34)		30.44 (13.96)
Second trimester		32.86 (14.81)		32.76 (14.49)
Third trimester		33.37 (14.65)		33.41 (14.09)
Last month of pregnancy		31.83 (16.20)		32.18 (16.38)
Gestational age		39.77 (1.40)		39.77 (1.39)
Season of birth				
Winter	105	28.77%		151
Spring	145	39.73%		175
Summer	71	19.45%		93
Autumn	44	12.05%		71
Pregnancy complications*				
No	294	80.55%	406	82.86%
Yes	71	19.45%	84	17.14

The smaller number of samples from the Rome center in the DNA methylation analysis was due to small number of samples with extracted DNA at the time when methylation analyses were conducted (eMethodsS1). *Includes gestational diabetes, intra-uterine growth restriction, (pre)eclampsia, low birth weight (<2500g) and preterm birth (gestational age<37), for more details see eTableS 2.

showed opposite directions in the first and the third trimester. Thirty-eight unique CpGs were mapped to gene promoters (Supplementary material 2). Only one DMR (not mapped to any known gene) located on the chromosome 6 (chr6:30095136-30095295) was associated with PM_{10} exposure, in particular with exposure during the second gestational week (see Table 2).

The pathway enrichment analysis using the CpGs that passed the FDR threshold in the discovery analysis, revealed no pathways associated with a p-value < 0.05 after adjustment for multiple comparisons. The lowest p-value was found for the KEGG pathway "insulin resistance" when analysing CpGs associated with PM_{10} exposure during the whole pregnancy (FDR 0.056), eTableS 5. The sensitivity analysis taking all CpGs with unadjusted p-value<0.0001 did not reveal pathways with a p-value < 0.05 (data not shown).

The replication analysis in the second Piccolipiù dataset was possible for 151 out of 284 unique CpGs (which was expected, since the replication dataset was analyzed with an array that has half of the targeted probes than the array used in the discovery dataset), Supplementary material 2. Of them, ten had p-values<0.1 (6 had p-value<0.05) and showed consistent direction of association. Eight out of the ten CpGs were associated with PM_{10} exposure during the second gestational week and two CpGs were associated with exposure during the whole pregnancy. The 10 CpGs were mapped to 5 genes *ITPR1*, *NPAS1*, *TMEM105*, *PRKCD* and *RPS27L*. The first gene (*PRKCD*) acts both as a tumor suppressor and as a regulator of cell cycle progression and apoptosis. The second one (*RPS27L*) is also involved in apoptotic processes probably via the regulation of the tumor suppressor gene p53. The other three genes have roles is ER stress-induced apoptosis (*ITPR1*); roles during late embryogenesis and postnatal development (*NPAS1*), and the gene *TMEM105* is an RNA gene previously associated with maternal nutrition. The 12 CpGs mapping to the DMR at chromosome 6 did not show consistent strength and direction of association in the replication dataset. All findings from the DMP and DLM analysis, including both the discovery and the replication analysis can be found in Supplementary material 2.

The DLM graphs from the discovery dataset, modeling CpGs identified in the second gestational week, also identified the beginning of the pregnancy as window of vulnerability, with strongest effects seen in the second gestational week. For half of them strong effect was also seen in the third gestational week, eFigure S5. On the other hand, the CpGs associated with PM_{10} during the whole pregnancy showed generally consistent direction of association throughout the pregnancy. The sensitivity analysis using daily exposures from days 8–35 showed consistent results with the weekly DLM analysis, eFigure S6. The DLM graphs from the replication dataset showed smaller effect sizes and wider confidence intervals, although the shape of the graph remained consistent, eFigure S7.

None of the identified DMPs/DMR was among the CpGs involved in TL maintenance or attrition (Wang et al., 2021). The DMPs were not strongly correlated with TL, including the top 10 replicated CpGs, eFigure S9.

We conducted the candidate CpG analysis on 4 out of 6 PM_{10}-related CpGs from the previously published meta-analysis that were available in our study. All 4 CpGs had weak/null associations and none had the same direction as in the original study eTable S8.

2. Telomere length analyses

There was no strong association between PM_{10} exposure in any of the predefined windows of interest and TL. The lack of strong association was confirmed by the following sensitivity analyses: removing potentially unreliable samples, adjusting for cell heterogeneity, DNA integrity and season of conception, eTableS 9. In the main DLM analysis (1–32 weeks), however, PM_{10} exposure between the 15th–20th gestational week was associated with shorter telomeres, while exposures between the 24th–29th week of gestation were associated with longer telomeres, Fig. 3. This association remained robust in the sensitivity analyses (data not shown). However, the association attenuated when extending the analyses to children that completed 39 weeks of gestation.

5. Discussion

The results provided by our analysis indicate that pregnancy, and particularly the early pregnancy period, represent a window of vulnerability during which PM_{10} exposure can influence DNA methylation

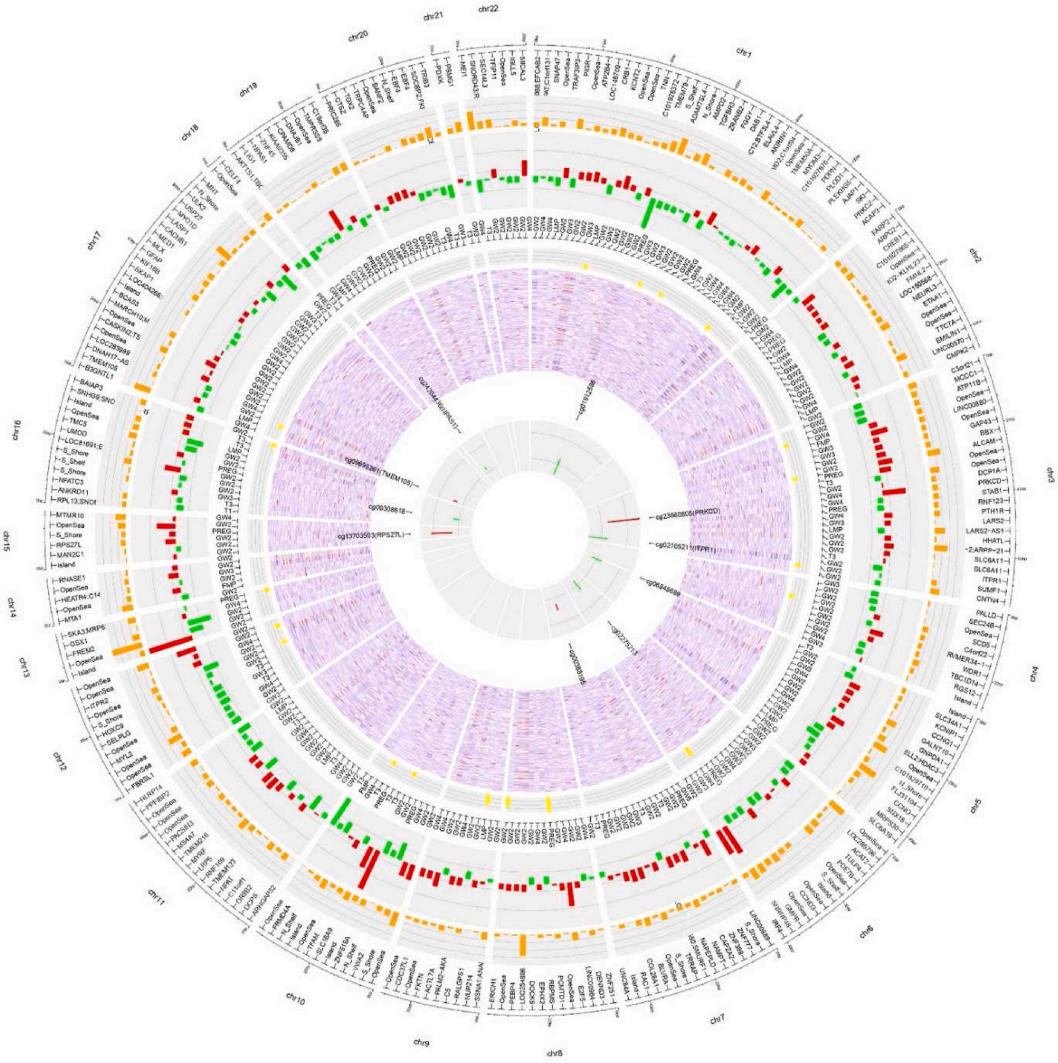

Fig. 2. Circos plot presenting the main results from the epigenome-wide differentially methylated probe (DMP) analysis. The Circos plot is organized in tracks. The first track shows 284 unique DMPs ordered according to genomic location, and labels them with the name of the gene they are mapped to or their genomic location in relation to a nearby CpG island (Island, Shore, Shelf and Open Sea) in cases when a DMP was not mapped to a specific gene. The –log 10 of the unadjusted p-values and the beta coefficients are plotted on the second and the third track, respectively. Hypermethylated DMPs are colored in red and hypomethylated in green. The fourth track shows the window of exposure where the DMPs was identified (in cases when the same DMP was identified in multiple windows the beta coefficient and the p-value were taken from the window with smallest unadjusted p-value). The fifth track annotates the CpG identified in multiple exposure windows. The sixth track is a correlation matrix. The seventh track annotates the DMPs replicated in a second Piccolipiù dataset and plots the beta coefficients from the replication analysis (hypermethylated DMPs are colored in red and hypomethylated in green). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Prenatal PM_{10} exposure and differentially methylated regions (DMRs) in cord blood.

Window of exposure	Region coordinates	N of CpGs	Coefmean	FDRmin
Gestational week 2				
Unmapped to gene	chr6:30095136-30095295	12	0.0044	7.49e-12

The coefficient represents mean DNA methylation change per 10μg/m3 increase in PM_{10}. The FDR value represents the FDR value for the CpG most strongly associated with the outcome.

patterns. TL was inversely associated with exposures in 15th-20th week of pregnancy and positively associated with exposures around the 25th week. These findings have biological plausibility and relevance, due to

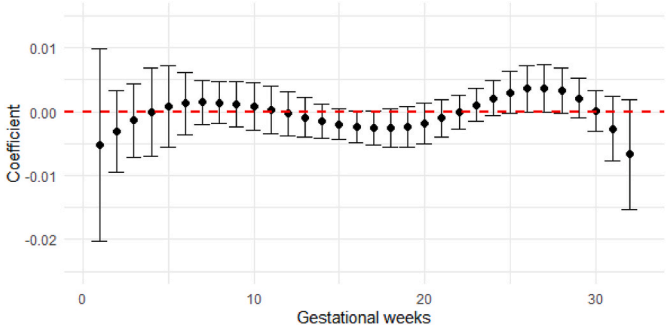

Fig. 3. Prenatal PM_{10} exposure and telomere length in cord blood. Week-specific (1–32 week) estimates and 95% confidence intervals from the lag distributed models analysis provided as a change in mean relative telomere length for a 10-unit increment in PM_{10} air pollution exposure.

the importance of the identified timing of exposure and the functional role of the identified DMPs, DMRs and TL.

Although there were DMPs associated with exposures during the whole pregnancy and late pregnancy (third trimester and last month of pregnancy), the majority of the DMPs identified in this study were associated with PM_{10} exposures in early gestation (weeks 2–4, with 50% of the DMPs identified in the periconceptional period, in particular week 2). Environmental influences in the period around conception period are known to influence epigenetic patterns. The oocyte is responsible for series of epigenetic modifications of the genome just prior and immediately after fertilization (Dean et al., 2003). The fertilization triggers genome wide loss in methylation, erasing almost all parental methylation patterns (paternal genome gets de-methylated quickly and actively, while the maternal genome is de-methylated passively). Then, at the time of implantation (that in most pregnancies occurs a week after fertilization), the entire genome undergoes a dramatic wave of *de novo* methylation with most loci becoming highly methylated, while CpG islands in genes important for totipotency and embryonic development remain unmethylated, creating a typical bimodal pattern. Post implantation, changes on a smaller scale occur, initiating cell differentiation (Grieger and Norman, 2020; Vineis et al., 2017). Since epigenetic marks tend to become fixed once the cells differentiate and are known to be maintained in subsequent cell divisions, changes in DNA methylation in response to environmental exposures in the period around conception and implantation have the potential to remain stable over the course of time and influence embryonal development and future health trajectories (Steegers-Theunissen et al., 2013).

In fact, DNA methylation is considered one of the possible mediators that link prenatal exposure to air pollution to newborns and childhood health outcomes, including low birth weight, as well as respiratory, cardio-metabolic and neurodevelopmental outcomes (Fossati et al., 2020; Vrijheid et al., 2016). In our study, the pathway "insulin resistance" was enriched among CpGs associated with PM_{10} exposure during the entire pregnancy. Furthermore, several of the replicated CpGs were mapped to genes previously associated with adverse exposures and/or birth outcomes. One of the replicated CpGs was mapped to a gene *ITPR1* whose methylation levels according to EWAS atlas (Li et al., 2019) were previously associated with prenatal arsenic (Rojas et al., 2015),prenatal phthalate exposure (Solomon et al., 2017) and NO_2 exposure in adults (Lichtenfels et al., 2018), as well as with preterm birth (de Goede et al., 2017), gestational diabetes (Weng et al., 2018) and maternal pre-pregnancy BMI (Sharp et al., 2017). The methylation of the *NPAS* gene, involved in embryogenesis and postnatal development, in one study was indicated as one of the mediators between prenatal smoking and birthweight (Hannon et al., 2019) and was associated with exposure to polybrominated biphenyl in adults (Curtis et al., 2019). The identified DMR at chromosome 6 (with unknown regulatory function) was also positively associated with third trimester lead exposure in a previous study (Rygiel et al., 2020). Three of the replicated CpGs were mapped to genes with roles in stress induced apoptosis: *ITPR1*, *PRKCD* and *RPS27L*, further suggesting DNA methylation involvement in responses linked to oxidative stress. In, addition, the CpGs cg23660805 (*PRKCD*) and cg13703503 (*RPS27L*) were mapped to their respective gene promotors and were hypermethylated in relation to PM_{10} exposure in pregnancy which is usually synonymous of decreased gene expression. According to GeneCards, *PRKCD* acts as tumor-suppressor in response to oxidative stress triggering DNA damage-induced apoptosis and has roles is B cell signaling and regulation of growth and cell differentiation, while activation of *RPS27L* also induces apoptosis by activating the tumor suppressor *p53*.

There was some evidence that air pollution exposure during pregnancy could influence telomere length. Although the findings were not conclusive when looking at children born at term with completed 39 weeks of gestation, our study suggests that the effect of PM_{10} on telomere length may be, at least partially, related to prematurity. Most of the studies regarding gestational exposure to air pollution and telomere length report overall inverse association between the two, but findings differ regarding the direction of association across different gestational windows (Song et al., 2019; Rosa et al., 2019; Martens et al., 2017; Nie et al., 2019). The plotted week-specific estimates from the DLM analysis revealed an M-shape graph similar to the one reported by Martens and colleagues (Martens et al., 2017). Both graphs show an inverse association between PM_{10} during mid-gestation: weeks 12–25 in the study by Martens and colleagues (Martens et al., 2017), and weeks 15–20 in our analysis. In both studies there is an indication that exposures later in the pregnancy might lead to longer telomeres, however this association is seen between 24th and 29th week in our study and between 30th and 35th week in the study by Martens and colleagues. It is known that disruption in the telomere length maintenance system leading to either short or long telomeres, conveys some sort of risk. Shorter telomeres increase the risk for many age-related diseases, while longer telomeres increase the risk for some types of cancer (Blackburn et al., 2015; Aviv and Shay, 2018) which calls for further analyses assessing the effect air pollution on telomere length during pregnancy.

The biological mechanisms that explain how air pollutants, and environmental exposures in general, influence locus-specific CpG methylation and TL is largely unknown. It is generally believed that they include both changes in genomic DNA sequences and non-genotoxic mechanisms. Excessive oxidative stress has a damaging effect on DNA, mainly due to the predisposition of the guanine base (G) to oxidation. Both CpG islands and TL are found within G-rich locations of the genome. Guanine base oxidation may interfere with the processes of DNA methylation at CpG islands by promoting DNA demethylation and increased gene expression (Menezo et al., 2016), while the presence of oxidized guanosine in the tandem DNA telomere repeats (TTAGGGn) impairs telomere maintenance, leading to telomere shortening (Singh et al., 2019). Air pollutants can increase or inhibit the activity of transcription factors as a mechanism of cellular defense. Their binding to the promoter may inhibit the activity the methyltransferases (resulting in hypomethylation) or allow their access leading to hypermethylation (Martin and Fry, 2016). Other mechanisms include reducing the availability of the substrate used for methylation (methionine) used in detoxification (Martin and Fry, 2016). DNA methylation may also interfere with the TL setting though methylation, although in our study no DMP/DMR were among those involved in TL maintenance and attrition (Wang et al., 2021), and the identified DMPs were not strongly correlated with TL.

Although there seems to be an abundant number of studies that report locus-specific changes in DNA methylation in relation to prenatal exposure to air pollution (Gruzieva et al., 2017, 2019; Breton et al., 2016; Plusquin et al., 2018; Ladd-Acosta et al., 2019; Abraham et al., 2018), epigenome-wide studies often suffer from lack of robust associations at single CpG level across cohorts (Pekkanen and Pearce, 2001; Breton et al., 2017). Even in the present study, we did not replicate findings for 4 PM_{10}-related CpGs emerging from a previous meta-analysis including 1949 children (Gruzieva et al., 2019). There may be several reasons for this lack of replication, such as misclassification of exposure, differences in laboratory conditions and other batch variability uncaptured by the variables chosen for adjustment, different pre-processing pipelines, different methods for estimating cell types, as well as a different setting-specific confounding patterns not accounted for in the analyses. However, in this study, we replicated, in an second Piccolipiù dataset, the association between PM_{10} exposure and DNA methylation at 10 CpGs. This could be due to fact that in our replication dataset we addressed some of the issues underlined previously, such as using the same method to estimate PM_{10} concentrations, running the pre-processing and filtering pipeline on raw data, estimating cell counts with the same method and adjusting for the same confounding factors. However, it does not indicate that the signal will be stable across different populations. The main and the replication dataset come from the same study population and might have similar confounding patterns. Future studies should replicate these findings in other cohorts paying

attention on specific windows of exposure during pregnancy.

Our study has several strengths. We measured two molecular markers, that capture different biological responses to cellular stress in a cohort exposed to high mean concentrations of PM_{10} with a wide interquartile range. We used daily PM_{10} exposure data based on machine learning method to obtain exposure estimates within smaller gestational windows of exposure and we reported the beginning of the pregnancy as potential window of vulnerability. In addition, we were able to test our top findings in another subsample of the same cohort and reported robust associations for 10 CpGs. We supported our finding of the beginning of the pregnancy as the most vulnerable period for PM_{10} exposure using a different statistical approach that includes, in the same model, the exposure-response relationship and the lag-response relationship. However, some limitations must be acknowledged. While we were able to reliably estimate air pollution exposure based on residential address, these estimates do not necessarily reflect the true individual exposure, and time-activity patterns might introduce exposure misclassification. Secondly, we studied only exposure to PM_{10} since daily exposure predictions for other pollutants, such as $PM_{2.5}$, were not available for the entire study period. Third, we relied on exposures during the gestational weeks 2–5 to capture the period around conception and implantation because we did not want to base our analyses on estimates calculated around an assumed time of fertilization, since the exact date of ovulation/conception is difficult to establish due to differences in menstrual cycle length (Grieger and Norman, 2020). Nevertheless, the relevance on the 8 replicated CpGs identified in the second gestational week (that reflects peri-conceptional exposures) was also supported by the DLM analysis. Fourth, we were not able to test all DMRs in the replication dataset due to the smaller number of probes targeted on the array used in the replication dataset, including DMPs identified in multiple exposure windows that might have been of particular relevance. Fifth, while we carefully chose the set of confounders included in the models, there is a possibility for residual confounding. Finally, we were not able to assess the stability of the identified epigenetic changes in later childhood.

Exposure to PM_{10} during the beginning of the pregnancy - a period of extensive epigenetic reprogramming, was associated with altered methylation in more than 250 DMPs and one DMR. Ten DMPs, which have relevant functions in response to oxidative stress and apoptosis, showed strong and robust associations in the replication dataset. The results from the TL analysis were consistent with previous findings and merit further exploration in future studies. The study underlines the importance of considering gestational windows outside of the predefined trimesters that may not always overlap with biologically relevant windows of exposure.

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Ethical approval

The study is based on data from the multicentric Italian birth cohort Piccolipiù set up to investigate the effects of exposures acting during prenatal and early post-natal life on infant and child health. Ethical approvals were obtained from the Ethics committees of the Local Health Unit Roma E (management center), of the Istituto Superiore di Sanità (National Institute of Public Health) and of each local center. Pregnant women with singleton pregnancy were eligible for inclusion if they were at least 18 years old, were scheduled to give birth in one of the selected hospitals participating in the study, had residence in the catchment area of the maternity center, ability to fill out the informed consent in Italian and had a telephone number to be reached at. All parents included in the study provided written informed consent at recruitment.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.112717.

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