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Prenatal particulate air pollution exposure and expression of the miR-17/92 cluster in cord blood: Findings from the ENVIRONAGE birth cohort

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ABSTRACT

Background: Air pollution exposure during pregnancy is an important environmental health issue. Epigenetics mediate the effects of prenatal exposure and could increase disease predisposition in later life. The oncogenic miR-17/92 cluster is involved in normal development and disease.

Objectives: Here, for the first time the potential prenatal effects of particulate matter with a diameter < 2.5 μm ($PM_{2.5}$) exposure on expression of the miR-17/92 cluster in cord blood are explored.

Methods: In 370 mother-newborn pairs from the ENVIRONAGE birth cohort, expression of three members of the miR-17/92 cluster was measured in cord blood by qRT-PCR. Expression of *C-MYC* and *CDKN1A*, a cluster activator and a target gene, respectively, was also analyzed. Multivariable linear regression models were used to associate the relative m(i)RNA expression with prenatal $PM_{2.5}$ exposure.

Results: $PM_{2.5}$ exposure averaged (10^{th}-90^{th} percentile) 11.7 (9.0–14.4) μg/m^3 over the entire pregnancy. In cord blood, miR-17 and miR-20a showed a −45.0% (95%CI: −55.9 to −31.4, $p < 0.0001$) and a –33.7% (95%CI: −46.9 to −17.2, $p = 0.0003$), decrease in expression in association with *first* trimester $PM_{2.5}$ exposure, and a –32.5% (95%CI: −45.6 to −16.3, $p = 0.0004$) and –23.3% (95%CI: −38.1 to −4.8, $p = 0.02$), respectively, decrease in expression in association with $PM_{2.5}$ exposure during the *entire* pregnancy. In association with *third* trimester $PM_{2.5}$ exposure, a reduction of −25.8% (95%CI: −40.2 to −8.0, $p = 0.007$) and −14.2% (95%CI: −27.7 to 1.9, $p = 0.08$), for miR-20a and miR-92a expression, respectively, was identified. Only miR-92a expression (-15.7%, 95%CI: −27.3 to −2.4, $p = 0.02$) was associated with $PM_{2.5}$ exposure during the *last month* of pregnancy. *C-MYC* expression was downregulated in cord blood in association with prenatal $PM_{2.5}$ exposure during the *first* trimester and the *entire* pregnancy, in the adjusted model.

Discussion: Lower expression levels of the miR-17/92 cluster in cord blood in association with increased prenatal $PM_{2.5}$ exposure were observed. Whether this oncogenic microRNA cluster plays a role in *trans*-placental carcinogenesis remains to be elucidated.

1. Introduction

Particulate air pollution is an important environmental health problem contributing to the increased burden of disease worldwide. One of its major components, particulate matter (PM) has been classified as carcinogenic to humans (Group 1) according to the International Agency for Research on Cancer (IARC). (Loomis and G. Y., Lauby-Secretan B, , 2013) The adverse effects of PM are more pronounced on vulnerable population groups such as pregnant women and neonates, (Parker, 2011) affecting both maternal and fetal health. (van den Hooven, 2012) In numerous epidemiological studies, prenatal PM exposure has been associated with neonatal outcomes including preterm births (Chang et al., 2011) and decreased newborn birth weight. (Fleischer, 2014) Recently, whole-genome microarray analysis in cord blood revealed altered gene expression in response to short- and long-term $PM_{2.5}$ exposure prior to birth. (Winckelmans, 2017) It has been

Abbreviations: $PM_{2.5}$, particulate matter with a diameter < 2.5 μm; miRNA, microRNA; *C-MYC*, cellular MYC; *CDKN1A*, cyclin-dependent kinase inhibitor 1A; BMI, body mass index; CI, confidence interval; IQR, Interquartile range

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suggested that *in utero* transcriptomic changes in response to environmental toxicants may predispose the fetus to increased disease susceptibility in later life. (Perera and Herbstman, 2011)

MiRNAs can fine-tune gene expression at the post-transcriptional level and they exert key roles in many biological processes in human development and disease. The miR-17/92 cluster is a well-studied miRNA cluster implicated in normal development, diseases (cardiovascular, immune, neurodegenerative, cancer) and aging. (Mogilyansky and Rigoutsos, 2013) This cluster encodes for six individual miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a). The members of this cluster act as oncogenes (Concepcion et al., 2012) and have an important function in several processes such as cell cycle regulation, proliferation, differentiation, angiogenesis, (Ncbi Resource Coordinators, 2017) apoptosis (Mogilyansky and Rigoutsos, 2013) and tumorigenesis. (Xiang and Wu, 2010) Overexpression of this cluster has been observed in a variety of human cancers, (Conkrite, 2011; Hayashita, 2005) whereas loss of its function has been linked to postnatal death and developmental defects in mice. (Ventura, 2008)

Little is known about the effects of *in utero* exposure to environmental stressors on the expression of the miR-17/92 cluster. Here, we investigate the potential alterations in the expression of the miR-17/92 cluster in cord blood in association with prenatal exposure to particulate matter. These oncogenic miRNAs might provide some more biological insights on the underlying mechanisms in early life in response to particulate air pollution exposure during pregnancy. We furthermore analyze the expression of *C-MYC*, a proto-oncogene known to induce expression of the miR-17/92 cluster (Tao et al., 2014) and a downstream target of the miR-17/92 cluster, cyclin-dependent kinase inhibitor type 1A (*CDKN1A*) which plays an important role in cell cycle progression (Deng, 2014) and inhibition of apoptosis. (Rodriguez and Meuth, 2006)

2. Material and methods

2.1. Study population

Participating mothers in the ongoing birth cohort ENVIR*ON*AGE (ENVIRonmental influence *ON* AGEing), (Janssen et al.,) were recruited at the hospital prior to delivery. The recruitment protocols are approved by the Ethics Committees of the Hasselt University and the South-East-Limburg hospital (ZOL), based on the declaration of Helsinki. After delivery, all participating mothers provided written informed consent and filled in study questionnaires. Detailed information on maternal age, parity, educational status, occupation, smoking status, alcohol consumption, place of residence, use of medication, newborn's ethnicity and paternal age were obtained. Smoking status was classified into three groups: *non-smokers* those who never smoked, *current-smokers* those who continue smoking during pregnancy and *past-smokers* those who quit smoking before pregnancy. Educational status was categorized into three levels: *low-level* with no or primary school diploma, *middle-level* with high school diploma and *high-level* with college or university diploma. Based on the origin (native country) of the newborn's grandparents, the ethnicity of the newborn was classified as *European-Caucasian* when he/she had more than two European grandparents and as *non-European* when the newborn had at least three non-European grandparents. Lastly, the birth weight, pregnancy maternal body mass index (BMI), gestational weight gain, method of delivery and data on health complications were provided by the hospital archives in which all these data were recorded. Our study population (n = 370) was recruited between February 2014 and December 2016.

2.2. Particulate air pollution exposure

Based on the residential address of the participating mothers, the average daily $PM_{2.5}$ exposure was measured. For this purpose we used a spatial temporal interpolation method combining land cover data obtained by satellite images for interpolating the pollutant concentrations measured by fixed-monitoring stations (n = 34) in combination with a dispersion model using emissions from line and point sources. (Lefebvre, 2013; Lefebvre, 2011) The performance of this model was evaluated by leave-one-out cross validation. In Flanders (Belgium), the validation statistics of this interpolation model gave a spatiotemporal explained variance of more than 80% for $PM_{2.5}$ exposure. (Lefebvre, 2011) The accuracy of the model has been demonstrated by showing that modelled $PM_{2.5}$ and black carbon (BC) at residence correlates with internal exposure to nano-sized BC particles measured in urine (Saenen et al., 2017) and in placental tissue. (Bové, 2019) In our study, different $PM_{2.5}$ exposure windows were used to investigate the association with m(i)RNA expression: trimester-specific time windows [*first trimester* (date of conception until 13th week), *second trimester* (14th until 26th week) and *third trimester* (27th week until delivery)], the *entire period* of pregnancy (date of conception until delivery) and the *last month* of pregnancy (30 days prior to delivery). If the participants moved during pregnancy (11%, n = 41), we also calculated the exposure based on these changes. The mean daily outdoor temperature was provided by the Belgian Royal Meteorological Institute (Brussels, Belgium), and was based on the mother's residential address.

2.3. Sample collection and RNA isolation

Umbilical cord blood was collected immediately after delivery in PAXgene Blood RNA tubes (Preanalytix GmbH, Feldbachstrasse, Switzerland). The PAXgene tubes were first stabilized for 24 h at room temperature and then stored at −20 °C for a longer time, based on the manufacturer's instructions.

Total RNA and miRNA were isolated from the whole blood stabilized in PAXgene Blood RNA tube using the PAXgene Blood miRNA isolation kit (Preanalytix GmbH, Feldbachstrasse, Switzerland) according to the manufacturer's protocol. RNA samples were quantified using the NanoDrop ND-1000 spectrophotometer (Isogen, Life Science, Belgium). RNA quality control was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). The extracted total RNA had an average (± SD) yield of 29.9 (± 15.7) μg with average $A_{260/280}$ ratio of 2.08 (± 0.04) and average $A_{260/230}$ ratio of 2.01 (± 0.15), and average (± SD) RIN of 9.1 (± 0.5). Samples were stored at − 80 °C for further processing.

2.4. Reverse transcription and relative miRNA expression analysis

Total RNA (10 ng) was reverse transcribed with TaqMan® Advanced miRNA cDNA Synthesis kit according to manufacturer's instructions. The cDNA was synthesized by 3′ poly(A) tailing and 5′ ligation of an adaptor sequence to extend the miRNA at each end prior to reverse transcription. CDNA was further amplified (pre-amplification step) using universal primers and TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA), in order to maximize the detection of miRNAs with low levels of expression. TaqMan Advanced miRNA assays were used to measure each miRNA target by RT-qPCR, using 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). For PCR, the standard thermal cycling conditions, including 1 cycle 95 °C for 20 s (enzyme activation), followed by 40 cycles at 95 °C for 3 s (denaturation) and at 60 °C for 30 s (annealing/extension), were used. In order to minimize the technical variation between the different runs of the same miRNA assay, inter-run calibrators (IRCs) were applied. An exogenous spike-in control (cel-miR-55) was used for normalization as recommended by the manufacturer. Amplification efficiencies of measured miRNA assays, including hsa-miR-17-5p (*Assay ID: 478447_mir*), hsa-miR-20a-5p (*Assay ID: 478586_mir*), hsa-miR-92a-5p (*Assay ID: 477827_mir*), and exogenous control, cel-miR-55-3p (*Assay ID: 478295_mir)*, were between 92 and 112% for all assays. The relative miRNA expression was calculated by $2^{-\Delta\Delta Cq}$ method using qBase plus software (Biogazelle, Belgium). All samples were analyzed in

triplicates, which were included only if their ΔCq was smaller than 0.5. Around 95% of the samples had a ΔCq < 0.5 in triplicates, while 5% of the samples had a ΔCq < 0.5 based on duplicates. We achieved coefficients of variation (CVs) of 6.9%, 1.4% and 1.7% for hsa-miR-17, hsa-miR-20a and hsa-miR-92a, respectively, across all measured samples. The other members of the miR-17/92 cluster, hsa-miR-18a and hsa-miR-19a, were not analyzed due to their very low expression levels in this tissue, while hsa-miR-19b was not analyzed in this study.

2.5. Target-miRNA relative expression analysis by qRT-PCR

Activator of the pathway *C-MYC* and downstream target *CDKN1A* gene expression were analyzed by qRT-PCR. First, total RNA (1.5 μg) was reverse transcribed to cDNA using the GoScript Reverse Transcription System (Promega, Madison, WI, USA) using a thermal cycler (TC-5000;Techne, Burlington, NJ, USA). The resulting cDNA was stored at –20 °C for downstream applications.

Samples were analyzed with a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) applying standard cycling conditions, as described previously, according to the manufacturer's protocol. Synthesized cDNA (10 ng), TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA) and PrimeTimeTM assay (Integrated DNA Technologies, Coralville, IA, USA) were used in PCR reactions. The target genes, *C-MYC (primer assay: Hs.PT.58.39142481, RefSeq number: NM_002467)* and *CDKN1A* (*primer assay: HS.PT.58.40874346.g, RefSeq number: NM_000389*) and the reference genes, *IPO8 (primer assay: Hs.PT.56a.40532361, RefSeq number: NM_001190995)*, *CYC1* (*primer assay: Hs.PT.56a.20696349.gs, RefSeq number: NM_001916)* and *TBP* (*primer assay: Hs.PT.56a.20792004, RefSeq number: NM_001172085)* were measured. Using SDS 2.3, the quantification cycle (*Cq*) values were provided and further processed to normalized relative gene expression values with qBase plus (Biogazelle, Zwijnaarde, Belgium). Inter-run calibrators (IRCs) were used as previously mentioned. Replicates (three per sample) were included when the difference in Cq values was < 0.5. The coefficient of variation (CV) for both *C-MYC* and *CDKN1A* measurements across all samples was 0.4%.

2.6. Statistical analysis

The relative m(i)RNA expression of miR-17, miR-20a and miR-92a and *C-MYC* and *CDKN1A* were log-transformed (log10) in order to increase the normality of their distribution. We used SAS software (Version 9.4, SAS Institute, NC, USA) for the statistical analysis. To explore the association of m(i)RNA expression with maternal exposure to particulate air pollution, based on the different $PM_{2.5}$ exposure windows, unadjusted and multivariable adjusted linear regression models were performed. The latter were corrected for an *a priori* selected set of covariates including newborn's sex, ethnicity (*European of Caucasian origin* or *non-European*), season and date of delivery, birth weight, gestational age, and maternal age, smoking behavior (*never*, *past* or *current smokers*), pre-pregnancy BMI, educational status (*low-*, *middle-* and *high-level*), parity (*first*, *second* or *higher-order birth*), and white blood cell counts. As previously suggested, (Wilson, 2017) in case of exploring the effects of $PM_{2.5}$ trimester-specific exposure on m(i)RNA expression, the models were corrected for other trimester specific exposures, as well. In these multi-trimester exposure models, no collinearity issues were observed, as the exposure between trimester were not strongly correlated with correlation coefficients (r) ranging from −0.23 to 0.11 (**Supplementary Table S1**). The estimated effects are presented as percentage (%) change in relative miRNA expression in cord blood per interquartile range (IQR) increment in $PM_{2.5}$ exposure windows, with 95% confidence intervals (CI). Significance level (*P*-value) was set at 5%. In *sensitivity analysis*, we explored the aforementioned associations by excluding from the main study population newborns with non-European origin (n = 53). Pearson correlation coefficients were calculated to assess the correlation between the measured miRNAs from the miR-17/92 cluster amongst each other as well as with the cluster activator *C-MYC* and the downstream target *CDKN1A*.

Table 1
General characteristics of the study population and particulate air exposure (n = 370), compared with those of all participants in the ENVIR*ON*AGE birth cohort enlisted between 2010 and 2018 (n = 1613).

Characteristic	Mean ± SD / Frequency (%)	
Maternal	Current study (n = 370)	ENVIR*ON*AGE cohort (n = 1613)
Age, *years*	29.7 ± 4.5	29.6 ± 4.5
Pre-pregnancy BMI, *kg/m²*	24.8 ± 4.9	24.6 ± 4.7
Smoking status		
Never-smoker	243 (65.7)	1050 (65.1)
Past-smoker	88 (23.8)	369 (22.9)
Current- smoker	39 (10.5)	194 (12.0)
Parity		
1	186 (50.3)	858 (53.2)
2	132 (35.7)	560 (34.7)
≥3	52 (14.0)	195 (12.1)
Education		
Low	41 (11.1)	180 (11.2)
Middle	140 (37.8)	578 (35.8)
High	189 (51.1)	855 (53.0)
Newborn		
Gestational age, *weeks*	39.2 ± 1.5	39.2 ± 1.6
Sex, *girls*	172 (46.5)	789 (48.9)
Birth weight, *g*	3,412 ± 466	3,398 ± 487
Ethnicity		
European-Caucasian	317 (85.7)	1418 (87.9)
Non-European	53 (14.3)	195 (12.1)
Seasonality at delivery		
Winter	77 (20.8)	379 (23.5)
Spring	98 (26.5)	422 (26.2)
Summer	81 (21.9)	365 (22.6)
Fall	114 (30.8)	447 (27.7)

3. Results

3.1. Study population

In Table 1 all characteristics of the mother-newborn pairs (n = 370) are indicated. Briefly, the mothers had an average (± SD) age of 29.7 (± 4.5) years and pre-gestational BMI of 24.8 (± 4.9) kg/m^2. Most of the mothers never smoked (65.7%), had a high educational level (51.1%) and had their first child (50.3%). The newborns' average gestational age was 39.2 (± 1.5) weeks and birth weight was 3,412 (± 466) g. Almost half of the newborns (46.5%) were girls, while the majority of them (85.7%) were from European or Caucasian origin. Column 2 shows the same characteristics for the entire ENVIR*ON*AGE cohort (n = 1613) recruited from February 2010 until December 2018, showing that our study population is representative for the entire cohort. The exposure data of different time windows (*trimester-specific* during pregnancy, *entire period* of pregnancy and *last month* before delivery) of $PM_{2.5}$ air pollution, during pregnancy or prior to delivery, are given in Table 2.

3.2. Associations of particulate air pollution with the miR-17/92 cluster

The distributions of the measured relative miRNA and mRNA expressions (log10-transformed) in cord blood are given in **Supplementary Figure S1**. Both unadjusted (**Supplementary Figure S2**) and adjusted analysis (Fig. 1) indicate that expression of miR-17, miR-20a and miR-92a in cord blood was inversely associated with $PM_{2.5}$ exposure during pregnancy.

Table 2
$PM_{2.5}$ exposure data (μg/m^3) across different time windows during pregnancy. Exposure data presented as mean $PM_{2.5}$ exposure with 10^{th} and 90^{th} percentiles and as median $PM_{2.5}$ exposure with interquartile range (IQR).

Time windows	Mean (10^{th} – 90^{th} perc.)	Median (IQR)
First trimester	11.9 (7.9–16.5)	11.3 (4.6)
Second trimester	11.5 (7.3–16.8)	10.4 (5.2)
Third trimester	11.7 (7.6–16.8)	10.9 (5.0)
Last month	11.7 (6.9–18.8)	9.9 (6.9)
Entire period of pregnancy	11.7 (9.0–14.4)	11.5 (3.0)

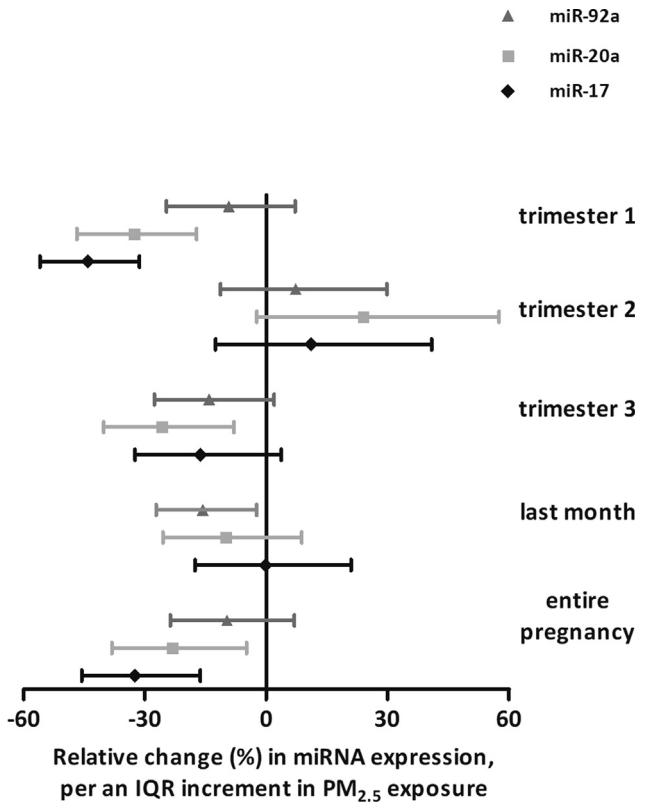

Fig. 1. Change (%) in relative miRNA expression for an IQR increment in the investigated $PM_{2.5}$ exposure windows, in adjusted analysis. The estimated effects are presented with 95% CI. Black line = miR-17, light grey with square = miR-20a, dark grey with triangle = miR-92a.

In the adjusted models, the expression of miR-17 (-45.0%, 95%CI: −55.9 to −31.4, $p < 0.0001$, and –32.5%, 95%CI: −45.6 to −16.3, $p = 0.0004$) and miR-20a (–33.7%, 95%CI: −46.9 to −17.2, $p = 0.0003$, and –23.3%, 95%CI: −38.1 to −4.8, $p = 0.016$) in cord blood was negatively associated with $PM_{2.5}$ exposure during the *first trimester* and the *entire* pregnancy, for an IQR increment in $PM_{2.5}$ exposure. While the expression of miR-20a (-25.8%, 95%CI: −40.2 to −8.0, $p = 0.007$) and miR-92a (-14.2%, 95%CI: −27.7 to 1.9, $p = 0.08$) in cord blood was inversely associated with an IQR increment in *third trimester* $PM_{2.5}$ exposure. Similarly, an IQR increase in *last month* of pregnancy $PM_{2.5}$ exposure was associated with a decrease in cord blood miR-92a expression (-15.7%, 95%CI: −27.3 to −2.4, $p = 0.023$). In Fig. 1, all estimated effects of the adjusted analysis for the different $PM_{2.5}$ exposure windows on the relative expression of miR-17, miR-20a and miR-92a in cord blood, are displayed. The estimates are presented as % change (95% CI) in relative miRNA expression in cord blood for an IQR increase in $PM_{2.5}$ exposure, adjusted for newborns' sex, ethnicity, season and date of delivery, birth weight, gestational age, and maternal age, smoking behavior, maternal pre-gestational BMI, education level of the mother, parity and white blood cell counts.

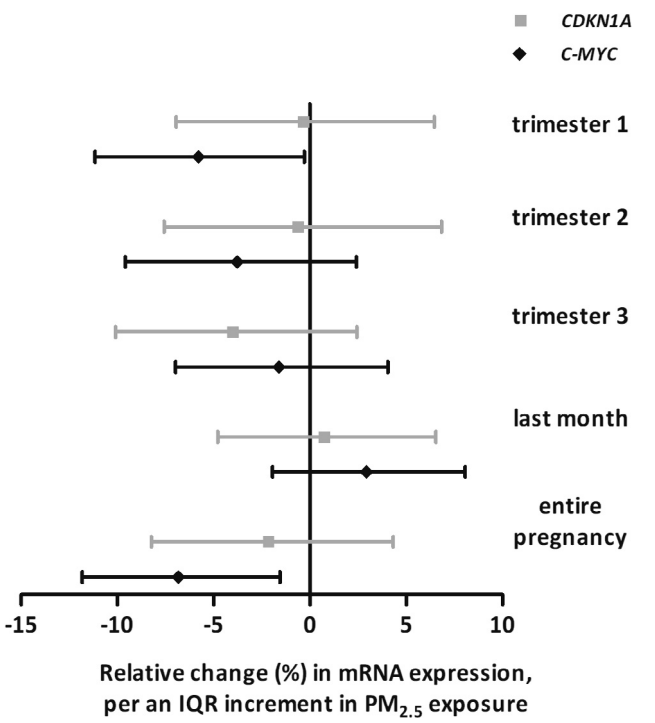

Fig. 2. Change (%) in relative mRNA expression for an IQR increment in the investigated $PM_{2.5}$ exposure windows, in adjusted analysis. The estimated effects are presented with 95% CI. Black line = *C-MYC*, light grey with square = *CDKN1A*.

3.3. Activator and target of miR-17/92 cluster

Next, we also measured the relative *C-MYC* and *CDKN1A* expression in cord blood as these genes are known either as an inducer (*C-MYC*) or a target (*CDKN1A*) of the miR-17–92 cluster. Fig. 2 shows the results from the adjusted analyses for prenatal $PM_{2.5}$ exposure in association with *C-MYC* and *CDKN1A* expression, with similar magnitudes of effect in the unadjusted analysis (**Supplementary Figure S3**). In the adjusted models, we corrected for the same set of potential covariates as previously mentioned, including newborns' sex, ethnicity, season and date of delivery, birth weight, gestational age, and maternal age, smoking behavior, maternal pre-gestational BMI, education level of the mother, parity as well as white blood cell counts. For an IQR increase in *first* trimester $PM_{2.5}$ exposure and in the *entire* pregnancy, a decrease of −5.9% (95% CI:-11.2 to −0.3, $p = 0.04$) and −6.8% (95% CI:-11.8 to −1.5, $p = 0.01$) in *C-MYC* expression in cord blood was observed, respectively. No significant associations were observed between *CDKN1A* expression and prenatal $PM_{2.5}$ exposure, in the adjusted model.

Moreover, we performed a sensitivity analysis on the relative expression of miR-17, miR-20a and miR-92a, and of *C-MYC* and *CDKN1A* in cord blood in association with $PM_{2.5}$ exposure in which newborns from non-European origin (n = 53) were excluded (***Supplementary Table S4***).

We checked the correlation for the different miRNAs belonging to the miR-17/92 cluster amongst each other and with *C-MYC* and *CDKN1A* by calculating Pearson correlation coefficients (Table 3). As expected, the three miRNAs miR-17, miR-20a and miR-92a were highly positively correlated. Only miR-17 expression was negatively correlated with *CDKN1A* expression in cord blood, whereas, the expression of all measured members of the miR-17/92 cluster was inversely correlated with *C-MYC* expression.

Table 3
Pearson correlation coefficients (r) for the miRNAs against each other and for the measured mRNAs (n = 370).

	miR-17		miR-20a		miR-92a	
	r	*P*-value	r	*P*-value	r	*P*-value
miR-17	–	–	0.86	< 0.001	0.67	< 0.001
miR-20a	0.86	< 0.001	–	–	0.53	< 0.001
miR-92a	0.67	< 0.001	0.53	< 0.001	–	–
CDKN1A	−0.10	*0.05*	−0.05	0.33	−0.07	0.15
C-MYC	−0.18	< 0.001	−0.18	*< 0.001*	−0.10	0.06

4. Discussion

Ambient particulate air pollution is the fifth most leading risk factor for human health based on the Global Burden of Disease Study. (GBDRF Collaborators, 2016) Inflammation and oxidative stress (Schins, 2004; Kelly, 2003) are two biological mechanisms which underlie the adverse effects of (prenatal) exposure to particulate air pollution. MiRNAs are master regulators of gene expression and have been suggested to serve a fundamental role in mediating biological processes in response to air pollution exposure. (Jardim, 2011) According to the developmental origins of health and disease (*DOHaD*) hypothesis, a disturbed early life environment may cause miRNA changes which can adversely influence developmental programming which can be disadvantageous in later life. (Perera and Herbstman, 2011)

Our data revealed lower expression of the measured members of the miR-17/92 cluster in cord blood with higher prenatal $PM_{2.5}$ exposure. These inverse associations were observed mainly during the *first* trimester and the *entire* period of pregnancy for miR-17 expression, during the *first* and *third* trimester and *entire* pregnancy for miR-20a expression, and lastly, during the *third* trimester for miR-92a expression. Early gestational stage from fertilization up to including implantation has been observed to be a critical period for methylation reprogramming and as observed by Janssen *et al.* to represent a sensitive window for the effects of $PM_{2.5}$ on placental methylation at birth. (Janssen, 2013) The exact reason of different windows of susceptibility for different miRNA targets as observed here is not known not only for miRNA and/or mRNA expression, (Tsamou, 2018; Saenen et al., 2015; Philipp, 2011) but also for other molecular markers such as telomere length, (Martens, 2017; Song, 2019) mitochondrial DNA, (Janssen, 2012) and global DNA methylation. (Cai, 2017; Maghbooli, 2018)

Consistent with our findings, lower levels of miR-20a and miR-92a in blood from adults were associated with short-term PM exposure, in TAPAS (Transportation, Air Pollution and Physical Activities) cohort. (Espín-Pérez, 2018) Based on these observations, a possible link between increased cancer risks and particulate matter exposure has been suggested.

Interestingly, it has been reported earlier that the expression of five members of the miR-17/92 cluster, including miR-20a and miR-92a, was significantly elevated in placentas of macrosomia, i.e. term babies with extremely high birthweight. (Li, 2015) Overexpression of individual miRNAs from the miR-17/92 cluster has been reported in human cancers, autoimmune and cardiovascular diseases, while underexpression was shown in neurodegenerative diseases (i.e. Alzheimer's disease). (Mogilyansky and Rigoutsos, 2013) Downregulation of members of the miR-17/92 cluster has also been observed in human replicative and aging models, suggesting a key role of this cluster in the relationship between cancer and aging. (Grillari et al., 2010) Upregulation of the miR-17/92 cluster inhibited the generation of reactive oxygen species (ROS) and DNA damage in human lung cancer. (Ebi, 2009) Thus, low levels of miR-17/92 cluster expression in response to $PM_{2.5}$ exposure during pregnancy might induce the generation of ROS and DNA damage, which could predispose the fetus to the development of diseases in later life.

In animal studies, lack of the miR-17/92 cluster was associated with smaller embryos and acute postnatal deaths, most likely due to defects in the development of lung and heart. This cluster is highly expressed in murine embryonic lung and decreases with the lung maturation. (Mendell, 2008)

Furthermore, a well-studied inducer and target of the miR-17/92 cluster, *C-MYC* and *CDKN1A*, (Mogilyansky and Rigoutsos, 2013; Fang, 2017; O'Donnell et al., 2005) were measured in cord blood from the same study population. *C-MYC* or *cellular-MYC* is a transcription factor mostly known to activate expression of many pro-proliferative genes through binding enhancer box sequences (E-boxes) and recruiting histone acetyltransferases (HATs). (Rahl and Young, 2014) *C-MYC* is known to affect miRNA expression, which is particularly important in the development of cancer. In B-cell lymphoma, *C-MYC* activates the miR-17/92 to induce cell growth, angiogenesis and metabolism. (Ventura, 2008; Inomata, 2009) Our results revealed that *C-MYC* expression was negatively associated with gestational $PM_{2.5}$ exposure, and slighlty ($r < -0.2$) inversely correlated with the measured members of the miR-17/92 members. Downregulation of *c-MYC* expression in cord blood has previously been shown to be associated with long-term $PM_{2.5}$ exposure in cord blood of 142 mother-newborn pairs. (Winckelmans, 2017) A human study showed that the miR-17/92 cluster can regulate the differentiation of primary trophoblasts. They demonstrated that *C-MYC* regulated members of the miR-17–92 cluster inhibit trophoblast differentiation by repressing hGCM1 and hCYP19A1, a process that potentially contributes to the pathogenesis of preeclampsia. (Kumar et al., 2013) Blocked *c-MYC* expression in rodents inhibited cell division and adversely affected fetal lung growth and differentiation. (Kellogg et al., 1992) In addition, *c-MYC* has been suggested to play a crucial role in angogenesis, lining the blood vessels of endothelial cells in mouse embryos. (He, 2008)

CDKN1A is a cyclin-dependent kinase inhibitor that is directly targeted by miR-17/92 and functions as an important cell-cycle regulator. (Inomata, 2009) It promotes cell proliferation and oncogenicity. (Roninson, 2002) Interestingly, *CDKN1A* is widely expressed at low levels in most of the tissues, while under DNA damage or other cellular stressors, its expression is increased in order to activate the cell cycle checkpoints, playing a crucial role in cell survival. (Wei, 2015) In our findings, higher *CDKN1A* expression was correlated with lower expression of miR-17 in cord blood, and was borderline significantly ($p < 0.1$) and positively associated with prenatal $PM_{2.5}$ exposure in unadjusted model, while these associations were no longer significant in the fully adjusted model. In a recent study, (Rossner, 2007) a positive association of *CDKN1A* protein levels with air pollution by carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) exposure measured in plasma from adults was reported.

To our knowledge, this the first study in which the effects of prenatal particulate air pollution exposure on the expression of the oncogenic miR-17/92 cluster in cord blood are investigated. Confirmation of an inducer and a target of this miRNA cluster was also performed. Remarkably, most of the significant findings remained consistent across all presented analyses. In particular, the exclusion of non-European newborns from our study population did not significantly alter our associations between m(i)RNA expression and prenatal $PM_{2.5}$ exposure.

Study limitations include the measurement of three out of six members of the miR-17/92 cluster, as the expression of the other members of the cluster could not be analyzed by qRT-PCR due to low PCR efficiency (data not shown). Our statistical models were corrected for a set of possible covariates, thus, we cannot exclude the possibility of other unknown and/or unmeasured variables to influence our findings. Moreover, other targets of the miR-17/92 cluster (i.e. *PTEN*, *TP53*) could also be of interest for further validation in association with PM exposure, as in this study one of the two measured targets, *CDKN1A*, was not associated with $PM_{2.5}$ exposure. The study is conducted on cord blood collected right after the delivery, which does not surely mirror the miRNA changes arising at earlier stages during pregnancy.

Furthermore, in order to confirm whether these changes in the studied m(i)RNAs at term are persistent at a later timepoint in life of the newborns, further follow-up should reveal whether the observed changes in the miR-17/92 cluster persist throughout childhood and outcome related consequences in later life.

5. Conclusions

The expression of miR-17, miR-20a and miR-92a, members of the oncogenic miR-17/92 cluster in cord blood at term was inversely associated with particulate air pollution in short- or long-term exposures prior to delivery. These changes were accompanied by a decrease in the miR-17/92 expression activator *C-MYC* in association with $PM_{2.5}$ exposure. These changes in cord blood might provide more insights into the underlying mechanisms upon prenatal particulate air exposure.

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.

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Appendix A. Supplementary data

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